Prevention of Posterior Capsular Opacification through Aldose Reductase Inhibition

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PURPOSE. The purpose of this study was to evaluate the effect of aldose reductase (AR) inhibition on posterior capsular opacification (PCO) with the use of a pig eye capsular bag model.

METHODS. Pig eye capsular bags were prepared by capsulorrhexis and cultured in medium without or with AR inhibitors for 7 days. Immunostaining was performed in paraformaldehyde-fixed capsular bags to determine the expression of proliferating cell nuclear antigen (PCNA), α-smooth muscle actin (SMA), β-crystallin, and intercellular adhesion molecule (ICAM)-1. The effect of AR inhibition on basic fibroblast growth factor (BFGF)-induced mitogenic signaling in cultured human lens epithelial cells (HLECs) was examined. Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and cell counting, the expression of α-SMA, β-crystallin, and ICAM-1 by Western blot and immunocytochemical analysis, protein kinases by Western blot analysis, and NF-κB activation by gel shift and reporter assays.

RESULTS. During culture of pig eye capsular bags, residual cells on both the anterior and the posterior capsule showed vigorous growth. Treatment with AR inhibitors significantly prevented the lens epithelial cell growth in capsular bags and expression of α-SMA, β-crystallin, and ICAM-1. HLECs showed a dose-dependent response to BFGF, proliferation at lower concentrations (<20 ng/mL) and differentiation/transdifferentiation at higher concentrations (>50 ng/mL). Inhibition of AR also prevented the BFGF-induced activation of ERK1/2, JNK, and NF-κB in HLECs.

CONCLUSIONS. Results suggest that AR is required for lens epithelial cell growth and differentiation/transdifferentiation in the capsular bags, indicating that inhibition of AR could be a potential therapeutic target in the prevention of PCO. (Invest Ophtalmol Vis Sci. 2009;50:752–759) DOI:10.1167/iovs.08-2522

Posterior capsular opacification (PCO), also called secondary cataract, is the most common postoperative complication of cataract surgery. Decrease in visual acuity resulting from PCO occurs in greater than 25% of subjects who undergo phacoemulsification surgery.1,2 With younger patients at higher risk. After cataract surgery, PCO develops because of the proliferation and differentiation or transdifferentiation of residual lens epithelial cells (LECs) at the equator and beneath the anterior lens capsule. Residual lens epithelial cells proliferate and migrate on top of the posterior capsule, under the intraocular lens, causing obstruction in vision. These cells may keep proliferating or may undergo normal fiber differentiation or transdifferentiation, a change in phenotype from an epithelial to a myofibroblastic/fibroblastic phenotype leading to opacification.3 Therefore, postsurgical pharmacologic inhibition of LEC proliferation, migration, and transdifferentiation is a potential opportunity to prevent PCO. In this context, several drugs that can block LEC proliferation and differentiation/transdifferentiation have been studied, but to date none have been shown to be clinically effective.4–7

Many studies have suggested the role of several cytokines and growth factors, such as IL-6,8 transforming growth factor (TGF)-β,9 fibroblast growth factor (FGF)-2,10,11 hepatocyte growth factor (HGF),12,13 and epidermal growth factor (EGF)14 in the development of PCO. After cataract surgery, the levels of these cytokines and growth factors are elevated in aqueous humor influencing proliferation, migration, and differentiation/transdifferentiation of LECs. Basic FGF or FGF-2 (BFGF), an important growth factor that establishes and maintains normal lens physiology, is constantly present in the normal lens milieu.15 BFGF regulates proliferation and promotes the differentiation of lens epithelial cells to lens fiber cells.16 Studies have shown that BFGF induces LEC proliferation at low doses and differentiation at higher doses, which may contribute to the development of PCO.3 Although the exact mechanism involved in BFGF-induced LEC differentiation is unclear, studies show the role of BFGF-stimulated activation of ERK1/2 and the expression of differentiation markers such as β-crystallin and filensin in LECs.17,18 On the other hand, TGF-β induces an epithelial mesenchymal transition (EMT) causing the residual LECs to transdifferentiate into spindlelike myofibroblastic cells that express α-smooth muscle actin (SMA).19 Hales et al.20 showed that TGF-β-induced opacification in the cultured lens was morphologically and biochemically similar to cataract. It has been suggested that immediately after cataract surgery, whose levels of active TGF-β are low, elevated levels of BFGF may induce LECs to proliferate. It could be speculated that when the level of active TGF-β increases, it inhibits BFGF-induced proliferation and stimulates PCO-specific changes such as EMT and transdifferentiation.8

Several reports also suggest that growth factor-induced reactive oxygen species (ROS) formation mediates lens epithelial cell growth that may cause PCO.14,21 and antioxidants such as caffeic acid and retinoic acid have been shown to prevent PCO.22–24 Further, in a recent study, redox-sensitive transcription factor NF-κB has been implicated in LEC proliferation and PCO.25 We have shown that aldose reductase (AR), a polyol pathway enzyme that reduces glucose and various lipid peroxidation-derived aldehydes and their glutathione conjugates,
mediates ROS signals initiated by growth factors, cytokines, high glucose, and bacterial endotoxin (lipopolysaccharide [LPS]) through the activation of NF-κB. We have shown that pharmacologic inhibition or genetic (siRNA and antisense) ablation of AR prevents the activation of ROS-sensitive transcription factors such as NF-κB and AP-1 in human lens epithelial cells and macrophages. We have also shown that LPS induced the expression of tumor necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 in human lens epithelial cells (HLECs) and rat eyes and that the inhibition of AR prevented it. Indeed, the association of AR with ROS-mediated signaling is evident by the observations that AR inhibition attenuates high glucose-induced oxidative stress and superoxide production in LECs and retinal pericytes. AR has also been implicated in the development of diabetic cataractogenesis caused by the increased flux of glucose through the polyol pathway that increases osmotic pressure, the decreased NADPH/NADH ratio that could increase oxidative stress, the activation of PKC that activates ROS signals, and the increased advanced glycation end product formation leading to protein alteration and tissue damage. We and others have shown that the inhibition of AR could prevent diabetic cataract in rodents. Further, AR is a growth response protein that is overexpressed during oxidative stress, diabetes, and cancer and in cells undergoing proliferation in vitro and in vivo. Thus, AR may play a role in the development of PCO, and the inhibition of AR could be a therapeutic target to prevent PCO. Here we have investigated the role of AR in LEC growth and differentiation/transdifferentiation in cultured pig eye capsular bags and cultured HLECs. Our results show that the inhibition of AR could significantly prevent LEC growth and differentiation/transdifferentiation in pig eye capsular bags explants and in HLEC, suggesting a potential therapeutic use of AR inhibitors in the prevention of PCO.

### Materials and Methods

#### Materials

- Dulbecco’s modified Eagle’s medium (DMEM) with 4 mM l-glutamine and 1 g/L glucose, phosphate-buffered saline (PBS), gentamicin solution, 0.25% trypsin/EDTA solution, and heat-inactivated fetal bovine serum (FBS) were purchased from Invitrogen-Gibco (Grand Island, NY).
- Antibodies against β-crystallin, filensin, intercellular adhesion molecule (ICAM)-1, and glyceraldehyde phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against α-SMA were from Research Diagnostic (Concord, MA), and antibodies against proliferating cell nuclear antigen (PCNA), phospho-p38, phospho-ERK1/2, phospho-SAPK/JNK, and total p38, ERK1/2, and SAPK/JNK were from Cell Signaling (Beverly, MA).
- AR inhibitors sorbinil and zopolrestat were gift from Pfizer (Groton, CT), and antibodies against bixinone–horse radish peroxidase (HRP) system (Dako, Carpinteria, CA) or they were incubated with respective fluorescein isothiocyanate (FITC)-labeled secondary antibodies. The samples were examined with an epifluorescence microscope (EPI-800; Nikon, Tokyo, Japan).
- Pig eye capsular bags were incubated for 7 days, as described. Lens epithelial cells were detached from capsular bags by incubation with 0.25% trypsin-EDTA solution. The trypsinized cells were harvested, washed with PBS, and suspended in PBS. To determine cell growth, equal volumes of cell suspension and trypan blue were mixed, and the cells that excluded tryp blue dye were counted manually by using a hemocytometer.

#### Determination of Lens Epithelial Cell Number in Pig Eye Capsular Bags

Pig eye capsular bags were incubated for 7 days, as described. Lens epithelial cells were detached from capsular bags by incubation with 0.25% trypsin-EDTA solution. The trypsinized cells were harvested, washed with PBS, and resuspended in PBS. To determine cell growth, equal volumes of cell suspension and trypan blue were mixed, and the cells that excluded tryp blue dye were counted manually by using a hemocytometer.

#### Cell Culture of HLECs

Adenovirus SV-40 viral DNA-transformed HLECs (HLE-B3) were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM supplemented with 20% heat-inactivated FBS and 50 μg/mL gentamicin and were grown in a humidified incubator at 37°C and 5% CO2. For the assessment of cell viability and the expression of α-SMA, β-crystallin, and ICAM-1, HLECs were pretreated with or without 20 μM AR inhibitors sorbinil or zopolrestat overnight in medium containing 0.5% FBS and then, without change of medium, stimulated with various concentrations (0–100 ng/mL) of BFGF for 24 hours. In experiments to investigate the effect of BFGF on NF-κB and MAPK activation, HLECs were starved in medium containing 0.5% FBS with or without AR inhibitors for 16 hours. The cells were washed with serum-free medium followed by incubation in serum-free medium containing 50 ng/mL BFGF, with or without AR inhibitor as before, for various time intervals.

#### Cell Viability Assays

HLECs were grown to confluence, harvested, and plated at 5000 cells/well in 96-well plates. The cells were grown-arrested by incubation in 0.5% FBS medium without or with AR inhibitor (20 μM) for 24 hours. After 24 hours, the cells were stimulated with BFGF (0, 10, 20, and 100 ng/mL) and incubated for an additional 24 hours. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described by us earlier. Briefly, after incubation of HLECs, 10 μL of 5 mg/mL MTT in PBS were added to each well and incubated further for 2 hours at 37°C. The medium was removed, the formazan granules obtained were dissolved in 100% dimethyl sulfoxide (DMSO), and absorbance at 562 nm was detected.
with an ELISA plate reader. Cell viability was also determined by cell counting with a hemocytometer, as described.

**Western Blot Analysis**

HLECs incubated under various conditions described were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer containing 50 mM HEPES [pH 7.6], 10 mM KCl, 0.5% NP-40, 1 mM, 1 mM phenylmethylsulfonyl fluoride, and 1:100 dilution of protease inhibitor cocktail (Sigma, St. Louis, MO) for 15 minutes at 4°C. The crude lysates were cleared by centrifugation at 12,000g for 10 minutes at 4°C. Aliquots of the lysates containing equal amounts of protein (40 μg) were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). The membranes were then incubated in blocking solution containing 5% wt/vol dried fat-free milk and 0.1% vol/vol Tween-20 in Tris-buffered saline. Subsequently, the membranes were incubated with antibodies against αSMA, β-crystallin, ICAM-1, phospho-p38, phospho-ERK1/2, phospho-SAPK/JNK, and total-p38, ERK1/2, and SAPK/JNK. The membranes were washed and probed with the respective HRP-conjugated secondary antibodies (Southern Biotechnology, Birmingham, AL) and visualized by chemiluminescence (Pierce Biotechnology, Rockford, IL). All blots were probed with GAPDH or β-actin as a loading control, and protein band intensities were determined by densitometric analysis by using Kodak Image Station 2000R.

**Electrophoretic Mobility Shift Assay**

HLECs were pretreated with or without AR inhibitors for 24 hours in serum-free medium, followed by treatment with BFGF (50 ng/mL) for an additional hour. Nuclear extracts were prepared as described earlier.38 Consensus oligonucleotides for NF-κB transcription factors were 5′-end labeled using T4 polyadenylate kinase. EMSA was performed as described earlier.59 The specificity of the assay was examined by competition with an excess of unlabeled oligonucleotide and supershift assays with antibodies to p65.

**NF-κB–Dependent Secretory Alkaline Phosphatase (SEAP) Expression Assay**

To examine NF-κB promoter activity in HLECs in response to BFGF treatment, cells (1 × 105 cells/well) were plated in a 24-well plate, starved for 16 hours in 0.5% FBS medium without or with AR inhibitors, and transfected with pNF-κB-SEAP2-control and pTAL-SEAP control plasmid (Clontech, Mountain View, CA) with the use of transfection reagent (Lipofectamine Plus; Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. After 6 hours of transfection, cells were stimulated with BFGF (50 ng/mL) for 48 hours. The cell culture medium were harvested and centrifuged at 5000 rpm, and supernatants were stored at −80°C. The medium was thawed and used for chemiluminescent SEAP assay with a reporter assay system (Great EscAPE SEAP, BD Biosciences, Palo Alto, CA) according to a protocol essentially as described by the manufacturer with 96-well chemiluminescence plate reader. All the suggested controls by manufacturers were used in the assay.

**RNA Interference Ablation of AR**

HLECs were grown to approximately 60% confluence in DMEM supplemented with 20% FBS in a six-well plate. The cells were incubated with medium (OptiMEM; Invitrogen) containing the AR-siRNA (AATCCGGTGCTCCACTCTC; scrambled siRNA (AAAATCTC-CCTGAAT CATACA; control) to a final concentration of 100 nM and the transfection reagent (RNAiFect; Qiagen, Valencia, CA) as described by us earlier.58 Briefly, for each well, 2 μg AR siRNA was diluted in serum-free medium to give a final volume of 100 μL and incubated with 6 μL transfection reagent (RNAiFect; Qiagen) for 15 minutes at room temperature. The transfection mixture was added to the respective wells, each containing 1900 μL complete medium (20% fetal bovine serum) and incubated for 24 hours. After 24 hours, the medium was replaced with fresh DMEM (serum free) for another 24 hours before stimulation with BFGF. Changes in the expression of AR were estimated by Western blot analysis using anti-AR antibodies.

**Immunocytochemical Analysis**

For immunocytochemical analysis, HLECs were plated in chambered slides and grown to approximately 70% confluence. The cells were growth arrested by incubation in 0.5% FBS medium for 24 hours without or with AR inhibitors. After treatment with BFGF for 24 hours, the cells were washed and quickly fixed in methanol (prechilled at −20°C) for 10 minutes. The slides were air dried and washed three times with PBS and blocked in the blocking solution containing 2% BSA, 0.1% Triton X-100, and 5% normal goat serum for 1 hour at room temperature. Slides were washed twice and incubated with antibodies against αSMA, β-crystallin, filensin, and ICAM-1 for 1 hour at room temperature. Subsequently, the slides were washed three times with PBS and stained using FITC-labeled secondary antibodies, washed, and mounted using mounting medium with DAPI (Vector Laboratories, Burlingame, CA). The sections were examined under epifluorescence microscopy (EPI-800 microscope; Nikon).

**Statistical Analysis**

Data were presented as mean ± SD, and P values were determined by unpaired Student’s t-test using commercial software (Excel; Microsoft, Redmond, WA). P < 0.05 was considered statistically significant.

**RESULTS**

**Prevention of Epithelial Cell Growth in Pig Eye Capsular Bags by AR Inhibition**

Phacoemulsification, the technique for cataract surgery in humans, was used to operate on enucleated pig eyes. After the lens was removed, a capsular tension ring was inserted in the capsular bag. The capsular bag was then cultured in complete growth medium without or with AR inhibitors. Lens epithelial cells first appeared after 2 to 3 days in culture near the equator and then migrated toward anterior and posterior regions of the capsule, covering it entirely in 7 days, and the capsule showed distinct folding in the central region (data not shown). As shown in Figure 1A, lens epithelial cells showed massive growth in the complete medium. Epithelial cells counted from three capsular bags in three subsequent batches of cultures showed a significant (approximately 74%) inhibition of cell growth by sorbini (Fig. 1A). Similar results were obtained with another structurally distinct AR inhibitor, fidarestat, which showed a dose-dependent response in inhibiting the growth of LECs in pig eye capsular bags (Fig. 1B). The most effective dose at which fidarestat significantly (P < 0.001) inhibited LEC growth was 30 μM (Fig. 1B).

Subsequent to observations that AR inhibition could block cell growth, we examined the status of the residual cells to determine whether they were still in cell division mode with the use of antiproliferating cell nuclear antigen (PCNA) antibodies. As shown in Supplementary Figures 1A and 1B, http://www.iovs.org/cgi/content/full/50/2/752/DC1, in capsular bags cultured without AR inhibitor a large number of cells were PCNA positive, indicating that they were still dividing; in AR inhibitor-treated capsular bags, fewer PCNA-positive cells were present. These results suggest that AR inhibition could block the growth/proliferation of residual cells after phacoemulsification of the lens.
showed similar inhibition in the expression of ICAM-1 with FGF were included, ICAM-1 expression was reduced by cultured with AR inhibitors. Similarly, when the AR inhibitors bags after 7 days of culture showed that a large number of cells expressed ICAM-1 protein at the anterior and posterior capsule. In AR inhibitor-treated capsular bags, the expression of ICAM-1 was markedly inhibited (Supplementary Figs. 2E, 2F). Our results thus indicate that AR inhibition could be successfully used to block the differentiation/transdifferentiation of epithelial cells subsequent to phacoemulsification of lens in cataract surgery, which could prevent PCO.

**Prevention of BFGF-Induced HLEC Growth by AR Inhibition**

To confirm our findings in the pig eye capsular bag model and to understand the molecular mechanisms by which AR inhibition prevents lens epithelial cell proliferation, we examined the effect of AR inhibitors on BFGF-induced proliferation of cultured HLECs. As shown in Figure 3A, BFGF induced significant increases in proliferation, compared with controls, at all doses greater than 10 ng/mL, and the response at 20 ng/mL BFGF was greater than at higher doses. Inhibition of AR by two structurally distinct inhibitors significantly (*P < 0.01*) blocked the cell growth induced by 20 ng/mL BFGF as determined by cell counting and MTT assay (Figs. 3A, 3B).

**Prevention of Expression of α-SMA, β-Crystallin, and ICAM-1 in HLECs by AR Inhibition**

We next examined the effect of AR inhibition on BFGF-induced expression of β-crystallin and α-SMA in HLECs. As shown in Figure 4A, treatment of HLECs with 50 ng/mL BFGF caused an approximately 4-fold increase in the expression of β-crystallin, and α-SMA and a 2.5-fold increase in the expression of adhesion molecule ICAM-1 and AR inhibitors significantly (approximately 90%) inhibited the increase (Fig. 4A). However, treatment with AR inhibitors alone did not cause any significant change in the expression of these proteins. Although sorbinil and zopolrestat are specific inhibitors of AR, to rule out any nonspecific effect of pharmacologic inhibitors, we ablated the AR message by transfecting the HLECs with AR-siRNA. As shown in Figure 4B, BFGF-induced increases in the expression of α-SMA and β-crystallin in untransfected (C) and scrambled siRNA oligo (Cs)-transfected HLECs but not in the AR-siRNA-transfected cells suggesting that AR plays a crucial role in the aberrant expression of α-SMA and β-crystallin in HLECs. Further, immunocytochemical analysis also demonstrates that AR inhibition significantly blocked BFGF-induced expression of α-SMA, β-crystallin, filensin, and ICAM-1 in HLECs (Supplementary Figs. 5A–5D, http://www.iovs.org/cgi/content/full/50/2/752/DC1).

**Prevention of BFGF-Induced Activation of NF-κB in HLEC by AR Inhibition**

Because the activation of NF-κB mediates the expression of various marker proteins and cytokines, we next examined the effect of AR inhibition on BFGF-induced NF-κB activation in HLECs. Treatment with BFGF caused an approximately threefold increase in the activation of NF-κB, as indicated by DNA binding capacity determined by EMSA (Fig. 5A). Inhibition of AR by sorbinil or zopolrestat significantly (>90%) prevented BFGF-induced NF-κB DNA binding capacity. However, sorbinil or zopolrestat alone did not affect the basal activity of NF-κB. We further confirmed our results with a more sensitive NF-κB-dependent reporter assay. As shown in Figure 5B, BFGF caused more than fivefold activation in the NF-κB-dependent reporter
SEAP activity that was significantly (approximately 60%; \( P < 0.01 \)) inhibited by AR inhibitors. These results suggest that the inhibition of AR could modulate BFGF-induced activation of redox-sensitive transcription factors, which could be responsible for preventing the growth and differentiation/transdifferentiation of LECs and thereby prevent PCO.

**Prevention of BFGF-Induced Phosphorylation of MAPKs by AR Inhibition**

MAPKs are known to regulate the phosphorylation and activation of NF-κB; therefore, we next examined the effect of AR inhibition on BFGF-induced activation of MAPKs. As shown in Figure 6, treatment with BFGF enhanced the phosphorylation of ERK1/2, MAPK, and SAPK/JNK in HLECs and pretreatment with the AR inhibitor prevented it. There was no change in the expression of total ERK1/2 and SAPK/JNK proteins. We did not observe any change in the phosphorylation of p38-MAPK in response to BFGF treatment (data not shown), suggesting that ERK1/2 MAPK and SAPK/JNK are the main mediators of growth factor–induced NF-κB activation in HLECs.

**Discussion**

After cataract surgery, the residual lens cells undergo proliferation and transdifferentiation or differentiation, resulting in PCO (see Introduction). ROS–mediated processes and stimulation by growth factors, including TGF-β and FGF, appear to play key roles in PCO development.9–11 Studies suggest that redox-sensitive transcription factors such as NF-κB play a crucial role in trauma-induced oxidative stress that could result in cataract formation.39–44 Various factors, including growth factors, cytokines, endotoxins, high glucose, and injury, are known to activate NF-κB.45–50 We have shown earlier that NF-κB activation is regulated by AR-catalyzed reduced glutathione–lipid aldehyde conjugates generally formed as a result of changes in the cellular redox status and ROS levels.46,47 Inhibition of AR prevents the AR-catalyzed reduction of glutathione–lipid aldehydes required for the activation of a signaling pathway that leads to the phosphorylation and nuclear translocation of NF-κB and the resultant inflammatory gene expression that promotes growth and proliferation.48–50
Our earlier studies show that the inhibition of AR could prevent exit from the cell cycle and accumulate in the G-phase. Similarly, in colon cancer cells (Caco-2), AR inhibition caused cells to undergo division of LECs in capsular bags. We have shown earlier that positive, suggesting that LECs continue to divide even after 7 days in culture showing a consistent pattern. Because proliferating nuclei express PCNA and incorporate BRDU during DNA replication, PCNA and BRDU-positive signal indicated that cells are in division phase. We observed that in capsular bags without the AR inhibitor, a large percentage of cells were positive for PCNA. In the AR inhibitor groups fewer cells were PCNA positive, suggesting that LECs continue to divide even after 7 days in culture and that the AR inhibitor blocked the cell division of LECs in capsular bags. We have shown earlier that in colon cancer cells (Caco-2), AR inhibition caused cells to exit the cell cycle and accumulate in the G-phase. Similarly, our earlier studies show that the inhibition of AR could prevent cytokine- and hyperglycaemia-induced cytotoxicity and the activation of redox-sensitive transcription factors in HLECs. In addition to cell growth, the transdifferentiation of LECs in myofibroblast-like cells is important for PCO. We observed that the inhibition of AR prevents oxidative stress-induced signals that cause the activation of NF-κB, leading to cell growth or death. Thus, our current and previous results strongly support our hypothesis that AR could regulate cell growth, and its inhibition can attenuate PCO formation.

Growth factors, especially TGF-β and BFGF, have been implicated in the development of PCO. Indeed, Nishi et al. have shown that cytokines such as IL-1, IL-6, and BFGF may be produced in vivo by residual LECs, causing postoperative inflammation and proliferation of residual lens epithelial cells. We observed that in HLECs, BFGF caused increased growth at all doses of 20 ng/mL and lower and differentiation/transdifferentiation at doses of 50 ng/mL and higher, as evidenced by an approximately fourfold enhanced expression of β-crystallin and α-SMA, respectively. The low-level expression of β-crystallin and α-SMA even in control HLECs not treated with BFGF (Figs. 4A, 4B) may suggest the presence of mixed populations of cells that could include differentiating and trans-differentiating cells and normal LECs. Consistent with this possibility, we also observed the presence of immunoreactivity for filensin in at least some cells in controls (Supplementary Fig. 3C(C)), which occurs only in lens fiber cells and not in normal LECs. A range of cell types such as these may be present in the capsular bag after surgery. However, it is significant that the inhibition and siRNA ablation of AR prevented the expression of these marker proteins in BFGF-treated PCO. Therefore, we used a pig eye capsular bag model to study the effects of AR inhibition on postsurgical events leading to PCO. Two different inhibitors of AR, namely sorbinil and fidarestat, were used in the experiments with pig eye capsular bags. Although the control capsular bags cultured without AR inhibitors showed robust proliferation, differentiation, or trans-differentiation of lens epithelial cells, the AR inhibitor-treated capsular bags showed significantly less cell growth. The cell growth event was similar in many batches of capsular bag culture showing a consistent pattern. Because proliferating nuclei express PCNA and incorporate BRDU during DNA replication, PCNA and BRDU-positive signal indicated that cells are in division phase. We observed that in capsular bags without the AR inhibitor, a large percentage of cells were positive for PCNA. In the AR inhibitor groups fewer cells were PCNA positive, suggesting that LECs continue to divide even after 7 days in culture and that the AR inhibitor blocked the cell division of LECs in capsular bags. We have shown earlier that in colon cancer cells (Caco-2), AR inhibition caused cells to exit the cell cycle and accumulate in the G-phase. Similarly, our earlier studies show that the inhibition of AR could prevent...
HLECs. Moreover, AR inhibition also blocked the phosphorylation of MAPKs, including that of ERK1/2, thereby preventing the activation of a molecular cascade that ultimately activates the transcription factor NF-κB. These observations are highly significant because HLECs have been shown to be dependent on ERK signaling for growth factor-induced cell proliferation.\textsuperscript{15,14,17,18} In addition, TGF-β has been implicated in LEC transdifferentiation and expression of α-SMA\textsuperscript{19,45} and induces cataract in cultured lens.\textsuperscript{20} Studies by other investigators have shown that BFGF exacerbated TGF-β-induced cataract in cultured lens.\textsuperscript{46} However, it is not clear how α-SMA is induced by BFGF in LECs. We predict that our preculture system with 10% to 20% FBS may contain various growth factors, including TGF-β, that could be responsible for the observed increase in α-SMA induction, and that FGF may not be responsible for the expression of α-SMA in LECs.

Several pharmacologic agents have been investigated for possible use in the prevention of PCO, including the nonsteroidal anti-inflammatory drug diclofenac, which has been shown to inhibit the proliferation of subconfluent cultures of LECs in a dose-dependent manner.\textsuperscript{5} Although the use of antioxidants (such as caffeic and retinoic acids) and corticosteroids has also been suggested,\textsuperscript{22–24,47} their effectiveness is marred by toxicity or by partial prevention of the PCO. Our results suggest that AR inhibitors block the signaling events that lead to LEC proliferation and differentiation/transdifferentiation, indicating that the inhibition of AR could be a novel therapeutic approach to prevent PCO. Over the years AR inhibitors have been clinically tested for the potential treatment of diabetic complications such as diabetic neuropathy and retinopathy. Initial studies showed hepatic and renal toxicity.\textsuperscript{48} However, structural modifications of AR inhibitors and dosage studies have led to reduced toxicity; in fact, the AR inhibitor epalrestat is marketed in Japan for the treatment of diabetic neuropathy.\textsuperscript{49} Furthermore, AR inhibitors such as fidarestat, zopolrestat, zenarestat, and minalrestat have been found to be safe and have passed US Food and Drug Administration phase-1 and phase-2 clinical trials; some are in ongoing phase-3 clinical trials for diabetic complications.\textsuperscript{49–51}

Although the precise series of events through which AR activates NF-κB remain unclear, we showed earlier that the AR-catalyzed reduced product of glutathione-4-hydroxynonenal, glutathionyl 1,4-dihydroxynonenone, could be the main causative factor for the activation of NF-κB through a number of kinases that eventually cause cytotoxicity,\textsuperscript{28} including the development of PCO after cataract surgery. Therefore, AR inhibitors may be therapeutic for the prevention of PCO. Further studies are needed to determine whether they are.

In summary, our results suggest that AR plays an important role in the development of PCO. Given that this enzyme mediates the molecular signaling events leading to the differentiation and transdifferentiation of LECs in porcine capsular bags and in in vitro cell culture studies, our results suggest that AR inhibition could be a novel pharmacologic approach to prevent the characteristic changes that cause PCO in humans after cataract surgery.

### References


![Figure 6. AR inhibition prevents BFGF-induced activation of MAPK in HLECs. Growth-arrested HLECs without or with the AR inhibitor zopolrestat (20 μM) were treated with BFGF (50 ng/mL) for indicated time periods. Cytosolic extracts containing equal amounts of protein were subjected to Western blot analysis using antibodies against phospho- and total ERK1/2, JNK/SAPK, and GAPDH. Representative blots from three analyses are shown.](image-url)


