Vascular endothelial growth factor (VEGF) may have a physiologic role in regulating vessel permeability and contributes to the pathophysiology of diabetic retinopathy as well as tumor development. We set out to ascertain the mechanism by which VEGF regulates paracellular permeability in rats. Intraocular injection of VEGF caused a post-translational modification of occludin as determined by a gel shift from 60 to 62 kDa. This event began by 15 min post-injection and was maximal by 45 min. Alkaline phosphatase treatment revealed this modification was caused by a change in occludin phosphorylation. In addition, the quantity of extracted occludin increased 2-fold in the same time frame. The phosphorylation and increased extraction of occludin was recapitulated in retinal endothelial cells in culture after VEGF stimulation. The data presented herein are the first demonstration of a change in the phosphorylation of this transmembrane protein under conditions of increased endothelial permeability. In addition, intraocular injection of VEGF also caused tyrosine phosphorylation of ZO-1 as early as 15 min and increased phosphorylation 4-fold after 90 min. In conclusion, VEGF rapidly increases occludin phosphorylation as well as the tyrosine phosphorylation of ZO-1. Phosphorylation of occludin and ZO-1 likely contribute to regulated endothelial paracellular permeability.

Tissues of the central nervous system, including the brain and retina, depend on intact blood-brain and blood-retinal barriers, respectively, to partition them from the systemic circulation. These barriers contribute to the maintenance of specific neural tissue environments by regulating ion concentrations, water permeability, and delivery of amino acids and sugars and by preventing exposure to circulating antibodies and immune cells. These requirements imply the need for regulation of the blood-brain/retinal barrier, which permits selective delivery of needed substrates in response to varying local tissue demands and systemic metabolic influences. Endothelial cells regulate the blood-brain/retinal barrier through a number of mechanisms including transporter activity, e.g. glucose transport via GLUT-1 and transcytosis. In this report we provide evidence for regulation of paracellular permeability by vascular endothelial growth factor (VEGF) through the rapid phosphorylation of tight junction proteins.

The endothelial cells of the blood-brain and blood-retinal barriers contain tight junctions that confer highly selective barrier properties to these vessels. Tight junctions contain at least seven proteins including occludin, zonula occludens 1, 2, and 3 (ZO-1, -2, or -3), cingulin, the 7H6 antigen, and symplekin (reviewed in Refs. 1–5). Recent studies of an occludin knockout mouse line revealed that claudins may also influence permeability (6), whereas other laboratories have identified novel isoforms of occludin by reverse transcription-polymerase chain reaction (7). Signaling molecules including Rac proteins, large G-proteins, and soluble tyrosine kinases have also been identified at tight junctions (reviewed in Refs. 1 and 2). Several lines of evidence suggest that occludin plays a crucial role in the function of tight junctions and control of vascular permeability. Hydrophobicity plots from several species predict that occludin spans the plasma membrane at four regions presenting two extracellular loops (8). Also, occludin tissue expression and content correlate well with barrier properties (9), and overexpression of occludin increases transendothelial electrical resistance in MDCK cells (10) and confers adhesiveness in fibroblasts (11). Finally, microinjection of occludin increases Xenopus oocyte barrier properties (12).

The molecular structure of ZO-1 indicates a functional capacity for multiple protein/protein interactions. It is a member of the membrane-associated guanylate kinase protein family of proteins (MAGuk proteins) and as such contains a region of homology to guanylate kinase. However, this region of homology lacks certain amino acids necessary for guanylate kinase activity, and no such activity has been demonstrated for ZO-1. Consistent with MAGuk family members, ZO-1 contains an SH3 domain, three PDZ domains, and a 90-amino acid domain unique to MAGuk family members (1, 13). ZO-1 also contains a leucine zipper region, a proline-rich region, and four PEST sequences that are associated with proteins that undergo rapid turnover. In vitro binding studies have revealed that ZO-1 can interact directly with occludin (14) as well as with ZO-2 and ZO-3 (15), and mutational analysis by Fanning et al. (16) has identified the sites of occludin interaction. Because of its mul-

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; ZO, zonula occludens; MDCK cells, Madin-Darby canine kidney cells; BREC, bovine retinal endothelial cells; BARC, bovine aortic endothelial cells; MAGuk, membrane-associated guanylate kinase; VVOs, vesiculo-vacuolar organelles; CAPS, 3-(cyclohexylaminopropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
VEGF-induced Tight Junction Protein Phosphorylation

tiple protein/protein interaction domains, current models place ZO-1 at the center of tight junction assembly and organization.

In a previous report we found that chronic incubation of bovine retinal endothelial cells (BREC) with VEGF decreased occludin content after 3–6 h, coinciding with changes in barrier permeability properties (17). Here we present the first evidence of occludin phosphorylation associated with a regulated increase in barrier permeability. VEGF causes a rapid phosphorylation of occludin both in BREC cultures and when injected into the vitreous cavity of rat eyes. VEGF also induces ZO-1 tyrosine phosphorylation when injected into the vitreous cavity. These rapid changes in the tight junction protein phosphorylation state may serve as a mechanism by which VEGF regulates endothelial barrier permeability. These observations are significant for understanding the physiologic regulation of vascular permeability as well as discerning the underlying mechanism of abnormal permeability associated with diabetic retinopathy and VEGF-producing tumors.

EXPERIMENTAL PROCEDURES

Materials—The following antibodies were used: polyclonal, rabbit antio-1, and anti-phosphotyrosine monoclonal antibody (4G10, Upstate Biotechnology Inc.). Cell culture reagents and chemicals were obtained through Sigma except minimum Eagle’s medium vitamins were from Mediatech, endothe-elial cell growth supplement from Collaborative Biochemical, antibo-otic/antimyotic from Life Technologies, Inc., and cell culture plastic ware from Falcon. For gel electrophoresis and transfer, the following reagents were used: polyacrylamide from Amresco, CAPS buffer from Research Organics, and nitrocellulose with 0.22-μm pore size (Micron Separations Inc.). Recombinant human VEGF 165 was obtained from R & D Systems Inc., and calf intestinal alkaline phosphatase was from New England Biolabs. Protease inhibitor tablet Complete™ (EDTA-free) was from Roche Molecular Biochemicals.

Animals—Male Sprague-Dawley rats (Charles River, MA) weighing approximately 175–200 g were maintained on a 12-h alternating light/dark cycle and received food and water ad libitum. All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology “Statement for Care and Use of Laboratory Animals.” Rats were anesthetized with ketamine/xylazine (50/0.5 mg/kg intramuscularly). VEGF, or vehicle alone, was injected into the vitreous cavity of the rat eye in 10 μl of saline, yielding a final concentra-
tion of ~25 ng/ml. The rats were sacrificed by decapitation, eyes were enucleated, and the retinas were removed under a dissecting microscope on ice and were then frozen in liquid nitrogen and stored at −70 °C.

Cell Culture—Primary BREC culture was carried out as described previously (18). The day before the experiment, the cells were stepped down from 20 to 10% fetal calf serum, or the cells were stepped down to serum-free medium with 103 nM hydrocortisone for 2 days. Recombinant human VEGF was added to the cell culture at 500 (12 μM), 50 (1.2 μM), or 5 ng/ml (0.12 μM). Cells were returned to the incubator for the indicated time and then washed twice with ice-cold phosphate-buffered saline containing phenylmethylsulfonyl fluoride (200 μM) and harvested in lysis buffer as described below.

Immunoprecipitation and Immunoblotting—The retinas were homogenized in 6 μl urea buffer (6 μl urea, 0.1% Triton X-100, 10 mM Tris pH 8.0, 1 mM diithiothreitol, 5 mM MgCl2, 5 mM EDTA, 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine (which had been shown previously to extract nearly 100% of ZO-1 (19)). 5-s pulses with a probe sonicator (Tekmar) were used to disrupt the cells. Cells were solubilized in 6 μl urea buffer with scraping or using a Triton/ deoxycholate/SDS buffer (100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 2 mM EDTA, 10 mM Hepes, pH 7.5, 1 mM benzamidine) along with Complete™ (EDTA-free), a protease inhibitor mixture tablet. Additionally, both buffers were brought to 1 mM NaVO4, 10 mM NaF, and 10 mM sodium pyrophosphate.

Western blotting of occludin allowed quantification of the protein as well as identification of a post-translational modification. Protein concentrations were determined using the Bio-Rad DC protein assay and an albumin standard curve. For immunoprecipitation, 100 μl of lysates were diluted 10-fold with lysis buffer without urea, and 5 μl of anti-ZO-1 antibody (R&D Systems Inc.) were added. Samples were incubated for at least 4 h at 4 °C, pelleted, washed twice with a buffer identical to the lysis buffer except containing only 1 mM urea, and finally washed once with 10 mM Tris, pH 8.0. Samples were resuspended in Laemmli sample buffer, separated on 7.5% SDS-polyacrylamide gels, and then transferred to nitrocellulose. Membranes were blocked with 3% albumin, and phosphotyrosine was detected by blotting with anti-phosphotyrosine antibody (1:1000 dilution) followed by secondary anti-mouse IgG antibody linked to alkaline phosphatase and enhanced chemifluorescence or by blotting with 125I-labeled protein A. Protein concentrations were determined to ensure that nearly equal protein was used for the immunoprecipitation, and a straight blot for ZO-1 content was carried out. Phosphotyrosine content was expressed as the amount of ZO-1 phosphotyrosine per ZO-1 protein. Bands were quan-
tified using volume analysis in ImageQuant software or by scanning autoradiograms and analyzing the images using NIH Image analysis software.

RESULTS AND DISCUSSION

Vascular permeability factor/vascular endothelial growth factor causes rapid post-translational modifications to the tight junction protein occludin. Previously, we demonstrated that VEGF treatment of BREC in culture decreased tight junction protein occludin content by 50% after 6 h (17). However, VEGF increases retinal vascular permeability to fluorescein in vivo within 10 min of injection into the vitreous cavity (20) and begins to increase water permeability in BAEC cultures within 20 min and in BREC by 1 h (21). Therefore, we set out to determine whether VEGF also induced rapid alterations in the tight junction proteins, which could account for the observed early changes in barrier permeability. Toward this end, VEGF was injected into rat vitreous cavity, and changes in occludin were observed through Western blot analysis. Rats were anes-
theitized, and VEGF was injected into one eye to a final concentra-
tion of 25 ng/ml while vehicle was injected into the con-
tralateral eye. The eyes were enucleated and the retinas extracted within 3 min. The retinas were homogenized in 6 μl urea buffer that was previously shown to extract nearly 100% of ZO-1 (19). This buffer extracted approximately 75–90% of the occludin when compared with the amount extracted from the insoluble pellet using Laemmli sample buffer. Western blotting of equal protein content revealed a series of bands from approximately 60 to 70 kDa and additional faint bands at approximately 80 kDa as compared with a negative control with no primary antibody (data not shown). Previous immunohistochemical staining of rat retinas in our laboratory revealed that occludin expression was restricted to the retinal vascula-
ture (17). After VEGF injection, an increase in occludin content was observed at 15 min, was maximal by 45 min, and began to decrease by 90 min, as seen in Fig. 1. The same observation was made from a separate set of animals (blot not shown). This increase probably represents the movement of occludin from an insoluble pool to a soluble pool or could be because of increased protein synthesis of occludin. Evidence for a change in occludin post-translational modification was observed as a change in occludin migration on the polyacrylamide gels. In the saline-

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Fig. 1. VEGF causes a change in retinal endothelial cell occludin migration on SDS-polyacrylamide gel electrophoresis. Adull, male Harlan Sprague-Dawley rats were anesthetized, and VEGF (V) was injected into the vitreous fluid of one eye to a final concentration of 25 ng/ml. As a control, saline (S) was injected into the contralateral eye. Retinas were harvested at the time points indicated and immunoblotted for occludin. On the gels, each pair of VEGF and saline injections represents the result from an individual animal. A, occludin migrates as two major bands of ~60 and ~62 kDa (α and β, respectively) as well as a series of slower migrating forms. VEGF caused an increase in the amount of occludin β relative to occludin α as well as an overall increase in the content of occludin that was extracted. Panel B depicts these changes in chart form. For each animal the relative increase in occludin α and occludin β was determined after VEGF injection; the means of 4 animals for each time point are shown. Statistical analysis was done by t test, where * indicates a difference at p < 0.05 and **, p < 0.005.

Fig. 2. Reduced occludin migration is caused by a phosphorylation event as revealed by alkaline phosphatase treatment. Retina lysates from injected eyes were incubated with 20 units of alkaline phosphatase for 1 h at 37 °C or incubated in buffer without alkaline phosphatase. Control, no incubation; Alk. Phos., alkaline phosphatase-treated samples; Con. Inc., control incubation without alkaline phosphatase; S, saline; V, VEGF. Alkaline phosphatase treatment caused a collapse of all bands to a single band with the fastest migration on the gel.

Fig. 3. Primary bovine retinal endothelial cells in culture respond to VEGF with occludin phosphorylation. BREC cultures were grown to confluence and treated with VEGF at the concentrations shown. Cells were harvested in the 6 M urea extraction buffer and Western blotted for occludin. A 15-min incubation with VEGF caused a shift in the migration of occludin from at least two bands of ~60 and ~62 kDa to only the slower migrating band. By 60 min, occludin distribution between the two bands returned to the control condition (A). Additionally, 15 min of VEGF treatment caused an increase in the amount of occludin that was extracted, which returned to control levels by 60 min (B). Extraction with an SDS/Tween-based detergent buffer yielded similar changes in the total occludin content extracted after VEGF treatment. The average of duplicate experiments is represented in panel C.
pared with in vivo conditions. Addition of VEGF caused a complete shift to the slower migrating band of ~62 kDa and a trend toward an increase in the amount of occludin extracted within 15 min. These changes were maintained until 30 min and returned by 60 min (Fig. 3, A and B). Alkaline phosphatase treatment of the lysates again caused occludin to migrate as a single band at ~60 kDa, whereas immunoprecipitation of occludin and phosphorytrosine blotting did not yield any evidence for occludin tyrosine phosphorylation (results not shown). These data suggest that VEGF causes occludin phosphorylation on serine or threonine residues. The same increase in extractability of occludin occurred when the experiment was conducted using a different extraction buffer containing 0.2% SDS, sodium deoxycholate, and Nonidet P-40 (Fig. 3C). The decrease in extractable occludin at the longer time points may represent a return of occludin to an insoluble pool or may represent an increase in occludin degradation.

The effect of VEGF on BREC was also assessed under serum-free conditions. While VEGF is a potent permeabilizing agent, hydrocortisone reduces vascular permeability and tissue edema. We found that endothelial cells could be maintained in culture without serum for 2 days in the presence of 103 nM hydrocortisone for 2 days. VEGF was added to the cultures at 1.2 nM and harvested at the time points indicated. Again, VEGF caused a shift toward the amount of phosphorylated occludin as revealed by an increase in the upper occludin band (A). Panel B reveals that the amount of occludin extracted also was increased with VEGF treatment and returned to control levels by 60 min.

FIG. 4. Primary bovine retinal endothelial cells incubated in the presence of hydrocortisone respond to VEGF with occludin phosphorylation. BREC cultures were grown to confluence and treated with 103 nM hydrocortisone for 2 days. VEGF was added to the cultures at 1.2 nM and harvested at the time points indicated. Again, VEGF caused a shift toward the amount of phosphorylated occludin as revealed by an increase in the upper occludin band (A). Panel B reveals that the amount of occludin extracted also was increased with VEGF treatment and returned to control levels by 60 min.2

FIG. 5. VEGF causes tyrosine phosphorylation of ZO-1 in rat retina. Adult male Harlan Sprague-Dawley rats were anesthetized, and VEGF (V) was injected into the vitreous fluid of one eye to a final concentration of 25 ng/ml. As a control, saline (S) was injected into the contralateral eye. Retinas were harvested at the time points indicated and homogenized in 6 M urea buffer. Samples were diluted 1 to 10 in buffer without urea and immunoprecipitated with an anti-ZO-1 monoclonal antibody. Proteins were resolved on 7.5% polyacrylamide gels, transferred to nitrocellulose, and blotted with anti-phosphotyrosine antibody. A, results from 15 and 45 min after VEGF injection. B, results from a separate experiment 90 min after a VEGF injection. C, quadruplicate samples from a 90 min VEGF injection were normalized to ZO-1 content and averaged. Tyrosine phosphorylation of ZO-1 increased 4-fold with VEGF injection and was significantly different according to the Mann-Whitney test; p < 0.03.

The observed increase in occludin phosphorylation and extractability in cells in culture again correlate with increased permeability. Previous experiments by Yaccino et al. (21) and Chang et al. reveal an increase in BAEC and BREC water permeability in response to VEGF, which started at about 20 or 60 min, respectively, after the addition of VEGF.2 In these experiments the water permeability continued to increase as long as the measurements were obtained (up to 4 h). Thus, if the observed correlation in occludin phosphorylation is indeed associated with the mechanism for increased water permeability, then the phosphorylation may be an initiating event that would precede additional changes in the tight junctions, yielding continued increased permeability. In a previous report we showed that VEGF treatment of BREC cultures reduced occludin content by 50% after 6 h (17); therefore, it is possible that occludin phosphorylation signals a degradation process. Conversely, occludin phosphorylation may initiate allosteric conformational changes allowing acute increases in permeability across the endothelial barrier, and degradation of the tight junction protein may then contribute to long term increased permeability.

To our knowledge this is the first observation of occludin phosphorylation associated with increased barrier permeability. Previously, Sakakibara et al. (22) reported occludin phosphorylation associated with decreased barrier permeability in Madin-Darby canine kidney cells. The investigators showed that a fraction of occludin in the Nonidet P-40 insoluble pool was hyperphosphorylated and migrated at an apparent molecular mass of ~82 kDa. Furthermore, this phosphorylated form of occludin was lost upon low calcium treatment, which caused increased permeability. Our observation differs from their report in several ways; occludin phosphorylation in response to
VEGF occurs in a time frame consistent with mediating increased barrier permeability, causes occludin to migrate at ~62 kDa, and represents the majority of the occludin protein after VEGF treatment. The increase in the occludin phosphorylation state migrating at 62 kDa could be because of an increase in kinase activity or because of a phosphatase active on hyperphosphorylated occludin. Determinations of the sites of phosphorylation as well as the kinases or phosphatases responsible are currently under way in our laboratory.

In addition to changes in the occludin phosphorylation state, VEGF induces tyrosine phosphorylation of ZO-1. In experiments identical to those described above, VEGF (final concentration 25 ng/ml) was injected into the vitreous cavity of anesthetized rats, and vehicle was injected into the contralateral eye. The eyes were enucleated, and retinas were removed and homogenized in urea extraction buffer as described above. A portion of the sample was diluted 1 in 10, and ZO-1 was immunoprecipitated. Precipitated proteins were separated on polyacrylamide gels, blotted, and probed for phosphotyrosine. As seen in Fig. 5A, VEGF induced an increase in ZO-1 tyrosine phosphorylation after 15 and 45 min. The top two arrows indicate the typical doublet of ZO-1 depicting the α and α isoforms at ~220 kDa, and the lower arrow may represent ZO-2, a protein of ~180 kDa. In a separate experiment the tyrosine phosphorylation of ZO-1 was observed at 90 min and quantitated relative to immunoprecipitated ZO-1 content. VEGF caused a 4-fold increase in ZO-1 tyrosine phosphorylation in rat retinas 90 min after intravitreal injection (Fig. 5, B and C). A role for ZO-1 tyrosine phosphorylation in tight junction regulation is not unprecedented; pervanadate and the more specific tyrosine phosphatase inhibitor phenylarsine oxide both increased the tyrosine phosphorylation state of a number of junctional complex proteins including ZO-1 and decreased trans-electrical resistance in MDCK cells (23). Temperature-sensitive v-Src transformed cells increased ZO-1 tyrosine phosphorylation at the permissive temperature and decreased trans-electrical resistance (24). Conversely, EGF stimulated the tyrosine phosphorylation of ZO-1 and was associated with the movement of ZO-1 from a diffuse cytoplasmic location to the plasma membrane in subconfluent A431 cells (25). Therefore, it appears that ZO-1 tyrosine phosphorylation is a component of tight junction regulation.

Diabetic retinopathy is a leading cause of blindness in the United States and is strongly correlated with vascular permeability (26). The cause of the vascular changes is still under debate. However, recent studies have revealed an increase in permeabilizing agents in the retina of both animal models and patients with diabetes. Studies by Aiello et al. have revealed that VEGF levels are increased in the vitreous fluid of patients with diabetic retinopathy (27) as well as in animal models of diabetes (20). Additionally, a soluble form of the VEGF receptor inhibited vascular angiogenesis in a hypoxia model of retinopathy (28). An increase in VEGF appears after 6 mo of experimentally induced diabetes in the rat (29), a time when vascular permeability is induced but vascular angiogenesis remains dormant. Experimental autoimmune uveoretinitis increases the expression of VEGF and transforming growth factor-β and also induces increased vascular permeability without stimulating angiogenesis (30).

The ability of VEGF to increase permeability in an endothelial barrier may arise from a number of distinct mechanisms that are not mutually exclusive. VEGF may stimulate transporer activity for specific molecules as was observed for GLUT-1 glucose transporter activity in BREC culture (31). Alternatively, VEGF may stimulate endocytosis and vesicular transport. Interestingly, VEGF stimulates the production of vesiculo-vacuolar organelles, or VVOs, that may act as a channel through venule endothelial cells (32, 33). However, the vasculature of the retina does not appear to contain many vesicles (34), and production of large VVOs in retinal blood vessels has not been reported in diabetes. Alternatively, VEGF may regulate paracellular permeability by regulating endothelial cell to cell junctions, a mechanism supported by the data presented herein. VEGF-induced changes in tight junction protein phosphorylation state may be a fundamental mechanism by which vascular permeability is regulated. These findings have importance for the understanding of physiologic regulation of vascular permeability for normal tissue function, and the observed changes in tight junction proteins may contribute to the tissue edema that results from acute inflammatory insults and chronic diseases, such as diabetic retinopathy and tumors.

REFERENCES

Vascular Endothelial Growth Factor Induces Rapid Phosphorylation of Tight Junction Proteins Occludin and Zonula Occluden 1: A POTENTIAL MECHANISM FOR VASCULAR PERMEABILITY IN DIABETIC RETINOPATHY AND TUMORS

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