Diabetes Can Alter the Signal Transduction Pathways in the Lens of Rats

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Diabetes is known to affect cataract formation by means of osmotic stress induced by activated aldose reductase in the sorbitol pathway. In addition, alterations in the bioavailability of numerous extralenticular growth factors has been reported and shown to result in various consequences. We have found that the basic fibroblast growth factor (bFGF) accumulates in the vitreous humor of 3- and 8-week diabetic rats. Consequently, the associating signal transduction cascades were severely disrupted, including upregulated phosphorylation of extracellular signal-regulated kinase (ERK) and the common stress-associated mitogen-activated protein kinases p38 and SAPK/JNK. Conversely, under diabetic condition, we observed a dramatic inhibition of phosphatidylinositol-3 kinase activity in lenses obtained from the same animal. Rats treated with the aldose reductase inhibitor AL01576 for the duration of the diabetic condition showed that the diabetes-induced lenticular signaling alterations were normalized, comparable to controls. However, treatment of AL01576 in vitro was ineffective at normalizing the altered constituents in extracted diabetic vitreous after the onset of diabetes. The effect of AL01576 in the high galactose-induced cataract model in vitro was also examined. Administration of AL01576 to lens organ culture normalized the aberrant signaling effects and morphological characteristics associated with in vitro sugar cataract formation. In conclusion, our findings demonstrate diabetes-associated alterations in the lens signal transduction parameters and the effectiveness of AL01576 at normalizing such alterations. The causes for these alterations can be attributed to elevated vitreal bFGF in conjunction with osmotic stress and associated attenuation in redox status of the lens. *Diabetes* 52: 1014–1022, 2003

Diabetes is known to cause cataract formation in the eye lens. Numerous studies have shown altered levels of various growth factors in the ocular fluids under diabetic condition. Boulton et al. (1) demonstrated elevated levels of basic fibroblast growth factor (bFGF) in the vitreous of patients with non–insulin-treated diabetes. Furthermore, Meyer-Schwickerath et al. (2) found increased vitreal levels of the insulin-like growth factor (IGF) and associated IGF binding proteins in human subjects with diabetes and neovascular eye disease. The gene expression of the angiogenic factor vascular endothelial growth factor (VEGF) was also found to be elevated in the retina of diabetic rats, providing a possible link to diabetic retinopathy (3). It is conceivable that the disruption of the retinal microvasculature or other vascularized tissues in the eye might leach such factors into ocular fluids (1,4–6), where an altered bioavailability of growth factors may influence the physiology and function of the lens.

Lens epithelial cells possess receptors to many growth factors (7), including epidermal growth factor, bFGF, IGF, platelet-derived growth factor, and transforming growth factor-β, all of which are associated with lens physiology (8–11). For example, exogenous addition of bFGF, IGF-1, or platelet-derived growth factor can induce differentiation and proliferation in cultured lens epithelial cells from rats or chicks (18,12–14). IGF-1 and insulin were shown to stimulate phosphatidylinositol-3 kinase (PI-3K), a survival factor, in bovine lens (15). In addition, we recently demonstrated that the lens, under various culture conditions, showed an intricate and comprehensive signal transduction (16) and signal intercommunication system involving mitogen-activated protein kinases (MAPKs) (17) that responded to various growth factors.

Vitreal growth factors are known to stimulate cellular calcium release, increase production of inositol phosphates, and induce cell proliferation and differentiation (18). However, the mechanisms underlying the involvement of growth factors in the lens intracellular signal transduction is poorly understood. Vivekanadan and Lou (19) demonstrated that lens epithelial cells responded to extracellular transmitters and generated intracellular signals from the metabolites in an active lens phosphoinositide cycle. Preliminary findings by Lou and colleagues (20,21) also showed a disparity of the status of phosphoinositide cycle in the lens under in vitro and in vivo hyperglycemic conditions. A stimulated phosphoinositide biosynthesis occurred in lens epithelia from either diabetic or galactosemic rats, but a depressed phosphoinositide

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ARI, aldose reductase inhibitor; bFGF, basic fibroblast growth factor; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-related protein kinase; IGF, insulin-like growth factor; MAPK, mitogen-activated protein kinase; PI-3K, phosphatidylinositol-3 kinase; VEGF, vascular endothelial growth factor.

biosynthetic status was found in lenses incubated with high glucose or galactose-containing medium. This suggested that the extralenticular environment may have been altered under the diabetic condition and may influence various intracellular signaling events in the lens.

To evaluate whether diabetes affects the MAPK signaling system in the lens through alterations in the bioavailability of vitreal growth factors in the eye, we used streptozotocin-induced diabetic rats as a model. Because of the importance of mitogenic bFGF in the eye (22–25), we hypothesized that any disruption or alteration in extralenticular bFGF concentration may affect the downstream MAPK signaling events in the lens epithelial cells during a diabetic disease state. This study examined the effect of the diabetic condition on the key signaling enzymes involved in the mitogenic extracellular signal-related protein kinase (ERK) and stress-responsive pathways (p38 and SAPK/JNK), and the interplay of the survival-associated pathway involving PI-3K. Whole lenses obtained from diabetic rats and normal lenses cultured in the presence of bFGF or high galactose-containing medium were analyzed. Results from this study show that the diabetic condition can alter vitreal bFGF levels and, consequently, can affect the downstream lens signaling cascades. This may ultimately contribute to an imbalance in lens metabolic homeostasis and even lead to the cataract formation.

RESEARCH DESIGN AND METHODS

Materials. Three-week-old male Sprague-Dawley rats were purchased from Taconic Laboratories (Germantown, NY). All procedures involving the live animals were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Medium-199 (TC199), and additional cell culture reagents and chemicals were purchased from VWR Scientific Products (Chicago, IL), unless otherwise noted. Protein BSA assay reagents were purchased from Pierce (Rockville, IL). The aldose reductase inhibitor (ARI) spiro-(2-flu-7H-fluorene-9,4′-imidazolidine)-2′-5′-dione (AL01576) was synthesized by Alcon Laboratories (Fort Worth, TX). PI and phosphatidylserine were purchased from Sigma. [γ-^{32}P]ATP was obtained from NEN (Boston, MA). ERK standard was obtained from BioMol (Plymouth Meeting, PA). The phospho-ERK 1/2 (E10) monoclonal antibody and the ERK (phospho-independent), phospho-Raf-1, phospho-MEK 1/2, phospho-p38, phospho-SAPK/JNK, phospho-PAK 1/2, the phospho-Akt polyclonal antibodies, and the ERK kinase activation assay kit (included phospho-Elk-1) all were obtained from Cell Signaling Technologies (Beverly, MA). bFGF was obtained from CalBioChem (San Diego, CA), and the monoclonal anti-bFGF antibody and the polyclonal antibody against the p85α subunit of PI-3K were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-p85α PI-3K monoclonal antibody, the enhanced chemiluminescence system components, including all horseradish peroxidase–conjugated secondary polyclonal antibodies, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A-Agarose beads was obtained from KPL (Gaithersburg, MD). Electrophoretic materials all were obtained from Bio-Rad Laboratories (Hercules, CA). All other chemicals were of analytical grade.

Induction of diabetes. Rats of 100 g body wt (3 weeks of age) received in the tail vein an intravenous injection of streptozotocin in citrate buffer at a dose of 70 mg/kg body wt. Some rats were treated with an ARI, AL01576, at 10 mg/kg body wt. AL01576 was administered orally for 8 weeks, concurrent with the inducement and duration of diabetes. A group of age-matched normal rats without diabetic inducement were used as controls. Three-week diabetic rats and age-matched controls were provided by Dr. George Rozanski (University of Nebraska Medical Center, Omaha, NE). Eight-week studies in the diabetic model and ARI treatments were conducted at the National Eye Institute (National Institutes of Health, Bethesda, MD). Only the diabetic rats that had blood glucose >300 mg/dl and showed no acute complications were included in the final analyses in this study. Rats were killed by CO_2 asphyxiation, and the lenses were surgically removed by posterior approach from enucleated eyes. Vitreous fluid was collected on parafilm sheets and pooled. All samples were immediately placed in liquid nitrogen and then transported to the University of Nebraska-Lincoln in dry ice.

Lens organ culture. Normal rats at 3 weeks of age were killed, lenses were removed, and vitreous was collected as described above. The organ culture was conducted under sterile conditions and followed the procedure as described previously (26). Whole lenses were cultured individually in a 24-well culture plate containing 1.5 ml of TC199 (containing 1% penicillin-streptomycin) for 24 h at 37°C in a humidified incubator in an atmosphere of 95% air and 5% CO_2. The culture conditions included TC199 medium alone (containing 30 mmol/l fructose as an osmotic control) or TC199 containing 30 mmol/l galactose, or TC199 containing 10 ng/ml bFGF or containing 20% normal rat vitreous, or 20% vitreous obtained from diabetic rats, all with or without the addition of 1×10^{-5} mol/l AL01576 or 5 μg/ml anti-bFGF neutralizing monoclonal antibody, or 20% vitreous obtained from AL01576-treated diabetic rats. bFGF was preneutralized in culture fluids with the anti-bFGF antibody for 1 h before addition to the culture medium and exposure to the lens. After the culture period, whole rat lenses were noted for morphological changes and weighed. Lens samples were frozen in liquid nitrogen for long-term storage or immediately processed for MAPK and PI-3K analysis assays.

Vitreal growth factor analysis. The concentration of bFGF in rat vitreous humor was determined using a Quantikine FGF-basic sandwich enzyme-linked immunosorbent assay (ELISA) obtained from R&D Systems (Minneapolis, MN), in accordance with the manufacturer's instructions. Vitreous from nondiabetic/control rats was diluted 50× and vitreous from diabetic rats was diluted 75–100× before the assay. Spectrophotometric analysis of the ELISA plates was recorded using a Dynatech MR700 microplate reader (Chantilly, VA).

MAPK analysis

Tissue preparation. Whole rat lens tissue samples were homogenized using a glass-glass Duall homogenizer in a buffered detergent lysis solution containing a cocktail of protease and phosphatase inhibitors, which was freshly prepared as described elsewhere (16). Individual rat lens was homogenized in 200 μl of lysis buffer. The supernatant was collected after centrifuging at 13,000*g* for 20 min. All procedures were conducted at 4°C. Total protein concentration was determined using BCA protein assay (27), following the protocol for microplate.

PAGE and Western immunoblot analyses. Fifty micrograms of total protein from the rat lens homogenate was separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane using the Bio-Rad minitrans blot electrophoretic transfer cell as described elsewhere (16). Experiments with >15 samples were carried out using Bio-Rad Criterion electrophoresis and transfer cell apparatuses. Phospho-p44/42 ERK (E10) (P-ERK) monoclonal antibody, phospho-p38 (P-p38), or phospho-SAPK/JNK (P-SAPK/JNK) polyclonal antibody was used to assay the activated MAPK profiles. An anti-MAPK 1/2 polyclonal antibody was used to assay total (phospho-independent) ERK. Membranes were also probed for phosphorylated Raf-1 (P-Raf-1), phosphorylated MEK (P-MEK 1/2), phosphorylated PAK (P-PAK2), and phosphorylated Akt (P-Akt) using the respective polyclonal antibodies. ERK MAPK activity was also assayed using a p44/42 MAPK assay kit in accordance with the manufacturer's instructions. Protein band intensities were quantified using Scion Image densitometric software package (Scion, Frederick, MD).

PI-3K analysis. Procedures for the analysis of PI-3K followed those that have been described elsewhere (15,16,28).

Tissue preparation. Lens tissue samples were homogenized as described above in lysis buffer to release the membrane-associated PI-3K. The supernatant was collected, and, for some experiments, the water-insoluble fraction was rehomogenized in 7 mol/l urea. The protein concentration of both fractions was determined using the BCA methods as discussed above.

Immunoprecipitation of PI-3K. The soluble proteins from whole rat lens homogenates (0.5–1.0 mg total protein) were immunoprecipitated using 5 μl of a polyclonal antibody against the p85α subunit of PI-3K, which also precipitates the associated p110 catalytic subunit of the PI-3K heterodimer. Lens cell extracts were incubated for 4 h at 4°C with constant agitation in a total buffered volume of 100 μl. Thirty microliters of Protein A-Agarose beads was added and incubated under identical conditions for an additional 2 h to precipitate the antibody-enzyme immunocomplex. Bound immunocomplexes were collected after a microcentrifugation pulse at 14,000 rpm for 30 s and washed to remove unbound complexes.

PI-3K assay. PI-3K activity was determined using 20 μg of PI as substrate in a total volume of 100 μl of a buffered solution containing 100 mmol/l $MgCl_2$ and 20 μg of phosphatidylserine. The reaction was initiated by the addition of [γ-^{32}P]ATP stock solution (0.88 mmol/l ATP containing 5 μCi per sample of [γ-^{32}P]ATP, 3,000 Ci/mmol, and 20 mmol/l $MgCl_2$). The reaction was continued for 10 min at 37°C and then terminated by adding 20 μl of 6N HCl. The radiolabeled lipid was extracted from the aqueous solution by adding 160 μl of chloroform/methanol (1:1 vol/vol). The reaction product PI-3[^{32}P] was

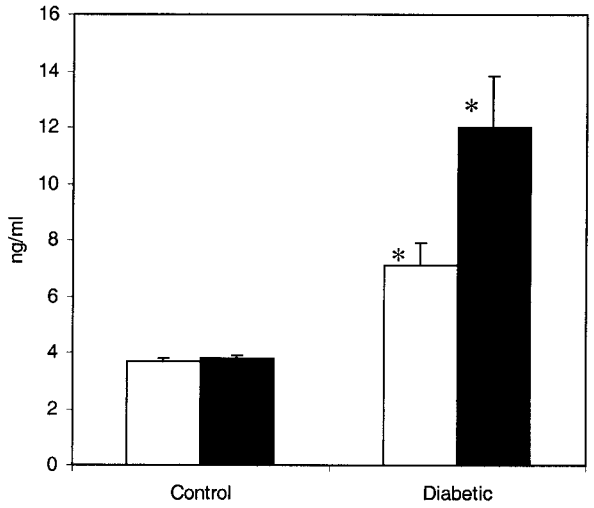

FIG. 1. Concentration of vitreal bFGF in normal and diabetic rats. For each group, vitreous was harvested from 15 rats at 3 weeks of age, after diabetes inducement (□) and 8 weeks of age, after diabetes inducement (■), and was pooled and analyzed by a bFGF-specific ELISA. Results are derived from three independent experiments. *$P < 0.05$ compared with controls. Each T-bar represents mean ± SE. Statistical analysis is based on the Student's t test: two-sample assuming equal variances.

separated by thin-layer chromatography using silica gel 60 linear K preadsorbent plates (Whatman, Clifton, NJ) pretreated with 1% potassium oxalate solution. The plates were developed by chromatography in chloroform/methanol/water/ammonium hydroxide (60:47:11.3:2 vol/vol/vol/vol). PI-3[^{32}P] bands were detected by exposure to X-ray film and quantified using Scion Image densitometric software. The amount of PI-3[^{32}P] product converted via PI-3K from PI substrate is indicative of PI-3K activity.

Western immunoblot analyses. Lens cellular homogenate (50 μg) was resolved on SDS-PAGE and Western transferred to nitrocellulose as described above. Blocked membranes were probed with an anti-p85α PI-3K monoclonal antibody. Some membranes were probed for P-Akt, the downstream target of PI-3K, for PI-3K activity. Protein bands were quantified as described above.

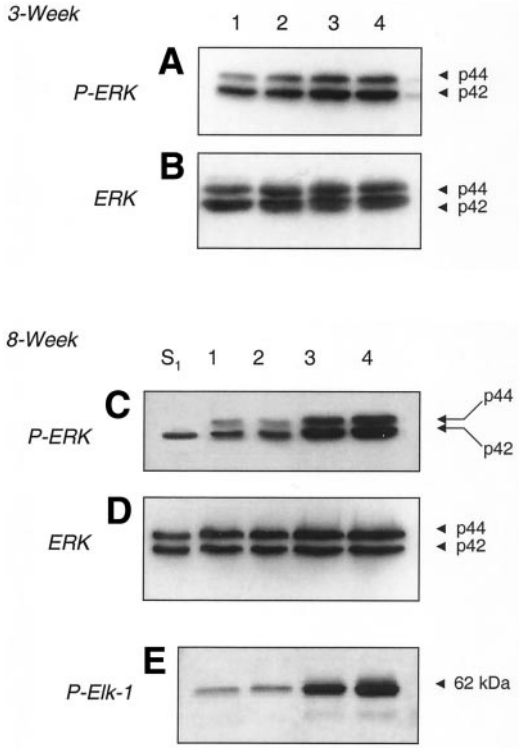

FIG. 2. Effect of the duration of the diabetic conditions on the activation of ERK in lenses from diabetic rats compared with age-matched controls using Western immunoblot analysis. Data represent three separate analyses using samples from different studies. Three-week diabetic conditions: changes in P-ERK.2 (*A*); total ERK protein (*B*). Eight-week diabetic conditions: changes in P-ERK (*C*); total ERK protein3 (*D*); the phosphotransferase activity of activated ERK as expressed by the analysis of P- Elk-1 (*E*). *Lanes 1* and *2* represent contralateral control left and right lenses, respectively; *lanes 3* and *4* represent contralateral diabetic left and right lenses, respectively; *lane S_1* represents ERK standard at 25 μg.

RESULTS

Glycemic status of the diabetic rats and the morphological changes in lenses. The diabetic rats showed typical slower weight gain in comparison with the control group (210 ± 36 vs. 365 ± 23 g body wt). The average blood glucose reached 502 ± mg/dl, compared with the normal control group of 96.7 ± 18 mg/dl at the end of the eighth week from the onset of diabetes inducement. A slight opacity was found in lenses from rats after 3-week diabetic inducement, and a typical cortical cataract was found in the 8-week group. The lens wet weight was slightly increased in the 3-week group and more so in the 8-week group (data not shown). The ARI-treated group showed no improvement in body weight gain (243 ± 24 g) or blood glucose level (482 ± 91 g/dl) at the end of the eighth week of study. However, the lenses in these ARI-treated diabetic rats remained clear throughout the study.

bFGF levels in the vitreal fluids of diabetic rats. For substantiating the previous reports that diabetic condition alters growth factor concentrations in ocular fluids (5,6,29), bFGF levels were assayed in the vitreous humor pooled from 15 diabetic rats. As shown in Fig. 1, the concentrations of bFGF were elevated proportional to the duration of diabetes at approximately twofold in the 3-week (7.1 ng/ml) and threefold in the 8-week (12.0 ng/ml) diabetic rats over the respective age-matched controls (3.75 ng/ml).

Effect of diabetic condition on activation of ERK in rat lens. The protein homogenates of individual whole lenses from either 3-week or 8-week diabetic rats and respective age-matched controls were separated on SDS-PAGE and analyzed for the changes in dually phosphorylated (activated) p42 and p44 ERK (P-ERK). As shown in Fig. 2*A*, P-ERK was increased in lenses from 3-week diabetic rats compared with the controls. Extending the diabetes to 8 weeks (Fig. 2*A*, *lanes 3* and *4*) further enhanced the activation of ERK over the age-matched controls (Fig. 2*C*, *lanes 1* and *2*). The veritable phosphotransferase activity of ERK was analyzed by measuring the phosphorylation of Elk-1, the downstream target of ERK. Note that the results of elevated Elk-1 activation in lenses of diabetic rats (Fig. 2*E*) correlate with the corresponding stimulated ERK signal shown in Fig. 2*C*. Samples used in Fig. 2*A* and *B* were also probed for phospho-independent (or total) ERK. The bands with equal intensity (Fig. 2*B* and *D*) signify the phosphorylation-state specificity of the activated ERK and the equal amount of protein samples applied on the gel.

Effect of diabetic condition on PI-3K activity in rat lens. The activity of PI-3K in the lens progressively diminished as a function of diabetes duration. Figure 3*A*

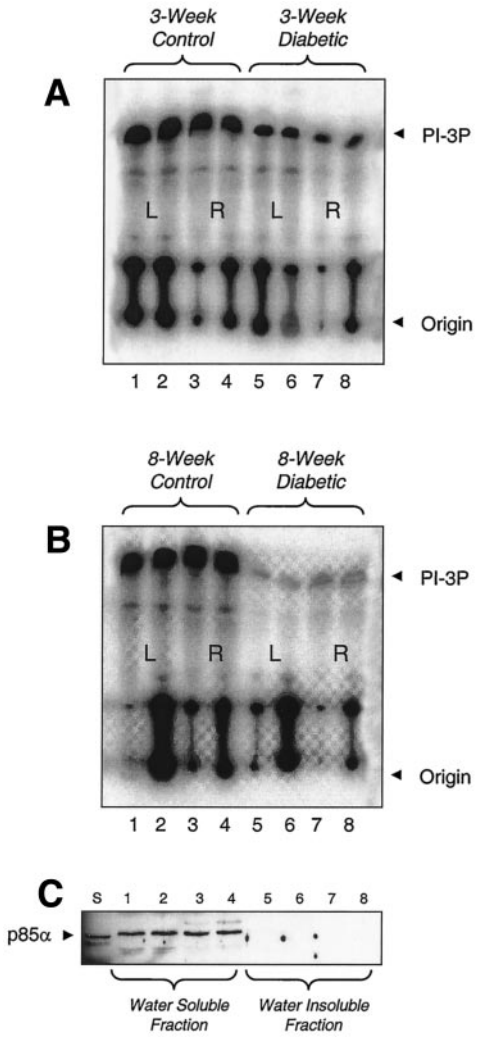

FIG. 3. Thin-layer chromatographic analysis of the activity of PI-3K in the rat lenses by probing for the radiolabeled product PI-3P (PI-3[^{32}P]). Data represent three separate analyses using samples from different studies. *A*: Analysis of PI-3K activity in the lenses of 3-week diabetic condition. *B*: PI-3K activity in the lenses of 8-week diabetic condition. *Lanes 1* and *2* and *lanes 3* and *4* represent contralateral left and right lenses from 3-week control animals, respectively, assayed in duplicate. *Lanes 5* and *6* and *lanes 7* and *8* represent contralateral left and right lenses from 3-week diabetic animals, respectively, assayed in duplicate. Bands near the origin represent unincorporated [γ-^{32}P]ATP. L, contralateral left lens; R, contralateral right lens. *C*: Immunoblot analysis of the same samples from *B*, probed for the p85α regulatory subunit of PI-3K, demonstrating that the structural PI-3K protein is retained in its active state in the water-soluble fraction of the lens homogenate. *Lanes 1-4* represent probing for p85 in the water-soluble fraction of the lens cellular homogenate; *lanes 5-8* represent probing for p85 in the water-insoluble (urea-soluble) fraction of the same lens cell homogenate; *lane S* represents the p85 standard protein.

shows the suppressed activity of PI-3K in the lens after 3 weeks of diabetes relative to the age-matched control, whereas Fig. 3*B* shows a more substantial suppression in the activity of PI-3K in the 8-week diabetic conditions.

It is possible that the observed loss of PI-3K activity may be attributed to signal transduction pathway cross-talk with an alternate signal that modulates the enzymatic activity. If so, then the PI-3K protein heterodimer would remain in an active structural conformation and associate with the water-soluble proteins. However, if the osmotic stress generated in a lens of a diabetic rat changes the

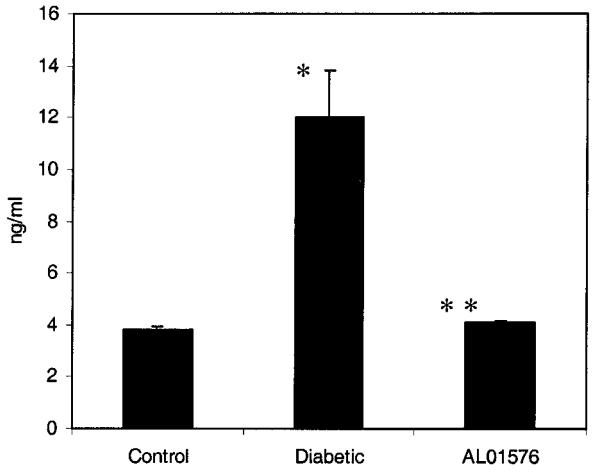

FIG. 4. Analysis of rat vitreal bFGF sampled from groups of control, diabetic, and diabetic with concomitant ARI administration. Concentration is measured in ng/ml from rats harvested at 8 weeks of age, after diabetes inducement, or age-matched controls by a bFGF-specific ELISA. Control samples averaged 3.75 ng/ml vitreal bFGF, whereas samples obtained from diabetic animals averaged 12.0 ng/ml. Diabetic rats that were treated with the ARI AL01576 demonstrated more normalized vitreal bFGF levels, averaging 4.1 ng/ml. Results are derived from three independent experiments. *$P < 0.05$ compared with controls; **$P > 0.05$, considered similar to the controls. Each T-bar represents the mean ± SE. Statistical analysis is based on the Student's *t* test: two-sample assuming equal variances.

protein solubility and impedes the functional activity (30), then PI-3K may precipitate and associate with the insoluble protein fraction. For clarifying this possibility, the water-insoluble protein fractions in the lenses used for Fig. 3*A* and *B* were solubilized in urea and separated on an SDS-PAGE gel, along with the respective water-soluble fractions. The immunoblots were probed with anti-p85α monoclonal antibody to assay the levels of the PI-3K protein. As shown in Fig. 3*C*, PI-3K enzyme remains exclusively in the cytosolic fraction in equivalent amounts, independent of the diabetic condition. This suggests that intrinsic signaling events may be attributing to the observed loss of enzyme activity.

Effect of ARI on the activation of MAPK signaling cascades in lenses of diabetic rats. During the 8-week duration of diabetes inducement, some rats were concurrently fed AL01576 (an ARI) to evaluate the possible involvement of the polyol pathway in the above observed alterations in lens MAPK signaling. As shown in Fig. 4, administration of AL01576 substantially normalizes the altered vitreal bFGF levels. The phosphorylation status of the members of the MAPK superfamily, including the mitogen-activated Raf-1-MEK-ERK cascade as well as the stress-associated MAPKs, the PAK-p38, and the SAPK/JNK cascades (31), known to present in the lens (16), was analyzed and is summarized in Fig. 5. AL01576 treatment (*lane 3*) essentially normalized the changes in lenses of diabetic rats (*lane 2*) to the basal levels found in the normal control lenses (*lane 1*), including P-Raf-1 suppression (Fig. 5*A*) and the elevations of P-MEK (Fig. 5*B*), P-ERK (Fig. 5*C*), P-PAK2 (Fig. 5*E*), P-p38 (Fig. 5*F*), and P-JNK (Fig. 5*G*). Total ERK remained unchanged (Fig. 5*D*).

Effect of ARI treatment on the activity of PI-3K in the lenses of diabetic rats. Figure 6*A* represents a thin-layer chromatographic analysis of the enzyme activity of PI-3K in

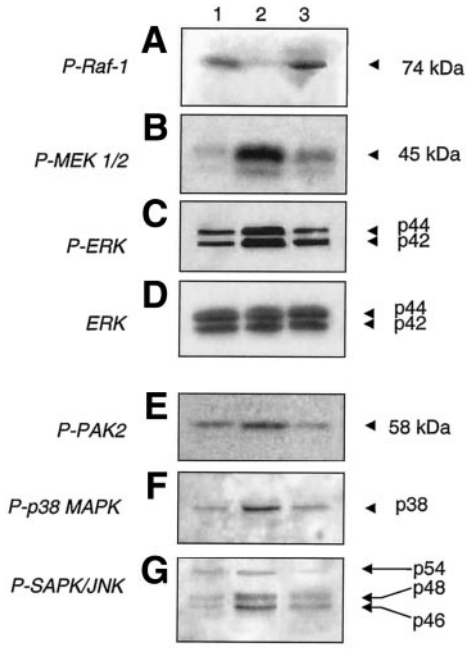

FIG. 5. Effect of ARI treatment on the members of the lens MAPK superfamily in 8-week diabetic rats. Diabetes-induced alterations in the phosphorylation status of the members of the MAPK superfamily were normalized in lenses from AL01576-treated diabetic rats. Data represent three separate analyses using samples from different studies. *A*: Immunoblot analysis of phospho-Raf-1. *B*: Immunoblot analysis of phospho-MEK1/2. *C*: Immunoblot analysis of phosho-ERK. *D*: Immunoblot analysis of total ERK. *E*: Immunoblot analysis of phosho-PAK2. *F*: Immunoblot analysis of phosho-p38 MAPK. *G*: Immunoblot analysis of phosho-SAPK/JNK. *Lane 1* represents a lens from a normal age-matched control rat; *lane 2* represents a lens from an 8-week diabetic rat; *lane 3* represents a lens from an 8-week AL01576-treated diabetic rat.

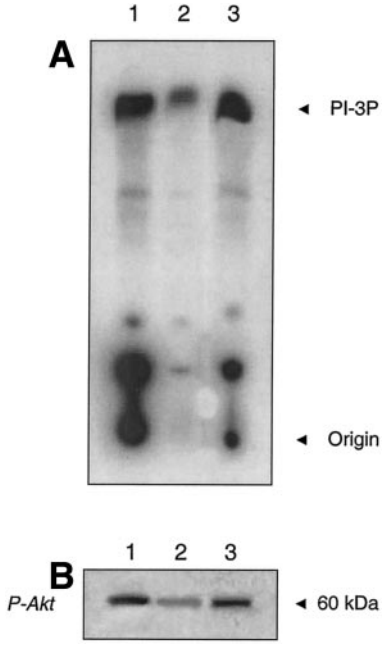

FIG. 6. The diabetes-induced suppression in the activity of lens PI-3K was normalized in lenses from AL01576-treated diabetic rats. Data represent three separate analyses using samples from different studies. *A*: The effect of ARI treatment on the activity of PI-3K in 8-week diabetic rat lens, as assayed by thin-layer chromatography. Bands near the origin represent unincorporated [γ-^{32}P]ATP. *B*: In these same tissue samples, Western immunoblot analysis of the phosphorylation status of Akt, a PI-3K target, also showed a comparable response to the measured veritable activity of PI-3K. *Lane 1* represents a lens from an age-matched control rat; *lane 2* represents a lens from an 8-week diabetic rat; *lane 3* represents a lens from an AL01576-treated 8-week diabetic rat.

the same tissue samples used for Fig. 5. The diabetic condition (*lane 2*) suppressed the activity of PI-3K relative to the control (*lane 1*) but was completely normalized with ALO1576 treatment (*lane 3*). P-Akt also showed a normalized profile by ALO1576 treatment, similar to that of PI-3K (Fig. 6*B*).

Effect of various organ culture conditions on the signal transduction parameters in the normal lens.

Effect of diabetic rat vitreous on the activation of the MAPK superfamily in normal rat lens. To characterize whether the vitreous is associated with the above observed alterations in the signaling components in the lens of diabetic rat, we cultured normal rat lenses for 24 h in the presence of pooled vitreous (20%) from 8-week diabetic rats, using the vitreous from age-matched normal rats as controls. Each study was conducted using the left lens (*L*) for the control group and the contralateral right lens (*R*) for the experimental group. Total ERK (Fig. 7*B*) was measured to ensure that equal amounts of protein were loaded on the gel.

The immunoblot data of Fig. 7*A* shows that when a normal lens was incubated in the presence of 20% vitreous from diabetic rats, the level of ERK phosphorylation (*lane 2*) was elevated as compared with the lens cultured in normal control vitreous (*lane 1*). AL01576 (1×10^{-5} mol/l) showed no effect when added to both of the vitreous-containing media (*lanes 3* and *4*). However, when the lens was exposed to vitreous taken from diabetic rats that were treated by AL01576 in vivo, the diabetes-associated alteration in P-ERK was normalized (*lane 6*) as compared with its control (*lane 5*). When the vitreous from diabetic rats was pretreated with anti-bFGF antibody, its stimulating effect for P-ERK was diminished (*lane 8*). Thus, the P-ERK signal was the same as the control (*lane 7*). The phosphorylation status of p38 (Fig. 7*C*) and SAPK/JNK (Fig. 7*D*) responded similarly to the culture conditions as observed in Fig. 7*A*.

Effect of high galactose and bFGF on the activation of the MAPK superfamily in normal rat lens. To confirm the above findings, we also cultured lenses for 24 h under high galactose (30 mmol/l)-containing medium to mimic the osmotic stress observed in diabetic conditions while lenses cultured in 30 mmol/l fructose-containing medium were used as controls (32,33). As shown in Fig. 7*A*, when a lens was exposed to high galactose (*lane 10*), the P-ERK was elevated above the control (*lane 9*), similar to the case between *lanes 2* and *1*. This stimulation was normalized when the lens was exposed to high galactose medium in the presence of AL01576 (*lane 12*) in which the P-ERK intensity was the same as the control (*lane 11*, normal medium with AL01576). When the lens was exposed to 10 ng/ml bFGF (*lane 13*), the intensity of P-ERK was extensively enhanced (compared with *lane 9*). This stimulation was completely eradicated when the bFGF-containing medium was pretreated with 5 μg/ml anti-bFGF neutralizing monoclonal antibody (*lane 14*).

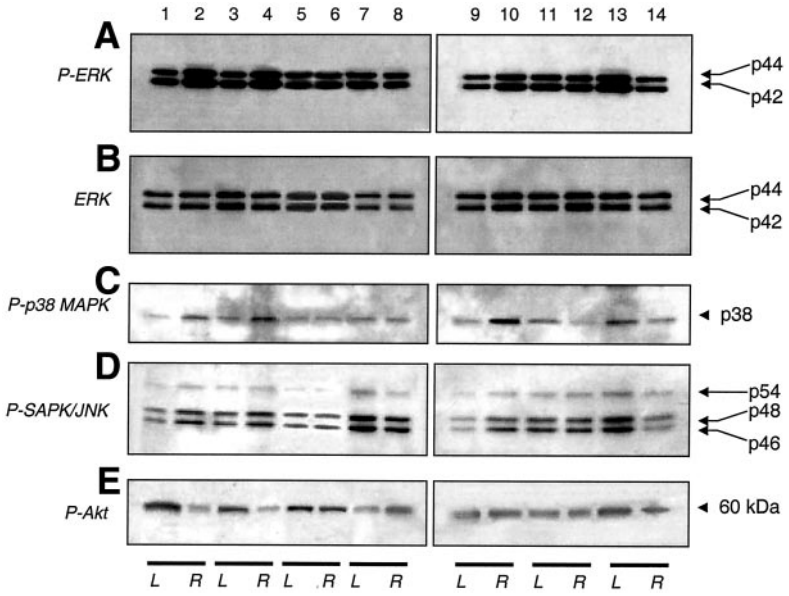

FIG. 7. Immunoblot analysis of the effects of normal control and diabetic vitreous on the signaling parameters of members of the MAPK superfamily and on P-Akt (PI-3K activity) in cultured normal whole rat lens (see text for details). Data represent three separate analyses using samples from different studies. L, contralateral left lens; R, contralateral right lens, for each pair of samples exposed to the given culture conditions. *A*: Immunoblot analysis of phospho-ERK. *B*: Immunoblot analysis of total ERK. *C*: Immunoblot analysis of phospho-p38 MAPK. *D*: Immunoblot analysis of phospho-SAPK/JNK. *E*: Immunoblot analysis of phospho-Akt. Vitreous-exposed culture conditions: *lane 1*, 20% control vitreous (8-week age-matched control rat); *lane 2*, 20% diabetic vitreous from an 8-week diabetic rat; *lane 3*, 20% control vitreous (8-week age-matched control rat) with the addition of 1×10^{-5} mol/l AL01576; *lane 4*, 20% diabetic vitreous from an 8-week diabetic rat with the addition of 1×10^{-5} mol/l AL01576; *lane 5* represents normal control vitreous and was compared with the contralateral sample in *lane 6*, exposed to vitreous from an AL01576-treated 8-week diabetic rat; *lane 7*, 20% control vitreous (8-week age-matched control rat) with the addition of 5 μg/ml anti-bFGF neutralizing monoclonal antibody; *lane 8*, 20% diabetic vitreous from an 8-week diabetic rat with the addition of 5 μg/ml anti-bFGF neutralizing monoclonal antibody. Control/simulated diabetic culture conditions: *lane 9*, normal control medium-199; *lane 10*, 30 mmol/l galactose-containing medium; *lane 11*, normal control medium-199 with the addition of 1×10^{-5} M AL01576; *lane 12*, 30 mmol/l galactose-containing medium with the addition of 1×10^{-5} mol/l AL01576; *lane 13*, 10 ng/ml bFGF-containing medium; *lane 14*, 10 ng/ml bFGF-containing medium with the addition of 5 μg/ml anti-bFGF neutralizing monoclonal antibody.

The stimulation profiles of other signaling components (Fig. 7*C* and *D*, *lanes 9–14*) also showed similar patterns as P-ERK, although P-p38 (Fig. 7*C*) was more responsive to high galactose (*lane 10*) than bFGF treatment (*lane 13*), whereas P-SAPK/JNK (Fig. 7*D*) responded more readily to bFGF treatment (*lane 13*) than to the treatment with high galactose (*lane 10*).

Effect of diabetic rat vitreous on the activation status of Akt (PI-3K activity) in normal rat lens. It has been shown that the phosphorylation status of one of the PI-3K targets, Akt, can serve as an indicator for the activity of PI-3K (34). We probed for P-Akt using the same samples analyzed in Fig. 7*A–D* and summarized the results in Fig. 7*E*. The response of P-Akt to medium containing vitreous correlated very well with ERK, p38, and JNK, except in most cases it was suppressed. High galactose- or bFGF-containing medium, however, seemed to enhance P-Akt slightly (*lanes 9–14*).

DISCUSSION

Results from this study illustrate a distinct disruption in the lens signaling cascades on multiple levels within the MAPK and PI-3K signaling components in response to the diabetic condition. We speculate that an elevated level of bFGF found in the vitreous of diabetic animals is likely one of the possible causes for such aberrant signaling parameters. Hyperglycemia is known to generate osmotic stress initiated from polyol accumulation in the cells, which induces membrane leakage (6,35). Under these conditions, some of the growth factors may have leaked into the vitreous and influenced the stimulation of particular signaling systems in the lens. Other causes may involve the osmotic stress and associated attenuation of redox status in the lens (31,36,37). Although we have not measured the lens sorbitol levels, the experimental protocol used in this study was similar to that of Kador et al. (38) in which sorbitol in the untreated diabetic rats (7 weeks) reached 0.27 μmol per lens and no sorbitol was detected in the clear lenses of the nondiabetic rats. The lenses from the same untreated diabetic rats showed 70–80% depletion of glutathione (GSH) levels. We believe that the lenses from untreated diabetic rats in our current study would have accumulated a high level of sorbitol with concurrent loss in GSH concentration.

bFGF may originate from retinal cells, such as the endothelial cells in the vasculature, and efflux into the vitreous humor under the diabetic condition. However, the elevated vitreal bFGF could be normalized (Fig. 4) when the diabetic animal was simultaneously treated with AL01576, which has also been shown to normalize the polyol pathway, prevent GSH loss, maintain ATP level, and eradicate the complications induced by diabetes (32,33, 38,39). Therefore, lenses from AL01576-treated diabetic rats showed signaling patterns comparable to those found in lenses from normal control rats (Figs. 5 and 6). The involvement of excess vitreal bFGF in altering these signaling components was validated from four findings in our study. First, the consistent and substantial elevation in activated MAPKs in the lenses of diabetic rats correlated with the vitreal bFGF concentrations and was dependent on the duration of diabetes (Figs. 1, 2, and 5). Second, sequestering active bFGF in the diabetic vitreous using a neutralizing antibody resulted in near-normal levels of phosphorylation of lens signaling components (Fig. 7, *lane 8*). Third, vitreous from AL01576-treated diabetic rats could no longer induce signaling changes in a normal lens (Fig. 7, *lane 6*). Fourth, AL01576 was unable to normalize signaling alterations induced in the lens under culture

conditions using vitreous from diabetic rats (Fig. 7, *lanes 3* and *4*). The effect of exogenous bFGF on the MAPK signaling components in a normal lens is clearly shown in the organ culture study (Fig. 7, *lane 13* compared with *lane 9*), similar to the earlier findings (16). The same figure also shows that sequestering bFGF by its antibody can normalize this alteration (*lane 14* versus *lane 13*).

The evidence that osmotic stress alone can alter the lens signaling of MAPKs is demonstrated in Fig. 7. Similar findings were also observed by Zatechka and Lou (16,17). AL01576 at a concentration (1×10^{-5} mol/l), which is known to eradicate osmotic stress and its related effects (32,33), could normalize the alterations induced by the presence of high galactose in the culture medium (Fig. 7, *lane 12* versus *lane 11*). The presence of 30 mmol/l fructose in the culture medium served as a suitable control for the group of galactose-induced osmotic stress (Fig. 7, *lane 1* versus *lane 2*) as fructose does not cause polyol accumulation and thus does not produce polyol-induced osmotic stress in the lens. Although this study did not focus on the element of oxidative stress in the lens of diabetic rat, we have reported that H_2O_2 alone can activate MAPKs (40,41). Purves et al. (42) also found that oxidative stress results in MAPK activation. The potential role of oxidative stress in the altered MAPK signaling in the lens of diabetic rat is not to be ignored.

Enhanced ERK activation may have deleterious effects on lens osmoregulatory mechanisms. Gong et al. (43) recently generated a constitutively active mutant of MEK in transgenic mice that substantially activates the corresponding ERK signal. The phenotype showed macrophthalmia, lens hydration, and opacity. They also observed upregulated GLUT-1 expression and high glucose levels in the lens. The transgenic mutant did not affect the other MAPKs or the membrane-associated lens proteins, including connexin, MP26, and GLUT-3. In certain cell types, GLUT-1 expression is regulated by the activation of the ERK pathway (44,45). Therefore, we speculate that the combined effects of excessive bFGF in the vitreous and the high level of ERK-mediated GLUT-1 expression result in an influx of glucose that is already high in the lens as a result of hyperglycemia, allowing the accumulation of sorbitol and enhanced osmotic perturbation and its associated occurrence of oxidative stress. This perpetual event would be detrimental to the health of a lens and is likely to contribute to the eventual cataract formation.

An additional consistent observation was noted at the level of PI-3K signaling, where a diabetes-induced suppression in the activity of PI-3K was observed and was dependent on the duration of the disease (Fig. 3*A* compared with *B*). The activated Raf (P-Raf), a key component of the ERK signaling pathway, was also diminished (Fig. 5*A*). The loss in P-Raf signal, which is in agreement with the finding of Takemoto (46), is likely linked to the weakened PI-3K because it has been shown that Raf is a critical target of PI-3K regulation (47). Weakened PI-3K and Raf activities could be a result of the cellular regulation of signal redundancy. Downregulating PI-3K may be necessary to avoid the imbalance of cell calcium homeostasis because the other regulator, phosphoinositide biosynthesis, was found to be enhanced under diabetic conditions (20,21).

The phenomenon that diabetes induced activations of MEK and ERK while simultaneously suppressing the activity of the upstream Raf signal is intriguing. The same lens also showed strong activation of p38 (Fig. 5*F*) and its upstream regulator, PAK (Fig. 5*E*). We speculate that PAK may be responsible for the activation of MEK (Fig. 5*B*) independent of Raf via cross-talk communication from an active stress-associated MAPK cascade. Several laboratories (48,49), including ours (17), have established cross-talk interaction at this level.

Studies in Tomlinson's laboratory (50) recently demonstrated that p38 was the major transducer of glucose in the cultured adult rat sensory neurons and also in the dorsal root ganglia of diabetic rat. Administering a p38 inhibitor in culture normalized such alteration. The same laboratory recently further demonstrated that feeding p38 inhibitor during the last 5 weeks after 7 weeks of untreated diabetes partially normalized sensory nerve conduction deficit, whereas ARI feeding blocked nerve dysfunction completely (50). This suggests that the PAK-p38 pathway is one of the main signaling defects causing pathophysiological consequences in diabetes. Our observations of p38 and PAK activation agree with these reports.

PI-3K is a known survival factor that generates antiapoptotic signals to resist stress (51). Conversely, activation of the SAPK/JNK and p38 MAPKs is associated with promotion of apoptosis (52). Inducement of lens epithelial cell apoptosis may be a mechanism to impede excessive cell proliferation in response to the enhanced ERK signal under diabetic condition. Progression of apoptosis is facilitated via caspase-mediated proteolysis of specific signaling components. Raf-1 is known to be one of the target components for suppressing the antiapoptotic ERK signals (53). This could provide an alternative explanation for the loss of P-Raf under diabetic condition in the present study. Caspase-mediated cleavage also activates MEKK and PAK to favor a proapoptotic response (54) and can subsequently activate the proapoptotic signals of the SAPK/JNK and p38 pathways. This may further explain the observed increase in P-PAK under diabetic conditions in this study.

In conclusion, we presented evidence in this study that diabetic condition induces signaling alterations in the lens. These alterations may be a secondary factor involved in the diabetic complication of the lens following the primary factors of increased glucose and glucose-derived cell stresses in both osmosis and oxidation. Our study also supports the hypothesis that the PAK-p38 cascade plays a key role in the regulation of lens cell signaling under diabetic conditions.

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