VEGF increases permeability of the blood-brain barrier via a nitric oxide synthase/cGMP-dependent pathway

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Mayhan, William G. VEGF increases permeability of the blood-brain barrier via a nitric oxide synthase/cGMP-dependent pathway. Am. J. Physiol. 276 (Cell Physiol. 45): C1148–C1153, 1999.—It appears that the expression of vascular endothelial growth factor (VEGF) is increased during brain injury and thus may contribute to disruption of the blood-brain barrier (BBB) during cerebrovascular trauma. The first goal of this study was to determine the effect of VEGF on permeability of the BBB in vivo. The second goal was to determine possible cellular mechanisms by which VEGF increases permeability of the BBB. We examined the pial microcirculation in rats using intravital fluorescence microscopy. Permeability of the BBB [clearance of FITC-labeled dextran of molecular mass 10,000 Da (FITC-dextran-10K)] and diameter of pial arterioles were measured in absence and presence of VEGF (0.01 and 0.1 nM). During superfusion with vehicle (saline), clearance of FITC-dextran-10K from pial vessels was minimal and diameter of pial arterioles remained constant. Topical application of VEGF (0.01 nM) did not alter permeability of the BBB to FITC-dextran-10K or arteriolar diameter. However, superfusion with VEGF (0.1 nM) produced a marked increase in clearance of FITC-dextran-10K and a modest dilatation of pial arterioles. To determine a potential role for nitric oxide and stimulation of soluble guanylate cyclase in VEGF-induced increases in permeability of the BBB and arteriolar dilatation, we examined the effects of N\(^\text{G}\)-monomethyl-L-arginine (L-NMMA; 10 \mu M) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 1.0 \mu M), respectively. L-NMMA and ODQ inhibited VEGF-induced increases in permeability of the BBB and arteriolar dilatation. The findings of the present study suggest that VEGF, which appears to be increased in brain tissue during cerebrovascular trauma, increases the permeability of the BBB via the synthesis/release of nitric oxide and subsequent activation of soluble guanylate cyclase.

fluorescein isothiocyanate-dextran; cerebral venules; pial arterioles; soluble guanylate cyclase; N\(^{\text{G}}\)-monomethyl-L-arginine; vascular endothelial cell growth factor

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF), also called vascular permeability factor, is an endothelial cell-specific mitogen (25). In addition to its mitogenic effect, VEGF has been shown to increase the permeability of the peripheral circulation and endothelial cell monolayers (2, 3, 7, 10, 20, 26, 30). Mechanisms that account for the effects of VEGF on vascular and endothelial cell permeability in the peripheral circulation are varied but appear to involve an increase in endothelial cell calcium influx (3), synthesis/release of nitric oxide (20, 28, 30) with subsequent activation of guanylyl cyclase (30) and protein kinase G (30), increased synthesis of platelet-activating factor (26), and/or an increased release of products via activation of the cyclooxygenase pathway (20). Thus it appears that VEGF is an important regulator of vascular permeability in the peripheral circulation.

VEGF also appears to play an important role in the cerebral circulation. VEGF mRNA is expressed in normal brain tissue (18), and the expression of VEGF increases during brain injury (21, 23) and in brain tumors (4, 8, 9, 24). Thus it is possible that VEGF may contribute to disruption of the blood-brain barrier (BBB) during brain injury and may be responsible for tumor angiogenesis and tumor-related increases in permeability of the BBB. Investigators have examined the effects of VEGF on permeability of cultured endothelium from brain microvessels (29, 31). These investigators (29, 31) report that VEGF produced an increase in the transport of small molecules (sucrose and fluorescein) across cerebral endothelium. However, mechanisms by which VEGF increases permeability of the BBB were not examined in these previous studies (29, 31).

No studies have used in vivo methodologies to directly examine the permeability of the BBB to VEGF and the role of a nitric oxide/cGMP-dependent pathway in alterations in permeability of the BBB in response to VEGF. Thus the first goal of this study was to determine the in vivo effect of VEGF on permeability of the BBB. The second goal of this study was to examine potential cellular mechanisms that may account for VEGF-induced increases in permeability of the BBB. Specifically, we examined the role of synthesis/release of nitric oxide and stimulation of soluble guanylate cyclase in VEGF-induced increases in permeability of the BBB in vivo.

METHODS

Preparation of animals. Male Wistar-Furth rats (n = 24) were anesthetized (Nactan; thiobutabarbital 100 mg/kg ip), and a tracheotomy was performed. The rats were mechanically ventilated with room air and supplemental oxygen. A catheter was placed in a femoral artery for the measurement of systemic blood pressure and to obtain blood samples. A catheter was placed in a femoral vein for injection of the intravascular tracer FITC-dextran (mol mass 10,000 Da; FITC-dextran-10K). All procedures were carried out following Institutional Animal Care and Use Committee approval.

To visualize the cerebral microcirculation, a cranial window was prepared over the parietal cortex using methods we have described previously (14–16). An incision was made in the skin to expose the skull. The skin was retracted with sutures and served as a "well" for the suffusion fluid. Inlet
and outlet ports were made in the skin to allow for the constant flow of suffusate across the cerebral (pial) microcirculation. Finally, a craniotomy was performed, the dura was incised, and the cerebral microcirculation was exposed. The suffusion fluid (artificial cerebrospinal fluid) was heated (37 ± 1°C) and bubbled continuously with 95% nitrogen and 5% carbon dioxide to maintain gases within normal limits. Blood gases were also monitored and maintained within normal limits. At the end of the experiment, all anesthetized rats were killed with an injection of saturated potassium chloride.

Permeability of the BBB. The permeability of the BBB was evaluated by calculating the clearance of FITC-dextran-10K (10^6 ml/s) by the area of parietal cortex exposed by the craniotomy, as we have described previously (14–16). Briefly, the suffusate fluid was collected in glass test tubes with the aid of a fraction collector, and we determined the concentration of FITC-dextran-10K in the suffusate fluid during topical application of vehicle (saline), VEGF (0.01 and 0.1 nM), VEGF (0.1 nM) in the presence of N^0-nitro-l-arginine (L-NMMA; 10 µM), and VEGF (0.1 nM) in the presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 1.0 µM). FITC-dextran-10K was infused intravenously (40 mg/ml at 0.06 ml/min), and arterial blood samples (~60 µl/sample) were drawn at various intervals throughout the experimental period. To quantitate the concentration of FITC-dextran-10K in the suffusate fluid and plasma samples, standard curves for concentration of FITC-dextran-10K vs. percent transmission were obtained with a spectrophotofluorometer (PerkinElmer model LS30). The standard (FITC-dextran-10K) was prepared on a weight-per-volume basis. The suffusate concentration was used as background, a standard curve was generated for each experiment, and each curve was subjected to linear regression analysis. The percent transmission for unknown samples (suffusate and plasma) was measured on the spectrophotofluorometer, and the concentration was calculated from the standard curve. The clearance of FITC-dextran-10K was calculated by multiplying the suffusate-to-plasma concentration ratio by the suffusate flow rate (14–16).

Pial arteriolar diameter. Diameter of pial arterioles was measured online using a video image shearing device (model 908, Instrumentation for Physiology and Medicine). We measured the diameter of the largest pial arteriole exposed by the craniotomy before and during application of vehicle, VEGF, VEGF in the presence of L-NMMA, and VEGF in the presence of ODQ. We report the maximum change in diameter of cerebral arterioles, which appeared to occur 40 min after the start of superfusion with VEGF. In addition, we also examined the effects of ODQ on nitroglycerin-induced responses of pial arterioles to determine the efficacy of ODQ on cGMP-mediated vasodilatation.

Experimental protocol. The first group of rats served as time controls (n = 5). Two hours after the preparation of the craniotomy, a constant injection of FITC-dextran-10K (40 mg/ml at 0.06 ml/min) was started. Thirty minutes after starting infusion of FITC-dextran-10K, we started a continuous suffusion of vehicle (saline) over the cerebral microcirculation. The clearance of FITC-dextran-10K was determined for the next 60 min during suffusion with vehicle. Diameter of pial arterioles was determined immediately before and 5 and 10 min after the start of suffusion with vehicle and every 10 min thereafter.

In a second group (n = 4) and third group (n = 5) of rats, we examined the effects of VEGF (0.01 and 0.1 nM, respectively) on the permeability of the BBB to FITC-dextran-10K and reactivity of cerebral arterioles. The protocol followed was similar to the one described above, with the exception that the cranial window preparation was suffused with VEGF instead of vehicle. Clearance of FITC-dextran-10K and diameter of cerebral arterioles were determined as described above.

In a fourth group (n = 4) of rats, we examined the role of nitric oxide in VEGF-induced increases in permeability of the BBB and reactivity of cerebral arterioles. In these studies, we suffused the cranial window preparation with L-NMMA (10 µM) 60 min before a topical application of VEGF (0.1 nM) and continued L-NMMA suffusion during VEGF application. Thus clearance of FITC-dextran-10K was determined while the preparation was suffused with VEGF in the presence of a continuous suffusion of L-NMMA. Diameter of cerebral arterioles was measured under control conditions, during topical application of L-NMMA, and during topical application of VEGF in the presence of L-NMMA.

In a fifth group (n = 6) of rats, we examined the role of soluble guanylate cyclase in VEGF-induced increases in permeability of the BBB and reactivity of cerebral arterioles. In these studies, the protocol followed was similar to that outlined for L-NMMA, with the exception that ODQ (1.0 µM) was suffused over the cranial window preparation. Clearance of FITC-dextran-10K and diameter of cerebral arterioles were determined as described above. In addition, to determine the efficacy of ODQ for soluble guanylate cyclase, we examined responses of cerebral arterioles to nitroglycerin before and after treatment with ODQ.

RESULTS

Response to VEGF. Clearance of FITC-dextran-10K remained relatively constant during suffusion with vehicle (Fig. 1). Clearance of FITC-dextran-10K also was not significantly increased by suffusion with the low dose (0.01 nM) of VEGF (Fig. 1). In contrast, there was a marked increase in clearance of FITC-dextran-10K during suffusion with the 0.1 nM concentration of VEGF (Fig. 1). The increase in clearance of FITC-dextran-10K compared to control conditions was statistically significant (P < 0.05 vs. response during suffusion with vehicle).

Fig. 1. Clearance of 10,000-Da FITC-dextran (FITC-dextran-10K), in 10^6 ml/s, during suffusion with vehicle, 0.01 nM vascular endothelial growth factor (VEGF), or 0.1 nM VEGF. Values are means ± SE. *P < 0.05 vs. response during suffusion with vehicle.
dextran-10K in response to VEGF occurred rapidly, i.e., within 8–10 min after the start of suffusion with VEGF, and continued to increase during the time course of the experiment. During suffusion with VEGF, we observed that extravasation of FITC-dextran-10K appeared to be localized around cerebral venules and veins.

As to arteriolar diameter, we report values at 40 min after the start of superfusion with vehicle or VEGF. This time appeared to be the time of the maximum change in arteriolar diameter in response to VEGF. Application of vehicle did not alter diameter of cerebral arterioles (Fig. 2; baseline diameter 30 ± 1 µm). In addition, application of VEGF (0.01 nM) did not alter diameter of cerebral arterioles (Fig. 2; baseline diameter 47 ± 3 µm). However, superfusion with VEGF (0.1 nM) produced a modest but significant increase in arteriolar diameter (Fig. 2; baseline 32 ± 5 µm).

Thus it appears that suffusion with VEGF (0.1 nM) produces a marked increase in permeability of the BBB to FITC-dextran-10K and a modest but significant increase in diameter of pial arterioles.

Response to VEGF in the presence of L-NMMA and ODQ. L-NMMA (10 µM) and ODQ (1.0 µM) significantly inhibited the clearance of FITC-dextran-10K during application of VEGF (0.1 nM; Fig. 3). The clearance of FITC-dextran-10K during suffusion with VEGF (0.1 nM) in the presence of L-NMMA was similar in magnitude to that reported during suffusion with vehicle (P > 0.05; Fig. 1).

Topical application of L-NMMA (10 µM) produced a modest but significant constriction of pial arterioles (11 ± 2% baseline diameter 60 ± 4 µm) before suffusion with VEGF. In addition, L-NMMA significantly inhibited dilatation of pial arterioles in response to VEGF (0.1 nM; Fig. 2; baseline diameter 53 ± 4 µm). Topical application of ODQ (1.0 µM) also produced a modest (7 ± 3%) decrease in baseline diameter (47 ± 3 µm) of cerebral arterioles. However, unlike that reported for L-NMMA, this change in baseline diameter in response to ODQ failed to reach statistical significance (P > 0.05). In addition, topical application of ODQ significantly inhibited dilatation of cerebral arterioles in response to VEGF (Fig. 2; baseline diameter 44 ± 2 µm) and significantly inhibited dilatation of cerebral arterioles in response to nitroglycerin (Fig. 4; baseline diameter 47 ± 3 µm).

Thus it appears that the synthesis/release of nitric oxide or a nitric oxide-containing compound with subsequent activation of soluble guanylate cyclase plays a significant role in VEGF-induced increases in permeability of the BBB and dilatation of rat pial arterioles.

**DISCUSSION**

The main findings of the present study are that in vivo application of VEGF to the cerebral microcircula-
tion produces an increase in permeability of the BBB to a molecule of moderate size (FITC-dextran-10K) and produces a modest dilatation of cerebral arterioles. In addition, this increase in permeability of the BBB and dilatation of cerebral arterioles in response to VEGF could be inhibited by application of L-NMMA, an enzymatic inhibitor of nitric oxide synthase, or ODQ, a novel inhibitor of soluble guanylate cyclase. Thus it appears that the actions of VEGF on the cerebral microcirculation involve the synthesis/release of nitric oxide with a subsequent activation of soluble guanylate cyclase.

Consideration of methods. The permeability of the BBB was evaluated by calculating the clearance of FITC-dextran-10K by the area of brain exposed by the craniotomy. This methodology has been used by our laboratory in many previous studies to evaluate the permeability characteristics of the BBB (14–16). No extravasation of FITC-dextran-10K was observed from the dura, skin, or bone during suffusion with vehicle and/or VEGF, and we suggest that increases in clearance of FITC-dextran-10K during topical application of VEGF represent changes in permeability that are occurring in pial vessels.

We evaluated changes in permeability of the BBB in response to two concentrations of VEGF (0.01 and 0.1 nM). Although studies have shown that VEGF mRNA is expressed by normal brain tissue (18) and that brain injury increases the expression of VEGF (21, 23), no studies to our knowledge have examined the concentration of VEGF produced during brain injury. Thus it is not possible for us to determine whether the concentrations of VEGF used in the present study actually represent that observed during brain injury. The choice of concentrations of VEGF used in the present study was based on previous studies that have examined the effects of VEGF on permeability of the peripheral circulation and/or monolayers of endothelium from the peripheral circulation (2, 3, 7, 10, 20, 26, 30) and in vitro studies that have examined the effects of VEGF on permeability of cerebral endothelium (29, 31).

We used L-NMMA to examine the role of nitric oxide in permeability of the BBB and dilatation of pial arterioles following topical application of VEGF. The concentration of L-NMMA (10 μM) used in the present study has been shown to be specific and efficacious in inhibition of the effects of nitric oxide or a nitric oxide-containing compound on cerebral arterioles (6, 11, 17), disruption of the BBB during acute hypertension (12), or disruption of the BBB during topical application of histamine (13). Thus we suggest that the concentration of L-NMMA used in the present study is appropriate for examining the effects of nitric oxide on the cerebral microcirculation.

We also examined the role of activation of soluble guanylate cyclase in disruption of the BBB in response to VEGF and dilatation of cerebral arterioles in response to VEGF and nitroglycerin by using ODQ. It appears that relaxation of vascular smooth muscle in response to nitric oxide is accomplished by activation of soluble guanylate cyclase and the production of cGMP (19, 22, 27). However, recent evidence suggests that nitric oxide may also affect blood vessels via a soluble guanylate cyclase-independent mechanism (1, 5). In the present study, we found that ODQ, which is a specific inhibitor of soluble guanylate cyclase in cerebral arterioles (27), significantly inhibited dilatation of rat cerebral arterioles in response to VEGF and nitroglycerin. Thus we suggest that endogenous (produced in response to VEGF) and exogenous (produced in response to nitroglycerin) effects of nitric oxide on cerebral vessels are mediated predominantly by activation of soluble guanylate cyclase.

Consideration of previous studies. Several previous studies have examined the effect of VEGF on the permeability of the peripheral circulation. These previous studies have shown that VEGF increases the permeability of isolated perfused microvessels of the coronary (30) and mesenteric (2, 3, 26) circulations, of the skin (20), of the trachea, bronchi, and pancreas (26), and of endothelial cell cultures (10, 26). Mechanisms that contribute to changes in vascular permeability of the peripheral circulation in response to VEGF are varied. Murohara et al. (20) suggested that VEGF-induced increases in permeability are related to the synthesis/release of nitric oxide, since inhibitors of nitric oxide synthase attenuate the VEGF-induced increase in vascular permeability. Wu et al. (30) also reported that inhibitors of nitric oxide synthase attenuate VEGF-induced increase in venular permeability of the coronary circulation. In addition, these investigators (30) found that inhibitors of guanylate cyclase and protein kinase G also prevent VEGF-induced increases in coronary venular permeability. Thus it appears that VEGF may modulate changes in vascular permeability of the coronary microcirculation via a pathway involving synthesis/release of nitric oxide with subsequent stimulation of guanylate cyclase and protein kinase G. Others have suggested that VEGF may increase vascular permeability via the release of prostaglandins (20), synthesis of platelet-activating factor (26), an increase in the flux of calcium across the endothelium (3), and/or a direct effect of VEGF on vascular endothelium (10). The present study extends these previous findings by examining the effects of VEGF on the permeability and reactivity of the cerebral microcirculation using in vivo methodologies.

Although previous studies have shown that VEGF is expressed by brain tissue under physiological (18) and pathophysiological (21, 23) conditions, few studies have examined the effects of VEGF on permeability of the BBB. Two studies have examined the effects of VEGF on the transport of small molecules across endothelium derived from brain microvessels (29, 31). Wang et al. (29) found that VEGF increased the permeability of cerebral endothelium to [14C]sucrose. In addition, these investigators report that the increase in transport of sucrose across cerebral endothelium was greater when VEGF was applied to the basolateral side of the endothelial membrane than when it was applied to the apical side (29). However, mechanisms by which VEGF increased the flux of sucrose across cerebral endothelium were not investigated in this previous study (29). A
more recent study by Zhao et al. (31) examined the flux of carboxyfluorescein (mol wt 376) across endothelial cells derived from brain capillaries during stimulation with VEGF. These investigators report that VEGF, when applied to the abluminal side of cerebral endothelium, produced an increase in the flux of carboxyfluorescein (31). However, mechanisms by which VEGF increased the flux of carboxyfluorescein across cerebral endothelium were not examined by these investigators (31). The findings of the present study complement the results of these two previous studies (29, 31). We report that VEGF increases the permeability of the BBB to a moderately sized molecule (FITC-dextran-10K) and dilates cerebral arterioles. The increase in the permeability of the BBB to FITC-dextran-10K results from VEGF increases the permeability of the BBB to FITC-dextran-10K. In addition, the findings of the present study extend those of previous studies (29, 31) by examining the potential cellular mechanisms by which VEGF increases the permeability of the BBB and cerebral arteriolar diameter.

In summary, we found that VEGF, when applied topically to the cerebral microcirculation, produces an increase in the permeability of the BBB to FITC-dextran-10K and dilates cerebral arterioles. The increase in permeability of the BBB and dilatation of cerebral arterioles in response to VEGF appear to involve a mechanism dependent on the synthesis/release of nitric oxide or a nitric oxide-containing compound and stimulation of soluble guanylyl cyclase. We suggest that the production of VEGF may contribute to the pathogenesis of disruption of the BBB and the development of cerebral edema during brain injury.

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