ERK1/2 Signaling Pathways Involved in VEGF Release in Diabetic Rat Retina

Xiaofeng Ye, Gezhi Xu, Qing Chang, Jiaweng Fan, Zhongcui Sun, Yaowu Qin, and Alice C. Jiang

PURPOSE. Vascular endothelial growth factor (VEGF) is one of the major factors promoting diabetic retinopathy (DR). A better understanding of the signaling pathway in VEGF regulation is of clinical importance to identify more precise therapeutic targets for diabetic retinopathy. The ERK1/2 signaling pathway has been shown to play a key role in some oncoma and hematologic diseases by mediating VEGF release. This research was conducted to determine whether the ERK1/2 signaling pathway also plays a major role in VEGF release in DR development.

METHODS. One hundred Sprague-Dawley (SD) rats were induced to diabetes by streptozotocin (STZ) injection and monitored at several time points (1, 2, 3, 4, 8, and 12 weeks) for ERK1/2 phosphorylation, Activator protein (AP)-1 activity and concentration, and VEGF protein and mRNA expression, using immunohistochemical and biochemical methods.

RESULTS. The ERK1/2 signaling pathway was rapidly activated 1 week after diabetes was induced. AP-1, the downstream transcription factor of ERK1/2, was also activated, and VEGF became highly regulated in a similar trend. U0126, an inhibitor of ERK1/2, also downregulated VEGF expression, in addition to ERK1/2 and AP-1 activity.

CONCLUSIONS. ERK1/2 signaling pathway is involved in VEGF release in diabetic rat retina; therefore, ERK1/2 may be a potential therapeutic target of DR. (Invest Ophthalmol Vis Sci. 2010;51:5226–5233) DOI:10.1167/iovs.09-4899

Diabetic retinopathy (DR) is a common complication of diabetes that is present to some degree in nearly all individuals with diabetes for >15 years, regardless of whether the diabetes is due to loss of insulin secretion (type 1) or to insulin resistance (type 2). DR is responsible for most visual impairment in diabetic patients and imposes a burden on the community and the individual. Current treatments for DR are performed by laser photoagulation or vitreous surgery, which prevent further loss of vision. However, these therapeutic modalities are ineffective against restoring diminished visual acuity. Since the development of laser surgery for DR, there have been no major advances in treatment for the disease, despite numerous clinical trials using a variety of drugs. These drugs include calcium dolyes, aspirin, aldose reductase inhibitors, and antihistamines. Therefore, more effective treatment options need to be explored.

Recently, much research has focused on vascular endothelial growth factor (VEGF) because this potent biogenic permeability factor plays an important role in the pathogenesis of vascular complications in diabetes and is one of the major factors promoting diabetic retinopathy. VEGF is elevated in the vitreous of people with DR and in animal models of diabetes and is sufficient to increase both vascular proliferation and permeability. In vivo, administration of neutralizing VEGF antibodies to experimental animals reverses high-glucose-induced vascular hyperpermeability, which is an early manifestation of endothelial dysfunction in diabetic patients. Accumulated evidence has suggested that molecular processes involved in vascular growth and vascular hyperpermeability are based on the inappropriate regulation of VEGF. VEGF thus represents an important target for therapeutic intervention in DR. Molecular pharmacology that directly inhibits the actions of VEGF has shown considerable promise but has not been satisfactorily effective in blocking the disease and development of microangiopathies. Because VEGF is important for maintaining significant neuroprotective and antiapoptotic functions, VEGF suppression therapy may produce unidentified adverse effects on the neural retina. Therefore, we should regulate, not suppress, VEGF release to treat DR. A better understanding of the signaling pathway in VEGF regulation is of clinical importance to identify more precise therapeutic targets for diabetic retinopathy. However, the precise mechanisms underlying VEGF regulation are far from being fully resolved.

Expression of VEGF is regulated at multiple levels: transcriptional, posttranscriptional, and translational. VEGF release is mediated by a complex array of signaling pathways and the integration of these different pathways results in the generation of a net signaling input. We believe there are several signaling pathways that are stimulated in diabetes and potentially cross-talk to help each other control VEGF release. We selected the ERK1/2 signaling pathway for study, because it has been verified to play a key role in some oncoma and hematologic disease to mediate VEGF release. Although DR does not fall under the scope of oncoma, the biological behavior of DR, proliferation, and blood neogenesis is similar to the behavior of oncoma. The activation of ERK1/2 is involved in regulating proliferation and protein synthesis by stimulating transcription factors that induce the activation of activator protein (AP)-1 and other growth responsive genes. Moreover, ERK1/2 is known to regulate AP-1 (c-fos/c-jun) activation and the promoter region of the VEGF gene contains the AP-1 binding consensus sequence. The VEGF

From the 1EENT Hospital, Eye Institute, the 2Institute of Brain Science, Fudan University, Shanghai, China; and the 3Department of Surgery, University of Wisconsin, Madison, Wisconsin.

1Present affiliation: School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin.


Submitted for publication November 12, 2009; revised March 13 and April 6, 2010; accepted April 8, 2010.

Disclosure: X. Ye, None; G. Xu, None; Q. Chang, None; J. Fan, None; Z. Sun, None; Y. Qin, None; A.C. Jiang, None.

Corresponding author: Gezhi Xu, EENT Hospital, Eye Institute, Fudan University, Fengyang Road 83, Shanghai, China, 200031; newnewye@163.com.
promoter region contains binding sites of several transcription factors, including AP-1. The AP-1 site is significant because AP-1 binding activity is critical for VEGF gene induction.\textsuperscript{19,21} We investigated whether ERK1/2 and the downstream transcription factor AP-1 take part in the mechanisms of VEGF release in diabetes retina.

We hypothesized that the ERK1/2 signaling pathway leads to AP-1 activation and increased production of VEGF by retina cells, which results in DR development. We examined the expression of phosphorylated-ERK1/2 and VEGF and explored whether the ERK1/2 pathway is responsible for modulating VEGF release in DR using immunohistochemical and biochemical methods.

**Materials and Methods**

**Inducing Diabetes in Rats**

One hundred male Sprague-Dawley (SD) rats weighing 250 ± 20 g were assigned at random to become diabetic or remain nondiabetic controls. Treatment of the animals conformed to the National Institute of Health Principles of Laboratory Animal Care and the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research, and local institutional guidelines. Diabetes was induced by a single injection of streptozotocin (60 mg/kg body weight) into the abdominal cavity. Normal rats were injected with citrate buffer (6 mL/kg) using the same method. Three days after injection, rats with blood glucose levels above 16.0 mM were included in the diabetes groups. Blood glucose and weight of animals were measured once a week for the 3 months of the study and just before the rats were euthanatized. Blood samples were obtained from the lateral vein of the tail. At 1, 2, 3, 4, 8, and 12 weeks after the onset of diabetes, the rats were euthanatized by an overdose of pentobarbital, the eyes were removed, and a paraffin-embedded section was made for immunohistochemistry, and the retina was isolated and frozen immediately in liquid nitrogen for biochemical measurements. Each group had eight or more rats. The same methods were used for the normal control group.

**ERK Inhibitor U0126 Injected into Vitreous Cavity**

For further investigation of the ERK1/2 pathway after diabetes, U0126, an ERK1/2 specific inhibitor, was injected into the vitreous cavity in 10 rats before the diabetes was induced. U0126 was first dissolved in DMSO and then diluted with PBS (U0126 or DMSO to PBS = 1:19). The final concentration of U0126 was 0.1 mM with a concentration of 5% of DMSO. Two microliters of this solution was injected into the vitreous cavity with a 32-gauge syringe (Hamilton, Reno, NV) over a 24-hour period just before diabetes was induced in the rats. Two microliters of solution without U0126 (DMSO to PBS = 1:19) were injected into the fellow eye vitreous and 24 hours later, these rats were induced to diabetes with STZ. One week after diabetes was induced, the rats were euthanatized by an overdose of pentobarbital, the eyes were removed, and the retinas were isolated and frozen immediately in liquid nitrogen for biochemical measurements. Age-matched normal rats served as the control and were handled by the same methods.

**Immunohistochemistry**

Eyes were fixed in methacarn solution (60% methanol, 30% chloroform, and 10% acetic acid). Five-micrometer-thick paraffin-embedded sections were used for staining. The sections were briefly immersed in xylene, hydrated through graded ethanol solutions, and incubated in 3% hydrogen peroxide for 5 minutes, to eliminate intrinsic peroxidase activity. Sections were then incubated for 10 minutes in 10% normal goat serum (NGS) in Tris-buffered saline (TBS; 50 mM Tris-HCl and 150 mM NaCl [pH 7.6]) to reduce nonspecific binding. After the sections were rinsed briefly with 1% NGS/TBS, they were incubated overnight at 4°C with either total-ERK1/2 antibody (1:100 tERK; Cell Signaling Technology, Beverly, MA) or phosphorylated-ERK1/2 antibody (pERK, 1:100; Cell Signaling Technology). The sections were incubated for 30 minutes in goat anti-mouse HRP-antiserum, and then for 1 hour in species-specific peroxidase antiperoxidase complex. 3,3′-Diaminobenzidine (DAB) was used as the chromogen with sections developed in 0.75 mg/mL DAB with 0.015% hydrogen peroxide in Tris buffer. All images were acquired using the same intensity and photodetector gain, to allow quantitative comparisons of relative levels of immunoreactivity between sections.

**Western Blot Analysis**

Western blot analyses of total-ERK1/2 (tERK; Cell Signaling Technology), phosphorylated ERK1/2 (pERK; Cell Signaling Technology) and VEGF (AbCam, Cambridge, UK) were performed using the respective antibodies. Whole retinas were dissected, snap-frozen in liquid nitrogen, and stored at −80°C. Retinas were removed from the eyes of the diabetes–induced and control rats at 1, 2, 3, 4, 8, and 12 weeks (n = 5 at each time point) after induction to diabetes. We also dislodged retinas from U0126 - and PBS-treated eyes (both n = 5). Retinas were homogenized in a lysis buffer (100 µL per retina) that contained 50 mM Tris, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 1 mM 5-nitrophenyl phosphate, 20 mM calyculin A, 1 mM Na-orthovanadate, 100 mM phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, and 10 µg/mL aprotinin. All procedures were performed at 4°C. Homogenates were centrifuged at 14,000g for 30 minutes, and the supernatant was collected. Protein concentrations were determined by the Bradford method, using bovine serum albumin (BSA) as a standard. Samples of supernatants were mixed 1:1 with 2× sample buffer and heated to 95°C for 10 minutes. SDS-polyacrylamide gel electrophoresis was performed in 12% gel with 5% stacking gel and with 0.25 M Tris-glycine (pH 8.3) as the electroyte 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 Tris-buffered saline (TBS; pH 7.6). The membranes were then separately incubated with dilutions of the polyclonal VEGF (1:500), tERK (1:1000), and p-ERK (1:1000) antibodies in blocking solution overnight at 4°C. Membranes were then 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 with a Western blot detection kit (ECL-Plus, Pierce Biotechnology, Rockford, IL). 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.

**Electrophoretic Mobility Shift Assay**

An electrophoretic mobility shift assay (gel-shift; EMSA) was performed to analyze the DNA binding activity of AP-1. Preparation of nuclear protein extracts was completed as follows: a single retinal tissue sample (−50–100 mg) was homogenized in 1.5 mL of homogenization medium, and, after low-speed centrifugation, the supernatant was saved as the postnuclear supernatant fraction. The pellet (crude nuclear fraction) was washed and resuspended in extraction solution, and the supernatant was collected by centrifugation and saved as the nuclear protein fraction. All extranuclear supernatant and nuclear extracts were stored at −80°C until use. The protein concentrations were determined with a Bradford protein assay kit. Probe labeling was performed as follows: The EMSA probe is a 21-bp double-stranded AP-1 consensus oligonucleotide (5′-GGCTTGTGACTCAGCCGGA-3′) labeled with biotin. EMSA was performed as described in the manufacturer’s manual,\textsuperscript{22} with a slight modification. All binding reactions between nuclear proteins and the labeled probe were analyzed by 2% to 20% gradient polyacrylamide gels. DNA-protein complexes were resolved by polyacrylamide gel electrophoresis using 6% nondenaturing gel at 180 V...
for 3 hours. Dried gels were exposed to an x-ray film. The images were scanned and analyzed (Bio-Imaging Analyzer; Bio-Imaging Research, Lincolnshire, IL). The relative AP-1 activity was determined based on the intensity of the shifted bands measured.

**Real-Time Quantitative PCR**

To determine whether diabetes has any effect on the mRNA levels of VEGF and AP-1, the mRNA of VEGF and c-fos and c-jun (two main factors in the composition of AP-1) were quantified by real-time PCR. The mRNA expression of VEGF and AP-1, the mRNA of VEGF and c-fos and c-jun (two main factors in the composition of AP-1) were quantified by real-time PCR. Each mRNA level was normalized to β-actin mRNA. Specific primers were designed based on blood glucose (<16.0 mmol/L) and weight. As expected, the blood glucose level was significantly raised in rats in the diabetes groups compared with the normal control group (P < 0.01, Fig. 1). The mean body weight of the normal control rats gradually increased, but in the diabetic groups, the body weight of some of the rats increased and that of others decreased to lower than the mean weight in the normal control group (P < 0.01, Fig. 1).

**Statistical Analysis**

All values are shown as the 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**

**Glucose and Weight**

After injection of STZ, induction of diabetes was confirmed based on blood glucose (>16.0 mmol/L) and weight. As expected, the blood glucose level was significantly raised in rats in the diabetes groups compared with the normal control group (P < 0.01, Fig. 1). The mean body weight of the normal control rats gradually increased, but in the diabetic groups, the body weight of some of the rats increased and that of others decreased to lower than the mean weight in the normal control group (P < 0.01, Fig. 1).

![Figure 1](http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). Real-time quantitative PCR was completed using SYBR green reagents in a 20-μL reaction in light cycler. The relative changes in gene expression were analyzed by using the 2-ΔΔCt method. Specificity of RT-PCR was checked by analyzing melting curves and by gel electrophoresis of the amplicons.

**Figure 2.** (A, B) In the normal control retinas, at every time point of the study (1, 2, 3, 4, 8, and 12 weeks), the total and phosphorylated levels of ERK1/2 isoforms were unchanged (P > 0.05; n = 3). (C) The levels of pERK/ERK in ERK1 and ERK2 were both increased after 1 week of inducing diabetes and peaked at 8 weeks. (D) At various time points after diabetes was induced, the ratio of pERK/ERK continued to increase compared with the control (P < 0.01; n = 3).
Western Blot Analysis of ERK1/2 Activation in Diabetic Rat Retina

To determine whether the ERK1/2 pathway was activated in the rat retina after high blood glucose was obtained, Western blot analysis was performed with anti-phosphorylated ERK1/2 antibody or anti-total ERK1/2 antibody. Two bands, ERK1 (44 kDa) and ERK2 (42 kDa), were observed with both antibodies. For quantitative analysis, pERK/tERK was measured by densitometry.

In the normal control retinas, at every point of the study (1, 2, 3, 4, 8, and 12 weeks), the total and phosphorylated levels of ERK1/2 isoforms were unchanged (P > 0.05, Fig. 2).

At various time points (1, 2, 3, 4, 8, and 12 weeks) after the STZ-induced high blood glucose, the protein level of total ERK1/2 (tERK) was unchanged (P > 0.05). In contrast, there was a marked increase in phosphorylated levels of ERK1/2 isoforms compared with the control (P < 0.01). ERK1/2 activation occurred within 1 week after diabetes was induced, and levels remained elevated at the other time periods. By 8 weeks, phosphorylated ERK1/2 levels achieved their peak. The time course and magnitude of increased ERK1/2 phosphorylation were similar to trends in the AP-1 DNA binding and VEGF release (Fig. 2).

Immunohistochemical Studies of ERK1/2 and Phosphorylated ERK1/2

To determine the point of fluctuation of the ERK1/2 activation in diabetic retina, immunohistochemical studies were performed with specific antibodies against phos-ERK1/2. Sections treated with secondary antibodies alone showed no immunoreactivity. ERK1/2 immunohistochemistry in normal retinas showed a low level of labeling, primarily restricted to the ganglion cell layer (GCL) of retina (Fig. 3A). In diabetic retinas, labeling occurred mostly in the inner nuclear layer (INL). ERK1/2 phosphorylation was observed in retinas examined for immunohistochemical staining at 1 and 8 weeks after...
diabetes was induced. There was little expression of phos-ERK1/2 in the normal retina (Fig. 3B). Phos-ERK labeling is apparent at 1 week in INL cell nuclei (Fig. 3C). A further increase in staining intensity occurred in the same pattern 8 weeks after diabetes was induced (Fig. 3D).

AP-1 DNA-Binding Activity in Diabetic Retina
To determine whether AP-1 activity was induced by high blood glucose, nuclear protein extracts from diabetic rat retinas were analyzed by EMSA at different time points after STZ-induced diabetes. Figure 4 shows the results of an EMSA for AP-1 of the VEGF promoter. In normal control rats there was no AP-1 activation. In contrast, diabetic retinas showed very distinct increased binding of the AP-1 consensus oligonucleotide, visualized as a distinct band of labeling (*P < 0.01).

Real-Time PCR Analysis of AP-1 mRNA
The variation of AP-1 concentration was found by detecting the mRNA of c-fos and c-jun. Both c-fos and c-jun mRNA levels of the rats with induced diabetes were lowered (*P < 0.05), in contrast to that in the normal control animals (Fig. 5).

Real-Time PCR and Western Blot Analysis of VEGF
We used real-time PCR to detect the expression of VEGF mRNA. The VEGF mRNA expression was significantly elevated in comparison with the control except at 8 weeks after diabetes induction. The first peak of mRNA levels occurred at 1 week, and the second at 4 weeks (Fig. 6).

The results of Western blot analysis of extracts of rat retina are shown in Figure 6. Densitometry of the VEGF immunoreactive band of approximately 25 kDa increased significantly from weeks 1 to 12 after STZ-induced diabetes. The first peak of VEGF expression occurred between weeks 2 and 3, and the second peak occurred at week 12 (Fig. 6).

ERK Inhibitor U0126 Downregulates VEGF in Diabetic Retina
Our studies demonstrate that expression of VEGF and VEGF mRNA increased with a tendency similar to the fluctuation of the phosphorylated ERK1/2 and the DNA binding activity of AP-1 after inducing high glucose levels. Next, we studied the effects of a specific inhibitor of ERK1/2, U0126, on the diabetes-induced phosphorylation of ERK1/2, activation of AP-1, and the expression of VEGF.

The retina of the U0126-treated diabetic group and the untreated diabetic group were subjected to real-time PCR, Western blot analysis, and EMSA analyses. The high blood glucose activation of ERK1/2 was reduced by the presence of U0126. U0126 also inhibited the AP-1 DNA binding activity and expression of VEGF (Fig. 7). These results demonstrate that U0126, an ERK1/2 inhibitor, could significantly attenuate diabetes-induced VEGF expression (*P < 0.05).

DISCUSSION
Our results indicate that three early biochemical events—activation of ERK1/2, AP-1 DNA-binding activity, and the
increase of VEGF—occur in retinas of diabetics. When we used the ERK1/2 inhibitor U0126, AP-1 DNA-binding activity and VEGF expression were significantly decreased ($P < 0.05$; $n = 3$). This decrease indicates that the activation of the ERK1/2 pathway plays some role in mediating the VEGF upregulation in diabetes.

ERK1 and ERK2 are 44- and 42-kDa proteins that are highly homologous and have a similar spatiotemporal organization. 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 and rapid nuclear translocation of activated ERK1/2 are essential for stimulating activation of ERK1/2. Our research shows that this occurred 1 week after diabetes was induced, demonstrating the rapid activation of ERK1/2.

Activated ERK1/2 can remain in the cytoplasm to phosphorylate specific substrates or may translocate into the nucleus. In the nucleus, ERK1/2 phosphorylates specific transcription factors, enhancing their stability and transcriptional activity that control cellular growth, differentiation, and survival. The ERKs work as mediators of signal transduction from the cell surface to the nucleus by targeting AP-1. The regulation of these transcription factors is therefore of critical importance in determining the response to various physiolog-
ical and environmental stimuli. AP-1 can effectively enhance the transcription of genes in response to a variety of stimuli. When we demonstrated that ERKs activate soon after high blood glucose levels, we also wanted to verify that the downstream substrate, AP-1, is activated. We know that transcription factors like AP-1, a composite dimeric TF implicated in growth control and neurodegeneration, can be measured by activity and concentration—concentration and activity—each of which can be modulated in diverse ways.

In vitro experiments indicate that VEGF is posttranscriptionally regulated by ERK, and accumulating evidence suggests that AP-1 is also engaged in VEGF function.

In this report, we have shown that VEGF and VEGF mRNA are strongly upregulated, and the tendency of mobile VEGF mRNA levels appear to correlate with that of increased phosphorylated ERK1/2 and activated AP-1 in the early stage of diabetes. In addition, an inhibitor of ERK1/2 can effectively downregulate the expression of VEGF and VEGF mRNA. Thus, we have demonstrated that ERK1/2 can upregulate the expression of VEGF by acting at the VEGF promoter and rapidly activate the AP-1 DNA binding activity, but not increase the level of AP-1. However, we found that the peak of protein expression of VEGF is at 2 to 3 weeks after the onset of diabetes, which was later than that of VEGF mRNA transcription. We believe this may be due to a higher sensitivity to the regulation of ERK1/2 in VEGF mRNA transcription than in the protein expression. In addition, several anti-VEGF forces that were also present in diabetes interfered in VEGF protein expression.

The results in the present study implicate a possible mechanism for VEGF release in DR. Activation of the ERK1/2 signal transduction pathways by stimuli of diabetes may augment the activity levels of their downstream effector, transcription factor AP-1. Furthermore, AP-1 can directly bind to VEGF genes and enhance VEGF expression. However, we cannot completely rule out the mechanisms of VEGF expression in diabetes, because there may be several signaling pathways involved. These observations provide a basis for future investigations on the potential therapeutic implications of ERK1/2 inhibitors to inhibit VEGF release in diabetic retinopathy and indicate that a more intensive examination of the mechanisms of ERK1/2 and AP-1 should be performed.

References


