Vascular Endothelial Growth Factor Induces Rapid Phosphorylation of Tight Junction Proteins Occludin and Zonula Occluden 1

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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. Intra-ocular 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, intra-ocular 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 Rab 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 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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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 antizo-1, and mouse anti-1 p-tyrosine polyclonal, rabbit anti-phosphorylated mononclonal antibody (4G10, Upstate Biotechnology Inc.). Cell culture reagents and chemicals were obtained through Sigma except minimum Eagle’s medium vitamins were from Mediatech, endothelial cell growth supplement from Collaborative Biochemical, antibiotics/antimycotic 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 table CompleteTM (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 concentration of ~25 ng/ml. The rats were sacrificed by decapitation, eyes 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 (19). The day before the experiment, the cells were stepped down to 10% fetal calf serum, or the cells were stepped down to serum-free medium with 103 mM hydrocortisone for 2 days. Recombinant human VEGF was added to the cell culture at 25 ng/ml while vehicle was injected into the contralateral 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 insulin pellet using Laemml 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). Western immunoblotting of retinal samples in our laboratory revealed that occludin expression was restricted to the retinal vasculature (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).

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). Here, 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 BAE 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 anesthetized, and VEGF was injected into one eye to a final concentration of 25 ng/ml while vehicle was injected into the contralateral 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). Western immunohistochemical staining of rat retinas in our laboratory revealed that occludin expression was restricted to the retinal vasculature (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).


D. A. Antonetti, unpublished observations.
FIG. 1. VEGF causes a change in retinal endothelial cell occludin migration on SDS-polyacrylamide gel electrophoresis. 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 immunoblotted for occludin. On the blots, each pair of VEGF and saline injections represents the result from an individual animal. A, occludin migrates as two major bands of approximately 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.

cubated in parallel without alkaline phosphatase (Fig. 2). This change in occludin migration pattern was observed for both saline- and VEGF-treated rat retinas. The single band observed with alkaline phosphatase incubation may have run faster than the fastest migrating band in the control samples and could be caused by a persistent phosphorylation of occludin, although a definitive conclusion could not be drawn. This rapid change in occludin phosphorylation and extractability correlates well with the ability of VEGF to increase vascular permeability. Experiments by Aiello et al. (20) revealed that VEGF caused fluorescein to leak into the retina from the vascular lumen starting at about 10 min after injection of hormone into the rat vitreous cavity.

The ability of VEGF to stimulate occludin phosphorylation and transiently increase the amount of occludin extracted was also observed in bovine retinal endothelial cells. BREC cultures were brought to confluence for 2 days, were stepped down from 20 to 10% serum overnight, and were then treated with VEGF for varying amounts of time before being harvested in urea extraction buffer. Untreated BREC yielded two bands of approximately 60 and 62 kDa when blotted for occludin (occludin α and β, respectively). The higher base-line level of occludin phosphorylation in the BREC cultures correlates with the greater permeability of retinal endothelial cells in culture com-
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 phosphotyrosine 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. These cells were treated with 1.2 nM VEGF and extracted in the urea buffer. Again, the shift in occludin migration from two bands, as seen after 0 and 5 min, to one band, after 15 and 30 min, was observed, and this effect partly reversed by 60 min. Additionally, the increased extractability of occludin was even more pronounced under these conditions (Fig. 4, A and B). Further studies of the effects of hydrocortisone on endothelial cell barrier properties are currently being completed.

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. 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. 5B 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 the 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 Asahi 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 angio genesis 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 transporter 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.
Vascular Endothelial Growth Factor Induces Rapid Phosphorylation of Tight Junction Proteins Occludin and Zonula Occluden 1: A POTENTIAL MECHANISM FOR VASCULAR PERMEABILITY IN DIABETIC RETINOPTH AND TUMORS

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