ERK1/2 Signaling Pathway in the Release of VEGF from Müller Cells in Diabetes

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PURPOSE. Diabetic retinopathy (DR) is one of the most serious complications of diabetes and has become a major blinding eye disease, but its treatment remains unsatisfactory. The ERK1/2 signaling pathway has been shown to participate in regulating secretion of VEGF in DR from our previous studies. The role of VEGF in the development of DR provides a target for treatment. Our present research focuses on Müller cells, a major source of VEGF secretion, to investigate the role of ERK1/2 signaling pathway on regulation of VEGF release in diabetes.

METHODS. Immunofluorescence was used to observe the ERK1/2 phosphorylation activity on early diabetic rat retinal Müller cells. Müller cells were stimulated by high glucose in vitro. Western blot and immunohistochemistry were used to determine ERK1/2 signaling pathway expression and phosphorylation. AP-1 DNA binding activity status was monitored by electrophoretic mobility shift assay (EMSA). ELISA and PCR monitored VEGF secretion. Inhibition of ERK1/2 phosphorylation with U0126 was observed for changes in VEGF secretion.

RESULTS. Phos-ERK1/2 was expressed on Müller cells early in diabetes. In vitro high glucose stimulation of Müller cells increased VEGF secretion with a peak at 24 hours. An ERK1/2 specific inhibitor, U0126, stopped the phosphorylation of ERK1/2, lowered AP-1 DNA binding activity, and reduced Müller cells secretion of VEGF under high glucose conditions.

CONCLUSIONS. ERK1/2 signaling pathway has some role in regulating Müller cells secretion of VEGF in DR. Targeting the ERK1/2 signaling pathway in Müller cells through intervention of the upstream signaling pathway or nuclear transcription factors of VEGF secretion could be a type of anti-VEGF treatment for DR. (Invest Ophthalmol Vis Sci. 2012;53:3481-3489) DOI:10.1167/iovs.11-9076

Diabetic retinopathy (DR) remains a leading cause of visual impairment among working-age individuals in developed countries, and can reach its more advanced stages in almost total absence of symptoms. The prevalence of DR is approximately 70% in patients with type 1 diabetes and 40% among those with type 2.

VEGF is a potent biogenic permeability factor that is elevated in the eyes of people with DR and in animal models of diabetes, and is sufficient to increase vascular proliferation and permeability. The main implication of VEGF overexpression is that it could mediate the compromised barrier properties of diabetic retinal vessels. VEGF is a major pathogenic factor and a therapeutic target for DR. However, the cellular mechanisms of VEGF remain virtually uninvestigated, making it difficult to design cellular target-based therapeutics for DR.

In the retina, VEGF is expressed mainly in the Müller cells, retinal pigment epithelium (RPE), endothelial cells, astrocytes, and ganglion cells. Among these, we believe that Müller cells may become key to treating DR for the following reasons. First, Müller cells are specialized radial glial cells that span the entire thickness of the retina, and ensheath all somata and processes of retinal neurons. Because the Müller cells are the dominant type of macroglial cells in the retina, their large role in supporting the neurons and their functions can be a possible cause for dysfunction of vascular and neural cells. Second, Müller cells are a primary target of proinflammatory signals that mediate retinal inflammation and vascular leakage in DR. Third, although microvascular cells have long been considered primary targets of chronic hyperglycemia in DR, early biochemical, physiologic, and morphologic alterations have been observed in nonvascular cells, including Müller cells, which have the earliest and most important roles. Fourth, and most noteworthy, Müller cells are activated in response to virtually every pathologic alteration of the retina—a reaction termed Müller cell gliosis. Occurring shortly after injury, gliosis is thought to represent a cellular attempt to protect the neural tissue from further damage by release of neurotrophic factors and antioxidants. Some of the factors released by activated Müller cells, such as VEGF, may have neuroprotective and detrimental effects, as VEGF induces vascular leakage and neovascularization, which may exacerbate disease progression. The importance of Müller cells in physiologic and pathologic conditions of the retina can be used as an entry point for treatment of DR.

Several signaling pathways take part in VEGF release in DR. Here, we focus on the ERK1/2 signaling pathway, because much of oncoma research demonstrates its role in VEGF release. Although DR does not fall under the scope of oncoma, the biological behavior of DR, including proliferation and vascular neogenesis, is similar to that of oncoma. The activation of ERK1/2 regulates proliferation and protein synthesis by stimulating transcription factors c-fos and c-jun, which induce the activation of activator protein-1 (AP-1).

Notably, the AP-1 binding consensus sequence is contained within the promoter region of the VEGF gene. This link is significant because AP-1 binding activity is critical for VEGF gene induction. Our earlier research
demonstrated that the ERK1/2 signaling pathway was activated rapidly 1 week after inducing diabetes. AP-1, the downstream transcription factor of ERK1/2, also was activated, and VEGF levels increased in a similar trend. U0126, an inhibitor of ERK1/2, down regulated VEGF expression in addition to ERK1/2 and AP-1 activity. Given the involvement of ERK1/2 signaling pathway in VEGF release in diabetic rat retina, it may be a potential therapeutic target of DR. We investigated whether the ERK1/2 pathway is activated specifically on Müller cells and whether its activation controls Müller cell release of VEGF in DR. If the ERK1/2 pathway is found to affect Müller cells’ release of VEGF, it may help us demonstrate that ERK1/2 and Müller cells are potential therapeutic targets of DR.

**Materials and Methods**

**Animals and Experimental Diabetes Induction**

Diabetes was induced in SD rats (250 ± 20 g, male) by administration of streptozotocin (60 mg/kg body weight) (Sigma, St. Louis, MO). Three days after induction of diabetes, the rats’ blood glucose levels were above 16.0 mM. Age-matched normal rats served as the control. Each group had 8 or more rats. After 1 or 8 weeks of diabetes, the rats were euthanized by an overdose of pentobarbital and the eyes were removed. Five eyes in every group were fixed for immunohistochemistry to observe for ERK1/2 activation in Müller cells. Treatment of the animals conformed to the National Institute of Health Principles of Laboratory Animal Care, the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research, and local institutional guidelines.

**Primary Müller Cell Culture, High Glucose Stimulation, and U0126 Treatment**

Müller cells were prepared from Sprague-Dawley rats of postnatal days 1–3. Briefly, isolated retinas were digested with 0.25% trypsin in 10% fetal calf serum and PBS for 45 minutes. Dissociated retinal cells then were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5.5 mM glucose and 20% fetal bovine serum (FBS). Removal of aggregates and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5.5 mM glucose and 20% FBS. After 15 minutes on ice, the cells were vortexed in the presence of 0.5% Nonidet P-40. The nuclear pellet then was collected by centrifugation and extracted with buffer B (20 mM HEPES [pH 7.9], 0.5% Nonidet P-40, 0.1 mM EGTA, 1 mM dithiothreitol [DTT] and 0.5 mM PMSF). For detection of the level of the indicated proteins, cultures were washed in D-hanks, scraped off the culture bottles with 50–100 μL sample buffer, and boiled for 5 minutes. Protein concentrations were determined by the Bradford method, using BSA as a standard. Samples of supernatants were mixed 1:1 with 2× sample buffer and heated to 95°C for 10 minutes. SDS-PAGE was performed in 12% gel with 5% stacking gel, and with 0.25 M Tris-glycine (pH 8.3) as the electrolyte buffer. Protein bands were electroblotted for 2 hours onto polyvinylidene difluoride (PVDF) membranes for incubation with antibodies. Nonspecific binding sites were blocked for 1 hour at room temperature with 5% nonfat dried milk powder and 0.05% Tween-20 in TBS (pH 7.6). The membranes then were incubated separately with dilutions of the total-ERK (1:1000) and phos-ERK (1:1000) antibodies (Cell Signaling Technology) in blocking solution overnight at 4°C. Membranes then were probed with the appropriate secondary antibodies conjugated with HRP (1:4000) at room temperature for 1 hour. For loading control, the membrane was incubated with monoclonal mouse anti-actin (1:5000) and revealed as just explained. Precision prestained standards were used as molecular weight markers. Signals were visualized using an ECL-Plus Western blot detection kit. Membranes were scanned with a digital scanner to quantify band optical density. Each experiment was repeated three times to confirm the consistency of the results.

**Western Blot Analysis**

Cells from the different treatment groups were harvested to study the phosphorylation of ERK1/2 by Western blot. Müller cells were grown in 75 mm plates, treated as described above. Afterwards, cells were resuspended and placed in homogenizing buffer (50 mM TrisHCl [pH 7.4], 1 mM phenylmethylsulfonil fluoride [PMSF] and 5 mM EGTA). For detection of the level of the indicated proteins, cultures were washed in D-hanks, scraped off the culture bottles with 50–100 μL sample buffer, and boiled for 5 minutes. Protein concentrations were determined by the Bradford method, using BSA as a standard. Samples of supernatants were mixed 1:1 with 2× sample buffer and heated to 95°C for 10 minutes. SDS-PAGE was performed in 12% gel with 5% stacking gel, and with 0.25 M Tris-glycine (pH 8.3) as the electrolyte buffer. Protein bands were electroblotted for 2 hours onto polyvinylidene difluoride (PVDF) membranes for incubation with antibodies. Nonspecific binding sites were blocked for 1 hour at room temperature with 5% nonfat dried milk powder and 0.05% Tween-20 in TBS (pH 7.6). The membranes then were incubated separately with dilutions of the total-ERK (1:1000) and phos-ERK (1:1000) antibodies (Cell Signaling Technology) in blocking solution overnight at 4°C. Membranes then were probed with the appropriate secondary antibodies conjugated with HRP (1:4000) at room temperature for 1 hour. For loading control, the membrane was incubated with monoclonal mouse anti-actin (1:5000) and revealed as just explained. Precision prestained standards were used as molecular weight markers. Signals were visualized using an ECL-Plus Western blot detection kit. Membranes were scanned with a digital scanner to quantify band optical density. Each experiment was repeated three times to confirm the consistency of the results.

**Immunofluorescence**

**Retina.** Eyes were fixed in methacarn solution (60% methanol, 30% chloroform, and 10% acetic acid). Paraffin sections 5 μ thick were cut and used for staining. Sections were immersed briefly in xylene, hydrated through graded ethanol solutions, and incubated in 3% hydrogen peroxide for 5 minutes to eliminate intrinsic peroxidase activity. Sections then were incubated for 10 minutes in 10% normal goat serum (NGS) in Tris-buffered saline (TBS; 50 mM Tris-HCL, 150 mM NaCl, pH 7.6) to reduce nonspecific binding. After rinsing briefly with 1% NGS/TBS, the sections were incubated overnight at 4°C with mouse anti-phos-ERK1/2 (1:200; Cell Signaling Technology) and rabbit anti-GS (1:1000; Sigma) to show Müller cells. The sections then were incubated in CY3-conjugated anti-mouse or FITC-conjugated anti-rabbit secondary antibodies (Beyotime, Haimen, China) for 30 minutes. Slides were examined by confocal microscopy (Leica TCS SP2, Wetzlar, Germany).

The specificity of the reaction was confirmed by omitting the primary antibody and using appropriate isotype-matched antibodies as negative controls. Images were collected from five sections per rat.

**Cell.** The fourth-passage cells were treated in either 5.5 or 30 mM glucose for 24 hours. Then, the cells were fixed in 4% paraformaldehyde in 1.01 M phosphate-buffered saline for 15–20 minutes. Cells were permeabilized with 0.3% Triton X-100 for 30 minutes and 5% goat serum for 40–45 minutes, and incubated in phos-ERK1/2 antibodies overnight at 4°C (1:200). Then, the cells were incubated in FITC-conjugated secondary antibodies for 30 minutes. Nuclei were stained using propidium iodide (PI). Slides were examined by confocal microscopy (Leica TCS SP2, Germany).

**Electrophoretic Mobility Shift Assay**

Cultured cells were collected by centrifugation, washed, and suspended in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT] and 0.5 mM PMSF). After 15 minutes on ice, the cells were vortexed in the presence of 0.5% Nonidet P-40. The nuclear pellet then was collected by centrifugation and extracted with buffer B (20 mM HEPES [pH 7.9], 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF) for 15 minutes at 4°C. All extranuclear supernatant and nuclear extracts were stored at −80°C until use. The protein concentrations were determined with a Bradford protein assay kit. For electrophoretic mobility shift assay (EMSA), 10 μL of a mixture of AP-1 (5′- CGCTTTGATGACTCAGCCGGAA-3′) oligonucleotide, T4 polynucleotide kinase 10× buffer, (γ32P)ATP, nuclear-free water, and T4 polynucleotide kinase were incubated for 30 minutes at 37°C. The reaction was stopped by adding 1 μL EDTA (0.5 M). After adding 89 μL Tris-EDTA (TE) buffer (10 mM Tris-HCl [pH 8.0], and 1 mM EDTA), unincorporated nucleotides were removed from the DNA probe by chromatography through a G-25 spin column. The nuclear extract and gel shift binding 5× buffer (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM EDTA).
DTT, 250 mM NaCl, 50 mM Tris-HCl [pH 7.5], and 0.25 mg/mL poly [dI-dC]) were incubated at room temperature for 10 minutes. After incubation, 1 µL of 10× gel loading buffer was added to each reaction. Reaction mixtures were electrophoresed on 6% polyacrylamide gels. To perform the competition assay, a 100-fold excess of unlabeled competitor primer was added to the EMSA reaction mixture. Dried gels were exposed to an x-ray film. The images were scanned and analyzed using the Bio-Imaging analyzer. The relative AP-1 activity was determined based on the intensity of the shifted bands measured.

ELISA

Müller cells were seeded onto 24-well culture plates at $5 \times 10^4$ cells per well, and incubated for 48 hours in medium with 20% FBS. The cells were treated with U0126 and then stimulated by 30 mM glucose for 8 to 48 hours as described above in medium containing 2% FBS. The conditioned medium was collected and centrifuged at 15,000 g for 10 minutes, and the VEGF concentration in the supernatants was assayed using a VEGF ELISA kit (Wuhan Boster Bio-Engineering Co., Ltd., Wuhan, China) following the manufacturer’s instructions. The experiment was performed in triplicate.

Real-Time PCR

The Müller cells were cultured as above and treated with U0126, then subjected to 5 or 30 mM glucose for 8 to 48 hours. After stimulation by glucose, RNA was extracted and reverse transcribed. The levels of β-actin and VEGF-A mRNA were analyzed by real-time quantitative PCR. Each mRNA level was normalized to β-actin mRNA. Specific primers were β-actin (GenBank accession number NM03114; forward primer 5’-TGACGTTGGACATCCGCAAG-3’; reverse primer 5’-CTGGAAGGTGGAGCAGCGAGG-3’), and VEGF-A (GenBank accession number NM031836; forward primer 5’-CCGCGACGCAGGTAAATGTTCC-3’; reverse primer 5’-GACGGTGACGATTGGTGTTGT-3’). Real-time quantitative PCR (RT-PCR) was completed in a 20 µL reaction using SYBR Green reagent. The relative changes in gene expression were analyzed using the 2(-ΔΔCT) method. Specificity of RT-PCR was checked by analyzing melting curves and by gel electrophoresis of the amplicons.

Statistical Analysis

All values are shown as mean ± SD. Differences between two groups were determined with paired or unpaired Student’s t-tests. ANOVA was
used for multiple comparisons. P < 0.05 was considered significant. Each experiment was repeated at least three times.

RESULTS

Phos-ERK1/2 Expressed on Müller Cells Early in Diabetes

Anti-GS and anti-phos-ERK1/2 antibodies were used as two markers of activated ERK1/2 to demonstrate activation in Müller cells early in diabetic retina. Double immunofluorescence showed that after only 1 and 8 weeks of diabetes, phos-ERK1/2-labeled cells were co-localized with GS antigen. The results showed that diabetes led to activation of ERK1/2 within retinal Müller cells (Fig. 1).

In Vitro Activation of ERK1/2 Signaling Pathway in Müller Cells Stimulated by High Glucose (Immunofluorescence and Western Blot)

Müller cells were treated with 30 mM glucose for 24 hours to determine whether the ERK1/2 signaling pathway is activated after high glucose stimulation. Immunofluorescence results showed increased phos-ERK1/2 expression in the ERK1/2-labeled cells were co-localized with GS antigen. The results showed that diabetes led to activation of ERK1/2 within retinal Müller cells (Fig. 1).

Figure 2. (A–C) Immunofluorescence results showed that few phos-ERK1/2 (green) are present in the propidium iodide-labeled nuclei (red) of normal control group cultured Müller cells. (D–F) Proliferation of Müller cells and increased phos-ERK1/2 (green) expression after 24 hours of high glucose stimulation. (G) An enlarged image of the white rectangle in (F).

Figure 3. (A, B) Western blot results showed that 30 mM high glucose stimulated Müller cells increased phos-ERK1/2 protein expression in contrast to the control group. The increases corresponded to increased glucose stimulation time (n = 3, P < 0.05), with a peak of phos-ERK1/2 at 48 hours of glucose stimulation. (C, D) 30 mM mannitol had no effect on the phosphorylation of ERK1/2, compared to 30 mM glucose (n = 3, *P > 0.05).
nuclei of high glucose stimulated Müller cells after 24 hours (Fig. 2).

Western blot results showed that longer durations of 30 mM high glucose stimulation led to increased phospho-ERK1/2 protein expression, with peak expression at 48 hours of stimulation ($n = 3$, $P < 0.05$). The ERK1 and ERK2 were not significantly different among each group. We used 30 mM mannitol as an osmotic control and found no effect on phosphorylation ($n = 3$, $*P > 0.05$, Fig. 3).

**FIGURE 4.** (A, B) EMSA results for AP-1 DNA binding activity. Normal controls showed no AP-1 activation. High glucose stimulated groups showed increased binding of the AP-1 consensus oligonucleotide, visualized as a distinct band of labeling ($n = 3$, $P < 0.01$). The activity of AP-1 DNA-binding increased as the stimulation time was prolonged. (C, D) 30 mM mannitol stimulated cells had no effect on AP-1 DNA-binding activity compared to the 30 mM glucose stimulated cells ($n = 5$, $*P > 0.05$).

**FIGURE 5.** ELISA results of VEGF protein secreted into the culture medium after 8–48 hours of stimulation with 30 mM glucose. In each case, high glucose elicited a relatively small, but significant increase in VEGF output of culture Müller cells. The peak of release occurred at 24 hours after stimulation, with VEGF levels significantly higher than the control group ($n = 5$, $P < 0.05$).

**FIGURE 6.** VEGF mRNA expression increased in high glucose groups compared to the normal group, and peaked at 24 hours. After 24 hours the VEGF mRNA expression decreased, but remained higher than the normal group ($n = 3$, $P < 0.05$).
To determine whether AP-1 activity of Müller cells was induced by high glucose, nuclear protein extracts from culture cells were analyzed by EMSA at different time points after stimulation. Figure 4 shows the results of an EMSA for AP-1 of the VEGF promoter. Normal controls showed no AP-1 activation. High glucose stimulated groups showed increased binding of the AP-1 consensus oligonucleotide, visualized as a distinct band of labeling (n = 3, P < 0.01). The activity of AP-1 DNA-binding increased with prolonged stimulation time. Mannitol (30 mM) stimulated cells had no effect on AP-1 DNA-binding activity (n = 3, P > 0.05, Fig. 4).

**In Vitro Müller Cells Increase VEGF Release (ELISA and PCR) after High Glucose Stimulation**

**ELISA.** VEGF protein secretion was measured by ELISA after 8 to 48 hours of stimulation with 30 mM glucose in culture media containing 2% FBS. In each case, high glucose exposure elicited a relatively small, but significant increase in VEGF output of cultured Müller cells. The peak of release occurred at 24 hours after stimulation, with VEGF levels significantly higher than in the control group (n = 5, P < 0.05).

**Real-Time PCR.** RT-PCR results showed that VEGF mRNA expression increased in high glucose groups compared to the normal group, and peaked at 24 hours. After 24 hours the VEGF mRNA expression decreased, but remained higher than in the normal group (n = 3, P < 0.05, Fig. 6).

**ERK1/2 Inhibitor U0126 Inhibits the Activity of AP-1 DNA Binding and Down Regulates VEGF Release after High Glucose Stimulation**

Müller cells were treated with low (0.01 mM) and high (0.02 mM) dosage U0126, and then stimulated with high glucose for 24 hours. The treated and untreated groups were subjected to Western blot, EMSA, ELISA, and RT-PCR analyses.

Western blot analysis showed that high glucose activation of ERK1/2 was reduced by the presence of U0126, with a correlation to dose (n = 3, P < 0.05). EMSA analysis showed that AP-1 DNA binding activity of U0126 treated groups decreased compared to the untreated group, with a correlation to dose (n = 3, P < 0.05, Fig. 8).

ELISA analysis showed that the secretion of VEGF decreased in U0126 treatment groups compared to untreated groups, with a correlation to dose (n = 5, P < 0.05, Fig. 9).
Our research shows that in this part of in vitro experiments, cells were treated with low (0.01 mM) and high (0.02 mM) concentrations of U0126, and then stimulated by high glucose for 24 hours. ELISA analysis showed that the secretion of VEGF in U0126 treatment groups decreased compared to untreated groups, with a correlation to dose ($n = 5$, $P < 0.05$).

Real-time PCR analysis showed that VEGF mRNA decreased in U0126 treated groups compared to untreated groups, with a correlation to dose ($n = 3$, $P < 0.05$, Fig. 10). These results showed that blockage of ERK1/2 signaling with the inhibitor U0126 abolished the effect of high glucose not only on activity of ERK1/2 and AP-1, but also of VEGF mRNA and VEGF release, indicating that activation of this pathway is necessary for high glucose to increase VEGF expression.

**DISCUSSION**

Our earlier research indicated that activation of the ERK1/2 pathway has some role in mediating VEGF upregulation in diabetes. Immunofluorescence results from our current study now demonstrate the presence of phosphorylated ERK1/2 on Müller cells of the retina early in diabetes. The result indicates that Müller cells may be a primary cellular target for interrupting the signals that mediate VEGF release in DR. While initial STZ toxicity or the resultant systemic immune response from islet cell apoptosis may be implicated in transient activation of the ERK1/2 pathway, our earlier study showed increases of phos-ERK1/2 in the retina occurring 1 to 12 weeks after diabetes was induced, with peak levels at 8 weeks. Therefore, we believe activation of the ERK1/2 pathway in our model is not likely to be due to a transient immune response.

Our in-vitro experiment results indicate that three early biochemical events—activation of ERK1/2, AP-1 DNA-binding activity, and the increase of VEGF—occur in Müller cells after high glucose stimulation. Significantly decreased AP-1 DNA-binding activity and VEGF expression with use of the ERK1/2 inhibitor U0126 shows that activation of the ERK1/2 pathway has some role in mediating the VEGF upregulation in Müller cells exposed to high glucose.

The ERK1/2 signaling pathway is involved in cell proliferation, differentiation, and transformation. ERK1/2 kinases belong to a large family of serine/threonine kinases that are activated through signaling pathways triggered by multiple extracellular signals. This activation is attained after dual phosphorylation of the protein kinase “activating loop” on threonine and tyrosine residues. Phosphorylation of ERK1/2 kinase and rapid nuclear translocation are essential for stimulating activation of ERK1/2. Our research shows that this occurred on Müller cells of diabetic rat retina. Müller cells show glial activation in response to a variety of pathologic reactions, and demonstrate a number of non-specific responses, including the ERK signaling pathway. This can be seen in early experimental retinal detachment, retinal ischemia-reperfusion, endotoxin-induced uveitis, and glaucoma. Our Western blot and immunofluorescence results confirm that after Müller cells are stimulated with high glucose, ERK1/2 phosphorylation is activated.

VEGF is a key factor in the development of DR. The observation that a significant, but not complete, reduction of VEGF in the retina results in lower detectable retinal degeneration suggests that appropriate doses of anti-VEGF agents may be important in the effective treatment of retinal vascular diseases. Previous studies have shown that VEGF mRNA and protein are expressed by retinal vascular endothelial cells, astrocytes, retinal pigment epithelial cells, Müller cells, and ganglion cells. Research by Bai et al. suggests that the retinal Müller cell-derived VEGF is a major contributor to ischemia-induced retinal vascular leakage, and pre-retinal and intra-retinal neovascularization. Müller cells’ secretion of VEGF is affected by many factors, such as high glucose, hypoxia, and insulin. Our results confirm that exposure to high glucose increases VEGF secretion by Müller cells with detectable increases in VEGF mRNA, and with peak levels at 24 hours. Surprisingly, levels of VEGF mRNA did not increase with greater phos-ERK1/2. The results puzzled us but perhaps can be attributed to the following factors. First, the ERK1/2 signaling pathway is not the only regulator of VEGF secretion. Second, ERK1/2 activation also may activate other pathways or factors that interfere with upregulation of VEGF. VEGF secretion is due to the crosstalk among multiple signaling pathways.

Earlier, we showed that ERK1/2 downstream nuclear transcription factor AP-1 DNA-binding activity appeared enhanced in the diabetic rat model. In this part of in vitro experiments, EMSA results confirmed that AP-1 DNA binding activity increased with high glucose stimulation, and decreased with U0126 treatment before stimulation.

U0126 is a MAPK inhibitor and MEK1/2 inhibitor. U0126 can inhibit selectively MEK1/2, thereby inhibiting MAP kinase (ERK1/2) phosphorylation and activation, and blocking signal transduction into the nucleus. From our in vitro experiments, we observed that U0126 also can inhibit AP-1 dependent gene transcription. Additionally, the partial reduction of VEGF secretion when ERK1/2 signaling pathway is inhibited suggests that the active ERK1/2 signaling pathway may, indeed, be involved in the secretion of VEGF on Müller cells.

The AP-1 EMSA results demonstrated that AP-1 binding activity increased with ERK1/2 phosphorylation, but not with VEGF release. We believe this discrepancy may be due to crosstalk between the signaling pathways that influence AP-1 and VEGF levels.

Interaction of multiple pathways regulates VEGF secretion in DR, and because this interaction has not been studied in...
depth, we do not clearly know the individual role of ERK1/2. Our research results may provide some helpful clues. In our present study, interference with the ERK1/2 signaling pathways was shown to decrease VEGF secretion and inhibit the activation of retina-specific Müller glial cells, which may provide a therapeutic role for DR. However, the use of U0126 or other inhibitors of ERK1/2 pathway for the treatment of DR has not yet been documented. We hope that further research in the mechanism of VEGF secretion and interference in signaling pathways can lead to the future development of anti-VEGF drugs as effective therapeutic strategies for DR.

References


