Antipermeability Function of PEDF Involves Blockade of the MAP Kinase/GSK/ β -Catenin Signaling Pathway and uPAR Expression

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PURPOSE. Pigment epithelium–derived factor (PEDF) is a potent inhibitor of vascular endothelial growth factor (VEGF)–induced endothelial permeability. The goal of this study was to understand the mechanism by which PEDF blocks VEGF-induced increases in vascular permeability.

METHODS. The paracellular permeability of bovine retinal endothelial (BRE) cells was measured by assaying transendothelial cell electrical resistance and tracer flux. Western blot analysis was used to show phosphorylation of VEGFR2, MAP kinases, and glycogen synthase kinase 3 (GSK3)- β . Confocal imaging and Western blot analysis were used to determine subcellular distribution of β -catenin. Real-time RT-PCR and Western blot analysis were used to quantify urokinase plasminogen activator receptor (uPAR) expression.

RESULTS. PEDF blocked VEGF-induced phosphorylation of extracellular signal-regulated kinase (ERK), p38 MAP kinase, the p38 substrate MAP kinase-activated protein kinase-2 (MAPKAPK-2), and GSK3- β , but it had no effect on the phosphorylation of VEGFR2. In addition, the VEGF-induced transcriptional activation of β -catenin and uPAR expression were blocked by PEDF and by inhibitors of p38 and MEK. Finally, the VEGF-induced increase in permeability was blocked by both PEDF and the same kinase inhibitors.

CONCLUSIONS. The data suggest that p38 MAP kinase and ERK act upstream of GSK/ β -catenin in VEGF-induced activation of the uPA/uPAR system and that PEDF-mediated inhibition of the VEGF-induced increase in vascular permeability involves blockade of this pathway. These findings are important for developing precise and potent therapies for treatment of diseases characterized by vascular barrier dysfunction. (*Invest Ophthalmol Vis Sci.* 2010;51:3273–3280) DOI:10.1167/iovs.08-2878

Pigment epithelium–derived factor (PEDF) is a 50-kDa glycoprotein expressed in many cell types, including retinal pigment epithelial cells, vascular endothelial cells, and pericytes. It

was first identified as a neurotrophic factor¹ and was later found to have antipermeability activity.² Patients with diabetic macular edema have been shown to have elevated VEGF and reduced PEDF levels in ocular tissue, suggesting that a balance between PEDF and VEGF is critical for preserving the blood–retinal barrier.^{3,4} PEDF has been shown to block retinal vascular permeability increases induced by VEGF, advanced glycation end products, and diabetic conditions.^{2,5,6} It also prevents retinal pigmented epithelium barrier dysfunction after oxidant treatment.⁷ Despite all the information available about the beneficial effects of PEDF, the mechanism of its protective action on blood–retinal barrier function is still not clear.

It has been shown that VEGF induces hyperpermeability of endothelial cell monolayers by activating the uPA/uPAR system (urokinase and its receptor) through transcriptional activation of β -catenin, thus increasing uPAR expression.⁸ The increase in uPAR expression in the retina has also been confirmed in a diabetic animal model.⁹ uPA is a serine protease that can be activated by binding to uPAR and catalyzes conversion of plasminogen to plasmin, which can degrade the extracellular matrix, activate latent growth factors such as TGF- β , and convert inactive–matrix metalloproteinase (pro-MMPs), including MMP-2 and -9, into their active forms.¹⁰ Furthermore, a pharmacologic inhibitor of the uPA/uPAR system has been reported to inhibit alteration of the blood–retinal barrier in a diabetic animal model.¹¹

β -Catenin is a component of the adherens junction complex. It links the intracellular domain of cadherin to actin filaments, the main component of the cytoskeleton. Under normal conditions, free β -catenin released from the junction complex is phosphorylated by binding to glycogen synthase kinase 3 (GSK3)- β and is targeted for ubiquitination and degradation.¹² Under certain stimulations, β -catenin escapes phosphorylation and degradation, accumulates in the cytosol, and translocates to the nucleus. In the nucleus, β -catenin acts as a transcription factor and works with other transcription factors such as T-cell factor/lymphoid-enhancing factor (TCF/LEF) to induce expression of uPAR.¹³

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that regulate gene expression and cell proliferation, differentiation, and survival. Two MAP kinase subtypes, p38 MAP kinase and extracellular signal-regulated protein kinase (ERK), are important regulators of endothelial cell proliferation and migration and are activated in endothelial cells treated with VEGF.¹⁴ Activation of p38 MAP kinase has also been reported in endothelial cells maintained in high glucose in vitro¹⁵ and in diabetic retinas in vivo.¹⁶ In general, p38 MAP kinase has been designated as stress-activated kinase and is known to block cell proliferation and to induce apoptosis in a variety of cell types.^{17,18} On the other hand, ERK1/2 kinase is mostly activated in response to mitogenic stimuli and has been associated with cell proliferation. However, ERK1/2 may cross-talk with the p38 activation pathway under certain inflammatory conditions.¹⁹ Both p38 MAP kinase and ERK have

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been shown to play a critical role in activation of the uPA/uPAR system.^{20–22}

The purpose of this study was to identify upstream mediators in VEGF-induced activation of the β -catenin-uPA/uPAR permeability pathway and to examine whether PEDF preserves the barrier function by blocking this pathway.

MATERIALS AND METHODS

Cell Culture, VEGF, PEDF, and MAP Kinase Inhibitors

Primary cultures of bovine retinal microvascular endothelial cells (BRE cells) were isolated according to methods established in our laboratory.²³ For this study, BRE cells were maintained in endothelial growth medium (EGM; Lonza Walkersville, Inc., Walkersville, MD) under an atmosphere of 95% air and 5% CO₂ in a humidified 37°C incubator and were used at passages 5 to 9. On 90% confluence, for all experiments, unless otherwise designated, culture medium was switched to a serum-free endothelial basal medium (EBM; Lonza Walkersville, Inc.), including 0.1% BSA, and the cultures were incubated for an additional 20 and 24 hours before the indicated treatments were applied. The National Cancer Institute (NCI) Preclinical Repository (Frederick, MD) kindly provided all VEGF used in the study. PEDF was purified as described previously.²⁴ p38 MAP kinase inhibitor (SB202190) and MEK inhibitors (U0126 and PD98059) were purchased from Calbiochem (San Diego, CA).

Endothelial Cell Permeability Assay

BRE cell barrier function was assessed by measuring changes in trans-endothelial electrical resistance (TER) using ECIS (electrical cell-substrate impedance sensing; Applied Biophysics, Inc., Troy, NY) and by measuring FITC-dextran flux across the BRE cell monolayers grown on porous membranes. Decreased TER and increased dextran flux across the cell monolayer are indications of increased paracellular permeability.

In ECIS, cells are grown in fibronectin-coated chambers of the eight-well electrode arrays and the electric currents passing through the monolayers are measured independently in each chamber.²⁵ The ECIS system is superior to the previously used manual methods in that, instead of the end-point TER being measured, the electrical resistance is monitored uninterruptedly at short intervals, before and during the treatments. In this study, BRE cells were plated at a density of 5×10^4 cells/chamber (8W10E), to obtain a confluent layer of cells after 2 days, at which time the cells were serum starved for 24 hours and then treated according to the experimental design. Data from the electrical resistance measurements (in ohms) were recorded over the experimental time course. Integrity of the endothelial monolayer was confirmed at the end of each experiment microscopically and also by a final TER measurement. This method was used to establish the optimal doses of the tested reagents used in all experiments. Resistance values for each chamber were normalized as the ratio of measured resistance to baseline resistance and plotted as a function of time.

For the tracer flux permeability assay, BRE cells were grown to confluence on fibronectin-coated membranes with 0.4- μ m pores (Transwell; Corning Costar, Acton, MA). VEGF (30 ng/mL) was added to both the upper and the lower chambers for 1 hour before the addition of 10 μ M FITC-dextran to the upper chamber of the inserts. Aliquots taken from the lower chamber at 5 hours after the addition of dextran were placed in a 96-well plate (black with clear bottoms, polystyrene; Corning Costar). Additional samples were also taken from the upper chamber at the end of the experiment and placed in the 96-well plate. The fluorescence intensity of the samples was quantified with a plate reader. The FITC-dextran that passed across the endothelial cell monolayer was normalized to the fluorescence reading from the upper chamber, and permeability was calculated as relative fluorescence units.

Real-Time Quantitative PCR

The method for quantifying specific gene expression by polymerase chain reaction was as previously described⁸ with some modifications. In brief, after the indicated treatments, the cells were removed from the dish with a Teflon-coated razor blade, and RNA was extracted with a commercially available kit (RNeasy; Qiagen, Inc., Valencia, CA) and stored in ethanol-precipitated form at -70°C . Aliquots of 2.0 μ g total RNA were reconstituted, and reverse transcription (RT) was performed with random primers (Omniscript RT Kit; Qiagen, Inc.). Quantitative PCR was performed with fluorescence-labeled probes (TaqMan; and the Step-One-Plus system; Applied Biosystems, Inc. [ABI], Foster City, CA). The probes and primers for uPAR and the internal marker HPRT (hypoxanthine-guanine phosphoribosyl transferase) were designed based on the bovine DNA sequences (bovine uPAR accession number: AF144762 and HPRT accession number: BC103248; GenBank, <http://www.ncbi.nlm.nih.gov/Genbank>; National Center for Biotechnology Information, Bethesda, MD). The PCR products were also examined by agarose gel electrophoresis.

Protein Extraction and Western Blot Analysis

Methods for cytosolic and nuclear protein extraction were as described before.⁸ Endothelial cells were grown in 100-mm-diameter dishes and treated as indicated. At the end of the treatment time, the cells were collected and extracted by different lysis buffers as follows: For cytosolic β -catenin analysis, a detergent-free isotonic buffer (10 mM Tris-HCl [pH 7.4], 140 mM NaCl, 2 mM DTT, 5 mM EDTA, 50 mM NaF, and 10% protease inhibitor cocktail containing AEBSF, bestatin, aprotinin, E-64, pepstatin-A, and leupeptin) was used. The cells were homogenized in ice-cold buffer in a tight-fitting homogenizer (Dounce; Bellco Glass Co., Vineland, NJ) and centrifuged for 5 minutes at 2000g. The supernatant was collected and centrifuged for 60 minutes at 100,000g to obtain the cytosolic (supernatant) fractions. For extraction of nuclear proteins, the cells were rinsed two times with ice-cold PBS containing 1 mM PMSF and 1 mM sodium orthovanadate, scraped in the same buffer, collected and centrifuged at 500g for 5 minutes. The cell pellet was suspended in a 20-fold pellet volume of hypotonic buffer (10 mM Tris-HCl [pH 6.8], 3 mM MgCl₂, 1 mM PMSF, and 1 mM sodium orthovanadate) and incubated for 15 minutes on ice. The suspension was added drop-wise into an equal volume of extraction buffer consisting of the hypotonic buffer and 0.2% NP-40. The mixture was allowed to rest in the ice bath for an additional 15 minutes and then sonicated for three short strokes of 1 second each at 4 MHz. The suspension was then layered over an equal volume of ice-cold solution of 350 mM sucrose in hypotonic buffer. The test tubes were centrifuged at 500g for 8 minutes to pellet the cell nuclei. The nuclear pellet was further washed in 1.2 mL of extraction buffer, incubated for 15 minutes on ice, and centrifuged at 500g. The nuclear pellet was dissolved in Tris-SDS buffer (1% SDS and 2 mM EDTA in 10 mM Tris; pH 6.8), boiled for 10 minutes, and clarified at 15,000g.

For preparation of the whole-cell extract, the cells were lysed in RIPA buffer (50 mM Tris [pH 7.4], 1 mM EDTA, 1% NP-40, 0.25% deoxycholic acid, and 150 mM sodium fluoride) containing 1 mM PMSF and clarified by centrifugation at 12,000g for 20 minutes.

Levels of uPAR protein in endothelial cell lysates are low and difficult to detect, but VEGF stimulation has been shown to induce the release of soluble uPAR into the culture medium.²⁶ Therefore, we measured levels of soluble uPAR in the medium conditioned by the treated BRE cells. Phenol red-free culture medium was collected after indicated treatment and concentrated by fivefold with centrifugal filters (Millipore, Billerica, MA). Protein contents were quantified by protein assay (DC; Bio-Rad Laboratories, Hercules, CA).

Samples equated for protein content were separated on 10% acrylamide gel and transferred to nitrocellulose membranes. β -Catenin protein bands were identified by using anti- β -catenin antibody (BD-Transduction Laboratories, San Jose, CA). Phospho-protein and total protein for GSK3- β , p38 MAP kinase, MAPKAPK-2, ERK, and VEGFR2 were detected with commercially available antibodies (Cell Signaling Tech-

FIGURE 1. Role of MAP kinase activity in VEGF-induced increases in paracellular permeability. The involvement of MAP kinase activity in VEGF-induced decreases in transendothelial electrical resistance (TER; **A**) or increases in tracer flux (**B**) was determined in BRE cells grown on fibronectin-coated electrode arrays or porous membranes. Cultures were maintained in serum-free medium overnight, pretreated with inhibitors of p38 MAP kinase (SB202190, 3 μ M) and MEK (U0126, 1.8 μ M) for 30 minutes, and then stimulated with VEGF (30 ng/mL) for 6 to 8 hours. In the ECIS system, decreased TER indicates increased permeability. For TER measurement, $n = 3$, $\#P < 0.05$ vs. control, $*P < 0.05$ vs. VEGF-treated group. For FITC-dextran flux, $n = 8-15$, $\#P < 0.01$ vs. control, $*P < 0.05$ vs. VEGF-treated group. For analysis of VEGF effects on MAP kinase activity (**C**), the BRE cells were maintained in serum-free medium overnight and then stimulated with VEGF (30 ng/mL) for 5 minutes. Cell lysates were subjected to SDS-PAGE and Western blot analysis for phosphorylation of p38 MAP kinase and ERK1/2.

nology Inc, Danvers, MA). uPAR protein was probed by an antibody from R&D Systems Inc. (Minneapolis, MN). Protein bands were visualized with a chemiluminescence system (ECL; Amersham, Buckinghamshire, UK), exposed to x-ray films, scanned for densitometry, and normalized to appropriate internal markers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a cytosolic protein internal marker, lamin A/C served as a nuclear protein marker, and β -COPI as a Golgi-membrane marker. BSA served as a loading marker for analysis of the conditioned medium.

Immunocytochemistry and Confocal Microscopy

The analysis of β -catenin redistribution in endothelial cells by immunocytochemistry was performed as described previously.⁸

Statistical Analysis

The comparisons between multiple groups and the overall differences were analyzed by ANOVA with post hoc comparison (two-tailed Student's *t*-test). $P < 0.05$ was taken as significant.

RESULTS

MAP Kinase Activity in VEGF-Induced BRE Cell Permeability

To evaluate the potential involvement of MAP kinases in the VEGF-induced signaling pathway leading to the permeability of endothelial cells, we tested the effects of MAP kinase inhibitors on VEGF-induced decreases in TER levels by using ECIS and on VEGF-induced increases in tracer flux by using FITC-dextran. BRE cells grown to confluent monolayers on fibronectin-coated electrode arrays or cell-migration membranes were serum starved for 24 hours before the indicated treatments. The cells were pretreated with a p38 MAP kinase inhibitor (SB202190, 3 μ M) and an MEK inhibitor (U0126, 1.8 μ M) for 30 minutes and then incubated with or without VEGF (30 ng/mL) for an additional 5 to 8 hours. Figures 1A and 1B show that both inhibitors blocked VEGF-induced TER decreases and tracer flux increases. This result indicates that p38 MAP kinase and ERK1/2 (the specific substrate of MEK) participate in intracellular signaling pathways leading to an increase in endothelial cell permeability. Western blot data (Fig. 1C) further confirm the activation of

p38 and ERK1/2 kinases in BRE cells by VEGF, which were readily blocked by the same inhibitors that blocked the permeability increase.

Activation of the GSK3- β -Catenin Signaling Pathway in VEGF-Treated Endothelial Cells

Transcriptional activation of β -catenin is increased when GSK3- β is deactivated by phosphorylation on the Ser9 site. To examine whether p38 MAP kinase and ERK1/2 are upstream of GSK3- β in the VEGF permeability pathway, BRE cells were treated with VEGF in the presence or absence of kinase inhibitors. Western blot data (Fig. 2A) showed that the p38 MAP kinase inhibitor (SB202190, 3 μ M) and MEK inhibitors (U0126, 1.8 μ M, and PD98059, 20 μ M) blocked GSK3- β phosphorylation on Ser9, suggesting that MAP kinases work upstream of GSK3- β to mediate the VEGFR2- β -catenin signaling pathway. This concept was tested by determining the effects of the inhibitors on subcellular localization of β -catenin. Figure 2 show that β -catenin was increased in the cytosol (Fig. 2B) and nuclei (Fig. 2C) of BRE cells 1 hour after VEGF stimulation. Pretreatment of the BRE cells with the inhibitors of p38 MAP kinase or ERK1/2 prevented the VEGF-induced accumulation of β -catenin in both the cytosol and nucleus. The total levels of β -catenin protein were not changed in any of the treatment conditions (data not shown).

Effect of Inhibitors of MAP Kinase on VEGF-Induced uPAR Expression

The cell fractionation study results suggest that inhibiting p38 MAP kinase or ERK1/2 blocks VEGF-induced β -catenin transcriptional activation. To test this concept, we determined the effect of the same treatments on uPAR gene expression by using quantitative real-time PCR (Fig. 3A) and Western blot analysis (Fig. 3B). Two hours after VEGF treatment, uPAR mRNA was increased by approximately twofold. When BRE cells were pretreated with inhibitors of p38 and MEK, uPAR mRNA was significantly lower than in the group treated with VEGF alone. Moreover, 6 hours after VEGF treatment, the level of uPAR protein released into the culture medium was increased and pretreatment with inhibitors of p38 MAP kinase or

FIGURE 2. Role of MAP kinase in VEGF-induced phosphorylation/deactivation of GSK3- β and redistribution of β -catenin. BRE cells were pretreated with inhibitors for 30 minutes as indicated and then stimulated with VEGF (30 ng/mL) for 5 minutes. To monitor the GSK3- β activity status (A), cell lysates were subjected to SDS-PAGE and Western blot analysis for phosphorylated GSK3- β on Ser9. $n = 4$, $\#P < 0.01$ vs. control, $*P < 0.01$ vs. VEGF-treated group. For analysis of the subcellular distribution of β -catenin, cell lysates were fractionated and membrane-free cytosolic (B) and nuclear (C) preparations were subjected to Western blot analysis for β -catenin content. GAPDH, lamin, and β -COP1 served as cytosolic, nuclear, and Golgi membrane markers, respectively. $n = 3$, $\#P < 0.05$ vs. control, $*P < 0.05$ vs. VEGF-treated group.

MEK prevented this effect. These data indicate that inhibiting p38 or ERK1/2 blocks the VEGF-induced increase in uPAR expression.

FIGURE 3. Blockade of VEGF-induced uPAR expression by MAP kinase inhibitors. For mRNA analysis, BRE cells were pretreated with inhibitors of p38 MAP kinase (SB202190) and MEK (U0126; PD98059) for 30 minutes and then stimulated with VEGF (30 ng/mL) for 2 hours. Total cellular RNA extraction was followed by reverse transcription and quantitative real-time PCR analysis (A). $n = 4$, $\#P < 0.001$ vs. control, $*P < 0.05$ vs. VEGF-treated group. For analysis of uPAR protein, serum-starved BRE cells were pretreated with inhibitors for 30 minutes and then stimulated with VEGF (30 ng/mL) for 6 hours. uPAR protein levels in conditioned medium were determined by Western blot analysis (B). $n = 3$, $\#P < 0.01$ vs. control, $*P < 0.01$ vs. VEGF-treated group.

Antipermeability Effects of PEDF on VEGF-Treated BRE Cells

Previous studies in retinal endothelial cells have shown that PEDF treatment blocks VEGF-induced increases in tracer movement across the barrier and normalizes distribution of the junction proteins occludin and ZO-1, suggesting that PEDF plays a role in preserving endothelial barrier function.⁶ Therefore, we performed experiments using the ECIS system, to determine the effects of PEDF on VEGF-induced decreases in TER levels in BRE cells. Our initial studies confirmed that PEDF blocks VEGF-induced decreases in TER levels (Fig. 4). The optimum inhibitory effect of PEDF in our experimental model was between 100 and 250 ng/mL. At this concentration range, PEDF showed no detrimental effect on BRE monolayers over a 24-hour incubation. At high concentrations (e.g., 2.5 μ g/mL), however, PEDF did not block VEGF and caused a decrease in TER levels when used alone (data not shown).

PEDF Blockade of the VEGFR2-Induced MAP Kinase-GSK- β -Catenin Signal Transduction Pathway

To test whether PEDF functions by targeting the MAP kinase-GSK- β -catenin pathway, we first performed experiments to rule out the possibility that PEDF blocks activation of VEGFR2 by VEGF. Our experiments with BRE cells showed that VEGF induced a prominent increase in phosphorylation of VEGFR2 which was not affected by PEDF pretreatment (Fig. 5A). Note that, when used alone at 250 ng/mL, PEDF had no effect on VEGFR2 phosphorylation status. On the other hand, PEDF treatment significantly inhibited VEGF-induced phosphorylation of p38 MAP kinase, ERK1/2, and GSK3- β (Figs. 5B-D). To demonstrate that the inhibitory effect of PEDF on p38 phosphorylation results in blocking the kinase activity of p38, we also examined the phosphorylation of a p38 MAP kinase-specific substrate, MAPKAPK-2. Figure 5B shows that PEDF blocked VEGF-induced phosphorylation of MAPKAPK-2, indicating that PEDF, indeed, blocks the activity of p38 MAP kinase.

FIGURE 4. Inhibition of VEGF-induced permeability by PEDF. BRE cells grown on fibronectin-coated electrode arrays were pretreated with PEDF for 30 minutes and then stimulated with VEGF (30 ng/mL). The effects on TER of the endothelial monolayer were demonstrated using the ECIS system. Decreased TER indicates increased permeability. $n = 3$, $*P < 0.05$ vs. control, $\#P < 0.05$ vs. VEGF-treated group.

The observation that PEDF blocks VEGF-induced phosphorylation of GSK3- β suggests that the antipermeability action of PEDF involves blockade of β catenin transcriptional activation. This possibility was tested by using cell fractionation studies to examine β -catenin cytosolic accumulation and nuclear translocation.

Data in Figure 6 show that β -catenin was increased in the cytosol and nuclei of BRE cells 1 hour after VEGF stimulation. Pretreatment with PEDF (100 or 250 ng/mL) prevented the increase of β -catenin in both cytosol and nuclei in a dose-dependent manner (Figs. 6A, 6B). Subcellular distribution of β -catenin was also examined by confocal immunolocalization analysis. In Figure 6C β -catenin is shown in green and the nucleus is stained red. After 1 hour of VEGF treatment, β -catenin immunoreactivity on the plasma membrane was decreased compared with that in the vehicle control cells, whereas staining in the cytosol was increased. PEDF pretreatment blocked the VEGF-induced accumulation of β -catenin in the cytosol and preserved its normal plasma membrane localization.

PEDF Blockade of uPAR Expression

The data from the cell fractionation studies suggest that PEDF blocks VEGF-induced β -catenin cytosolic accumulation and nuclear translocation. We next determined the effect of these same treatments on uPAR gene expression by using quantita-

tive real-time PCR (Fig. 7A) and Western blot analysis (Fig. 7B). Two hours after VEGF treatment, uPAR mRNA was increased by approximately twofold. When BRE cells were pretreated with PEDF, uPAR mRNA was significantly lower than in the VEGF-treated group. Moreover, 6 hours after VEGF treatment, the level of uPAR protein released into the culture medium was increased, and pretreatment with PEDF prevented this effect. These data indicate that PEDF blocks VEGF-induced increases in uPAR expression.

DISCUSSION

To understand the intracellular signaling of PEDF as it relates to vascular permeability, we have explored, step by step, a particular VEGF-induced intracellular signaling pathway responsible for increasing endothelial cell permeability. We present data suggesting that VEGF induces activation of the GSK- β -catenin pathway, that activation of the uPA/uPAR system⁸ requires the activity of p38 MAP kinase and ERK1/2, and that the antipermeability function of PEDF involves the blockade of this pathway.

The correlation between tissue levels of PEDF and endothelial cell barrier function has been studied extensively. Research on ocular tissue from diabetic patients as well as diabetic animal models has shown that the imbalance between VEGF

FIGURE 5. Inhibition of VEGF-induced phosphorylation of MAP kinases and GSK3- β but not VEGFR2 by PEDF. BRE cells were pretreated with PEDF for 30 minutes and then stimulated with VEGF (30 ng/mL) for 5 minutes. Cell lysates were subjected to SDS-PAGE and Western blot analysis for phosphorylation of VEGFR2 (A), MAP kinase (B, C), and GSK3- β (D). $n = 4$, $\#P < 0.01$ vs. control, $*P < 0.01$ vs. VEGF-treated group. When used alone, PEDF at 250 ng/mL had no effect on VEGFR2 phosphorylation.

FIGURE 6. Inhibition of VEGF-induced subcellular redistribution of β -catenin by PEDF. BRE cells were pretreated with PEDF for 30 minutes and then stimulated with VEGF (30 ng/mL) for 1 hour. The cell lysates were fractionated and membrane-free cytosolic (**A**) and nuclear (**B**) preparations were subjected to Western blot analysis for β -catenin contents. GAPDH, lamin, and β -COP1 served as cytosolic, nuclear, and Golgi membrane markers, respectively. $n = 3$, $\#P < 0.05$ vs. control, $*P < 0.05$ vs. VEGF-treated group. BRE cells grown in chamber slides were pretreated with PEDF for 30 minutes, stimulated with VEGF for 1 hour, and analyzed with immunocytochemistry and confocal microscopy to demonstrate the redistribution of β -catenin from cell membrane to cytosol (**C**). β -Catenin was stained green with fluorescent-labeled secondary antibody and the nuclei were stained with propidium iodide.

and PEDF strongly correlates with disruption of the blood-retinal barrier.^{3,4} PEDF has also been shown to abrogate the increase in retinal vascular permeability induced by different factors such as VEGF and advanced glycation end product (AGE) or under experimental diabetes conditions, as well as to prevent oxidant-induced barrier dysfunction in retinal pigment epithelial cells.^{2,5-7} Our data showed that PEDF prevented VEGF-induced decreases in endothelial cell TER levels, which is consistent with the previous reports that PEDF possesses antipermeability function. Moreover, the inhibitors of p38 MAP kinase and MEK mimicked PEDF's antipermeability effects and prevented VEGF-induced permeability. This finding suggests that these kinases play a role in signaling pathways by which VEGF induces increased permeability and by which PEDF blocks the VEGF signal.

It has been reported that PEDF decreases VEGF expression under hypoxic conditions and induces proteolysis of VEGF receptor-1 beginning after 2 hours of VEGF treatment.^{27,28} However, these studies addressed relatively late-stage events in the VEGF/PEDF signaling process. In understanding the function of PEDF in regulating VEGF-mediated intracellular signaling and transcriptional events related to the initiation of vascular permeability, it is important to study early events in cells

treated with VEGF in the presence of PEDF. Our data show that PEDF blocked VEGF-induced phosphorylation/activation of MAP kinases and phosphorylation/deactivation of GSK3- β , which resulted in the blockade of β -catenin transcriptional activation and in turn blocked VEGF-induced uPAR expression and increase in permeability.

A previous study has shown that PEDF blocks the binding of radio-labeled VEGF to retinal endothelial cells.²⁷ However, our study showed that VEGF-induced phosphorylation of VEGFR2 was not affected by PEDF. Further study is necessary to clarify the reason for this apparent discrepancy.

p38 MAP kinase and ERK are downstream targets of cytokines and oxidative stress. They have also been shown to regulate critical steps in the angiogenesis process, such as endothelial cell proliferation, migration, and survival.²⁹⁻³¹ Activation of p38 MAP kinase and ERK has been reported in endothelial cells stimulated with VEGF, in endothelial cells maintained under high glucose conditions, and in retinas of diabetic animal models.^{9,21,32,33} Under diabetic conditions, blood-retinal barrier breakdown is thought to occur because of diabetes-induced oxidative stress and increased activity of cytokines including VEGF, both of which can activate p38 MAP kinase.^{9,34} Several studies have shown that p38 MAP kinase

FIGURE 7. Blockade of VEGF-induced uPAR expression by PEDF. For mRNA analysis, BRE cells were pretreated with PEDF for 30 minutes and then stimulated with VEGF (30 ng/mL) for 2 hours. Total cellular RNA was extracted, and samples were used for reverse transcription followed by quantitative real-time PCR (A). $n = 4$, $\#P < 0.001$ vs. control, $*P < 0.05$ vs. VEGF-treated group. For analysis of uPAR protein, serum-starved BRE cells were pretreated with PEDF for 30 minutes and then stimulated with VEGF (30 ng/mL) for 6 hours. Levels of soluble uPAR in the conditioned medium were determined by Western blot analysis (B). $n = 3$, $\#P < 0.01$ vs. control, $*P < 0.01$ vs. VEGF-treated group.

and ERK play a role in VEGF-induced vascular hyperpermeability.^{33,35–37} Moreover, treatments with p38 inhibitor (SB202190) and MEK inhibitor (U0126) have been shown to block the vascular permeability increase in experimental diabetes.³⁸

In our study, we tested whether MAP kinases are involved in the VEGF signaling pathways that lead to permeability increase and whether the antipermeability function of PEDF is implemented by blocking the actions of MAP kinase. In this study, we first showed that PEDF blocked phosphorylation/activation of p38 MAP kinase and ERK in a way that was comparable with the effects of specific inhibitors of p38 MAP kinase and MEK. Of importance, the MAP kinase inhibitors, mimicking PEDF function, preserved the barrier function of the endothelial cells, suppressed uPAR expression, blocked β -catenin transcriptional activation, and inhibited phosphorylation/deactivation of GSK3- β . These data together suggest that MAP kinases and the uPA/uPAR system are critical mediators of VEGF signaling that leads to endothelial barrier dysfunction and that PEDF prevents VEGF-induced permeability increase through blockade of MAP kinase/GSK3- β -catenin pathway, hence suppression of uPAR expression. This result is consistent with those in previous studies showing that p38 MAP kinase and ERK play a role in uPAR expression and activation of the uPA/uPAR system.^{20,22,39,40}

PEDF is a multifunctional protein that influences a variety of cell types by its different molecular domains. For example, the

antiangiogenic and antiapoptotic effect of PEDF on endothelial cells has been localized to its residues 24–57 and 78–94.⁴¹ On the other hand, the antipermeability effect of PEDF has been attributed to residues 78–121.² Moreover, PEDF-induced differentiation of neuronal cells is mediated by residues 58–101.⁴¹ These studies may suggest that PEDF uses distinct intracellular signaling mechanisms to exert different functions through different molecular domains.

Studies have shown that PEDF activates MAP kinases in various cell types with some contradictory outcomes. For example, prolonged PEDF treatment induces the apoptosis of human umbilical vein endothelial cells (HUVECs) through the activation of p38 MAP kinase.⁴² PEDF has also been shown to inhibit oxidative stress-induced apoptosis of retinal pigment epithelial cells by a process involving the activation of ERK.⁴³ These discrepancies may be due to differences in cell types or the experimental conditions used. Alternatively, since full-length PEDF is a multifunctional molecule with various functional domains, its effect on the MAP kinase signaling pathway may vary depending on the functions of different molecular domains in various cell types.

Study results suggest that activation of the metalloproteinases MMP-2 and -9 can regulate PEDF levels under hypoxic conditions or in VEGF treatment.⁴⁴ Together with our previous findings showing that VEGF activates the uPA/uPAR system, which in turn can activate MMPs,^{8,9} this activation of MMPs could explain the decreased PEDF levels seen in diabetic models with elevated VEGF levels.

Considering the beneficial effects in vascular barrier dysfunction shown in animal models, PEDF has been pushed for a phase I clinical trial on age-related macular degeneration (AMD). Knowing the pathways by which PEDF exerts its antipermeability effects will allow us to identify more precise and potent molecular targets for the treatment of diseases characterized by vascular barrier dysfunction.

References

1. Steele FR, Chader GJ, Johnson LV, Tombran-Tink J. Pigment epithelium-derived factor: neurotrophic activity and identification as a member of the serine protease inhibitor gene family. *Proc Natl Acad Sci U S A*. 1993;90(4):1526–1530.
2. Liu H, Ren JG, Cooper WL, Hawkins CE, Cowan MR, Tong PY. Identification of the antivasopermeability effect of pigment epithelium-derived factor and its active site. *Proc Natl Acad Sci U S A*. 2004;101(17):6605–6610.
3. Funatsu H, Yamashita H, et al. Vitreous levels of pigment epithelium-derived factor and vascular endothelial growth factor are related to diabetic macular edema. *Ophthalmology*. 2006;113(2):294–301.
4. Patel JJ, Tombran-Tink J, Hykin PG, Gregor ZJ, Cree IA. Vitreous and aqueous concentrations of proangiogenic, antiangiogenic factors and other cytokines in diabetic retinopathy patients with macular edema: implications for structural differences in macular profiles. *Exp Eye Res*. 2006;82(5):798–806.
5. Yamagishi S, Nakamura K, Matsui T, et al. Pigment epithelium-derived factor inhibits advanced glycation end product-induced retinal vascular hyperpermeability by blocking reactive oxygen species-mediated vascular endothelial growth factor expression. *J Biol Chem*. 2006;281(29):20213–20220.
6. Zhang SX, Wang JJ, Gao G, Shao C, Mott R, Ma JX. Pigment epithelium-derived factor (PEDF) is an endogenous antiinflammatory factor. *FASEB J*. 2006;20(2):323–325.
7. Ho TC, Yang YC, Cheng HC, Wu AC, Chen SL, Tsao YP. Pigment epithelium-derived factor protects retinal pigment epithelium from oxidant-mediated barrier dysfunction. *Biochem Biophys Res Commun*. 2006;342(2):372–378.
8. Behzadian MA, Windsor LJ, Ghaly N, Liou G, Tsai NT, Caldwell RB. VEGF-induced paracellular permeability in cultured endothelial cells involves urokinase and its receptor. *FASEB J*. 2003;17(6):752–754.

9. El-Remessy AB, Behzadian MA, Abou-Mohamed G, Franklin T, Caldwell RW, Caldwell RB. Experimental diabetes causes breakdown of the blood-retina barrier by a mechanism involving tyrosine nitration and increases in expression of vascular endothelial growth factor and urokinase plasminogen activator receptor. *Am J Pathol*. 2003;162(6):1995-2004.
10. Blasi F, Carmeliet P. uPAR: a versatile signalling orchestrator. *Nat Rev Mol Cell Biol*. 2002;3(12):932-943.
11. Navaratna D, Menicucci G, Maestas J, Srinivasan R, McGuire P, Das A. A peptide inhibitor of the urokinase/urokinase receptor system inhibits alteration of the blood-retinal barrier in diabetes. *FASEB J*. 2008;22(9):3310-3317.
12. Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. beta-Catenin is a target for the ubiquitin-proteasome pathway. *EMBO J*. 1997;16(13):3797-3804.
13. Mann B, Gelos M, Siedow A, et al. Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc Natl Acad Sci U S A*. 1999;96(4):1603-1608.
14. Rousseau S, Houle F, Landry J, Huot J. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene*. 1997;15(18):2169-2177.
15. Srinivasan S, Bolick DT, Hatley ME, et al. Glucose regulates interleukin-8 production in aortic endothelial cells through activation of the p38 mitogen-activated protein kinase pathway in diabetes. *J Biol Chem*. 2004;279(30):31930-31936.
16. Kondo T, Kahn CR. Altered insulin signaling in retinal tissue in diabetic states. *J Biol Chem*. 2004;279(36):37997-38006.
17. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem*. 1996;271(34):20608-20616.
18. Molnar A, Theodoras AM, Zon LI, Kyriakis JM. Cdc42Hs, but not Rac1, inhibits serum-stimulated cell cycle progression at G1/S through a mechanism requiring p38/RK. *J Biol Chem*. 1997;272(20):13229-13235.
19. Houliston RA, Pearson JD, Wheeler-Jones CP. Agonist-specific cross talk between ERKs and p38(mapk) regulates PGI(2) synthesis in endothelium. *Am J Physiol Cell Physiol*. Oct 2001;281(4):C1266-C1276.
20. Huang S, New L, Pan Z, Han J, Nemerow GR. Urokinase plasminogen activator/urokinase-specific surface receptor expression and matrix invasion by breast cancer cells requires constitutive p38alpha mitogen-activated protein kinase activity. *J Biol Chem*. 2000;275(16):12266-12272.
21. Lee KH, Choi EY, Hyun MS, Kim JR. Involvement of MAPK pathway in hypoxia-induced up-regulation of urokinase plasminogen activator receptor in a human prostatic cancer cell line, PC3MLN4. *Exp Mol Med*. 2004;36(1):57-64.
22. Bessard A, Fremin C, Ezan F, Coutant A, Baffet G. MEK/ERK-dependent uPAR expression is required for motility via phosphorylation of P70S6K in human hepatocarcinoma cells. *J Cell Physiol*. 2007;212(2):526-536.
23. Behzadian MA, Wang XL, Jiang B, Caldwell RB. Angiostatic role of astrocytes: suppression of vascular endothelial cell growth by TGF-beta and other inhibitory factor 23(s). *Glia*. Dec 1995;15(4):480-490.
24. Duh EJ, Yang HS, Suzuma I, et al. Pigment epithelium-derived factor suppresses ischemia-induced retinal neovascularization and VEGF-induced migration and growth. *Invest Ophthalmol Vis Sci*. 2002;43(3):821-829.
25. Tiruppathi C, Malik AB, Del Vecchio PJ, Keese CR, Giaever I. Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. *Proc Natl Acad Sci U S A*. 1992;89(17):7919-7923.
26. Chavakis T, Willuweit AK, Lupu F, Preissner KT, Kanse SM. Release of soluble urokinase receptor from vascular cells. *Thromb Haemost*. 2001;86(2):686-693.
27. Zhang SX, Wang JJ, Gao G, Parke K, Ma JX. Pigment epithelium-derived factor downregulates vascular endothelial growth factor (VEGF) expression and inhibits VEGF-VEGF receptor 2 binding in diabetic retinopathy. *J Mol Endocrinol*. 2006;37(1):1-12.
28. Cai J, Jiang WG, Grant MB, Boulton M. Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. *J Biol Chem*. 2006;281(6):3604-3613.
29. Matsumoto T, Turesson I, Book M, Gerwins P, Claesson-Welsh L. p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. *J Cell Biol*. 2002;156(1):149-160.
30. Yu Y, Sato JD. MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. *J Cell Physiol*. 1999;178(2):235-246.
31. Yilmaz A, Kliche S, Mayr-Beyre U, Fellbrich G, Waltenberger J. p38 MAPK inhibition is critically involved in VEGFR-2-mediated endothelial cell survival. *Biochem Biophys Res Commun*. 2003;306(3):730-736.
32. McGinn S, Saad S, Poronnik P, Pollock CA. High glucose-mediated effects on endothelial cell proliferation occur via p38 MAP kinase. *Am J Physiol Endocrinol Metab*. 2003;285(4):E708-E717.
33. Verin AD, Liu F, Bogatcheva N, et al. Role of ras-dependent ERK activation in phorbol ester-induced endothelial cell barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol*. 2000;279(2):L360-L370.
34. Doly M, Droy-Lefaix MT, Braquet P. Oxidative stress in diabetic retina. *Exs*. 1992;62:299-307.
35. Lal BK, Varma S, Pappas PJ, Hobson RW 2nd, Duran WN. VEGF increases permeability of the endothelial cell monolayer by activation of PKB/akt, endothelial nitric-oxide synthase, and MAP kinase pathways. *Microvasc Res*. 2001;62(3):252-262.
36. Issbrucker K, Marti HH, Hippenstiel S, et al. p38 MAP kinase: a molecular switch between VEGF-induced angiogenesis and vascular hyperpermeability. *FASEB J*. 2003;17(2):262-264.
37. Breslin JW, Pappas PJ, Cerveira JJ, Hobson RW 2nd, Duran WN. VEGF increases endothelial permeability by separate signaling pathways involving ERK-1/2 and nitric oxide. *Am J Physiol Heart Circ Physiol*. 2003;284(1):H92-H100.
38. Poulaki V, Qin W, Joussen AM, et al. Acute intensive insulin therapy exacerbates diabetic blood-retinal barrier breakdown via hypoxia-inducible factor-1alpha and VEGF. *J Clin Invest*. 2002;109(6):805-815.
39. Yu J, Bian D, Mahanivong C, Cheng RK, Zhou W, Huang S. p38 Mitogen-activated protein kinase regulation of endothelial cell migration depends on urokinase plasminogen activator expression. *J Biol Chem*. 2004;279(48):50446-50454.
40. Yoon SY, Lee YJ, Seo JH, et al. uPAR expression under hypoxic conditions depends on iNOS modulated ERK phosphorylation in the MDA-MB-231 breast carcinoma cell line. *Cell Res*. 2006;16(1):75-81.
41. Filleul S, Volz K, Nelius T, et al. Two functional epitopes of pigment epithelial-derived factor block angiogenesis and induce differentiation in prostate cancer. *Cancer Res*. 2005;65(12):5144-5152.
42. Chen L, Zhang SS, Barnstable CJ, Tombran-Tink J. PEDF induces apoptosis in human endothelial cells by activating p38 MAP kinase dependent cleavage of multiple caspases. *Biochem Biophys Res Commun*. 2006;348(4):1288-1295.
43. Tsao YP, Ho TC, Chen SL, Cheng HC. Pigment epithelium-derived factor inhibits oxidative stress-induced cell death by activation of extracellular signal-regulated kinases in cultured retinal pigment epithelial cells. *Life Sci*. 2006;79(6):545-550.
44. Notari L, Miller A, Martinez A, et al. Pigment epithelium-derived factor is a substrate for matrix metalloproteinase type 2 and type 9: implications for downregulation in hypoxia. *Invest Ophthalmol Vis Sci*. 2005;46(8):2736-2747.