Hypoxia induces permeability in brain microvessel endothelial cells via VEGF and NO

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Fischer, Silvia, Matthias Claus, Marion Wiesnet, Dieter Renz, Wolfgang Schaper, and Gerhard F. Karliczek. Hypoxia induces permeability in brain microvessel endothelial cells via VEGF and NO. Am. J. Physiol. 276 (Cell Physiol. 45): C812–C820, 1999.—In this study, an in vitro model of the blood-brain barrier, consisting of porcine brain-derived microvascular endothelial cells (BMEC), was used to evaluate the mechanism of hypoxia-induced hyperpermeability. We show that hypoxia-induced permeability in BMEC was completely abolished by a neutralizing antibody to vascular endothelial growth factor (VEGF). In contrast, under normoxic conditions, addition of VEGF up to 100 ng/ml did not alter monolayer barrier function. Treatment with either hypoxia or VEGF under normoxic conditions induced a twofold increase in VEGF binding sites and VEGF receptor 1 (Flt-1) mRNA expression in BMEC. Hypoxia-induced permeability also was prevented by the nitric oxide (NO) synthase inhibitor Nω-monomethyl-L-arginine, suggesting that NO is involved in hypoxia-induced permeability changes, which was confirmed by measurements of the cGMP level. During normoxia, treatment with VEGF (5 ng/ml) increased permeability as well as cGMP content in the presence of several antioxidants. These results suggest that hypoxia-induced permeability in vitro is mediated by the VEGF/VEGF receptor system in an autocrine manner and is essentially dependent on reducing conditions stabilizing the second messenger NO as the mediator of changes in barrier function of BMEC.

blood-brain barrier; endothelial barrier function; hyperpermeability; nitric oxide; vascular endothelial growth factor

CEREBRAL ENDOTHELIAL CELLS form the blood-brain barrier (BBB), which regulates the homeostasis of the central nervous system in vivo. The barrier properties of the BBB result from the presence of tight intercellular junctions between endothelial cells, the low number of pinocytic vesicles, and the absence of fenestrations (5, 6). Electrical resistance measurements can be used to document the tightness of the intercellular junctions. In vivo, Crone and Olesen (12) reported a value of 1,800 Ω·cm² in frog brain endothelial cells, whereas in vitro resistance measurements on monolayers of cultured brain capillary endothelial cells yielded values ranging from 60 to 300 Ω·cm² (16, 20, 24, 47).

Pathological conditions like local ischemia or brain tumors have been associated with disruption of the BBB leading to the development of vasogenic brain edema (1). Several factors are produced during hypoxia, including vascular endothelial growth factor (VEGF), which is also known as vascular permeability factor (14, 23, 28, 49, 54). VEGF stimulates cell growth and migration in vitro (17, 52), stimulates angiogenesis in vivo (10, 34), and increases the vascular permeability (15, 43, 53, 61, 62). In tumors, where VEGF production is localized to ischemic areas (41, 54), VEGF was found to be responsible for the enhanced permeability in blood vessels of solid tumors (3) and for the fluid accumulation induced by ascites tumors (51). Thus VEGF is a likely candidate for the development of ischemia-induced vasogenic brain edema (11).

VEGF binds and activates two phosphotyrosine kinase receptors on endothelial cells, the VEGF receptor 1 or fms-like tyrosine kinase (Flt-1) and the VEGF receptor 2 or kinase insert domain-containing receptor (KDR) (18). VEGF-induced endothelial hyperpermeability is caused by a direct action on endothelial cells (26) and is suggested to be mediated by enhanced transcytosis, gap formation between endothelial cells (9, 11), and the induction of fenestrations in nonfenestrated endothelium (43, 44). The downstream signaling cascade of VEGF-induced microvascular permeability also was demonstrated to involve nitric oxide (NO) as a second messenger (61). Several studies have implicated NO in the regulation of microvessel permeability (25). NO also has been demonstrated to induce reversible perturbations in a cell culture model of the BBB (27). The mechanism by which VEGF causes NO release in human endothelial cells was shown to involve activation of tyrosine and phosphoinositide 3 kinases (39). In cultured microvascular endothelial cells isolated from coronary postcapillary endothelium, VEGF triggers, via kinase-derivative receptor activation, the NO synthase (NOS)/guanylate cyclase pathway to activate the mitogen-activated protein kinase (40).

Using an in vitro model of the BBB consisting of porcine brain-derived microvascular endothelial cells (BMEC), we demonstrate that autocrine-produced VEGF mediates hypoxia-induced permeability and that addition of recombinant VEGF during hypoxia can further increase the permeability significantly. However, addition of recombinant VEGF to normoxic cultures did not increase permeability at all concentrations tested, indicating a different response of endothelial cells to VEGF in hypoxic vs. normoxic conditions. Therefore, we compared receptor expression during hypoxia and normoxia and, furthermore, explored the putative signaling role of NO.
MATERIALS AND METHODS

Cell culture. Capillary endothelial cells were isolated from porcine brains as described previously (36). The gray matter was digested with dispase II (0.5 mg/100 ml) for 3 h at 37°C. Capillaries were isolated after centrifugation with 18% dextran. The pellet was resuspended in medium 199 (M199) and treated with collagenase-dispase (0.1 mg/ml) for 5 h at 37°C. Endothelial cells were obtained after Percoll gradient centrifugation. Cells were seeded on petri dishes coated with collagen G (Biochrom, Berlin, Germany) or on rat tail collagen-coated polycarbonate Transwell inserts with a pore size of 0.4 µm (Gera & Gärte). The respiratory activity of cells did not change the content was always <1%. In vitro hypoxia model. To induce hypoxia, confluent monolayers of BMEC were washed once with PBS (pH 7.4). M199 without FCS in the absence or presence of added compounds was placed into a special chamber system containing a disposable hydrogen-generating system (GasPak Plus system). Becton Dickinson). Chambers were closed and placed inside an incubator at 37°C. After 3 h of hypoxia, the oxygen content in the culture medium was reduced to a constant concentration of 4–5% vol/vol. The concentration of oxygen in the culture incubation medium after different periods of hypoxia was determined using a digital oxygen meter (Schott Gera). The activity of cells did not change the oxygen content in the culture medium significantly. The pH of the medium was unchanged during up to 24 h of hypoxia. As a control, cultures always were incubated under normoxic conditions for the same length of time.

Assay of endothelial monolayer permeability. After seeding of BMEC onto polycarbonate membranes, maximal resistance values across the BMEC monolayer ranging from 70 to 120 Ω cm², which correspond to values determined by Erben et al. (16), were obtained after 6–8 days of culture (19). Chambers showing resistance values of >100 Ω cm² were used for measurements of the passage of [3H]inulin (Amersham, Buchler, Germany) across the BMEC monolayer. The chambers, consisting of an apical part containing the filter membrane inserts with the cell monolayer and a basolateral part, were washed three times with PBS, and 0.146 nmol (0.8 µCi) of [3H]inulin in 400 µl M199 without serum was added to the apical chamber, and 600 µl of medium were added to the basolateral chamber. Recombinant VEGF 165, a polyclonal antibody (Ab) against human VEGF (PreproTech, London, UK), rabbit anti-goat IgG (Sigma), N°-monomethyl-l-arginine (l-NMMA), 8-bromo guanosine 3',5'-cyclic monophosphate (8-BrcGMP; Biotrend, Cologne, Germany), sodium nitroprusside (SNP; Sigma), α-lipoic acid (thiotocaid, Merck, Darmstadt, Germany), or the combination of vitamin C and vitamin E acetate (Sigma) was added to both chambers at the start of the experiment, and chambers were incubated under normoxic or hypoxic conditions at 37°C. The appearance of [3H]inulin in the basolateral chamber was measured after different periods of time by scintillation counting of small aliquots of the basolateral buffer. Results were expressed as the ratio of the inulin concentration determined after different incubation times in the lower chamber and the total concentration of [3H]inulin added to the upper chamber at the start of the experiment (C_L/C_total). During the course of the experiment, chambers were kept at 37°C and care was taken to ensure that fluid levels in the apical and basolateral chambers were equal. The amount of inulin that crossed the cell-free membrane was not changed during hypoxia or in the presence of the test substances used.

Studies of [125I]-labeled VEGF binding to BMEC cultures. Radioiodination of VEGF165 was performed using the Chloramine T method as modified for VEGF (42), and specific activities of 3–4 × 10^6 counts/min (cpm)/ng were achieved. For radioligand binding experiments, confluent BMEC grown in 24-well culture plates were either treated with VEGF (5 ng/ml) or cultured under hypoxic conditions in the absence or presence of VEGF (5 ng/ml) and/or a polyclonal Ab to VEGF (4 µg/ml) for 6 h. In control experiments, cultures were grown under normoxic conditions for the same length of time. After the incubation period, culture medium was aspirated and the BMEC were washed twice with Hank’s balanced salt solution (HBSS). To remove bound VEGF, cells were incubated for 3 min on ice with acid wash buffer (10 mM HCl and 0.2 M NaCl, pH 2.1), followed by two washes with HBSS. Cells were incubated with 0.05 nM [125I]-VEGF in HBSS containing 20 mM HEPES and 0.1% gelatin, with or without a 100-fold excess of unlabeled VEGF for 2 h at 4°C. After the incubation period, cultures were washed four times with HBSS and trypsinized using 0.5% trypsin and 2 mM EDTA, and cell-bound [125I]-VEGF was measured in a gamma-counter (Packard, Frankfurt, Germany). Specific binding of [125I]-VEGF was calculated from the difference of [125I]-VEGF measured in wells incubated with [125I]-VEGF alone and subtracted from the radioligand binding activity determined in all cultures incubated with [125I]-VEGF in the presence of 100-fold molar excess of unlabeled material. The specific binding determined in normoxic cultures was set to 100%.

Extraction of total cellular RNA and Northern blot analysis. When cells reached confluency, plates were washed once with PBS and cultured in the absence or presence of VEGF (5 ng/ml) and/or anti-VEGF Ab (4 µg/ml) under normoxic or hypoxic conditions for the indicated times. For the isolation of RNA, cells were washed once with PBS and harvested directly into guanidinium-thiocyanate buffer, and RNA was isolated as described by Chomczynski and Sacchi (8). For Northern blot analysis, 15 µg of total RNA were denatured at 65°C in loading buffer containing formamide and ethidium bromide and were subsequently electrophoresed on a 1% wt/vol agarose gel containing 2.2 M formaldehyde. RNA was transferred with 125I-VEGF alone and subtracted from the activity determined in all cultures incubated with [125I]-VEGF in the presence of 100-fold molar excess of unlabeled material. The specific binding determined in normoxic cultures was set to 100%.

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of $1 \times 10^6$ cpm/ml and incubated overnight. Filters were washed twice in 2× SSC-0.1% SDS at room temperature for 15 min each and once at 42°C for 20 min and were exposed to Kodak XAR films at −70°C for 1–4 days. An 18S cDNA probe (kindly supplied by Dr. I. Oberbäumer) was used to rehybridize membranes for reference purposes. Quantitative analysis was performed using a PhosphorImager SF (Molecular Dynamics). To correct for differences in RNA loading, the signal intensity for each sample hybridized to the FLt-1 or KDR cDNA probe was divided by that for each sample hybridized to the 18S cDNA probe. The specificity of the FLt-1 and KDR cDNA probes was controlled by preparing RNA from mouse tissue and human umbilical endothelial cells. The sizes of the mRNAs for the FLt-1 and KDR receptors in these tissues (M. Minne, unpublished results) were the same as detected in BMEC and described by us.

Measurements of cGMP concentrations. When cells reached confluency, plates were washed once with PBS and cultured in the absence or presence of VEGF (5 ng/ml), anti-VEGF Ab (4 µg/ml), SNP (1 µM), L-NMMA (100 µM), or α-lipoic acid (2 µM) under normoxic or hypoxic conditions for 6 h. cGMP was extracted from the cells by rapid aspiration of medium, washing with ice-cold PBS, and the addition of ice-cold ethanol to 65% (vol/vol). The cells were harvested and centrifuged at 2,000 g for 10 min at 4°C. Supernatants were transferred to fresh tubes and air dried. The cGMP concentration of the cell extracts was determined by using a $[^3H]$cGMP assay system from Amersham. cGMP concentration was normalized to protein content as determined by using the Coomassie protein assay kit (Pierce, Rockford, IL).

Statistical analysis. Results are expressed as means ± SE. The unpaired Student’s t-test or ANOVA and subsequent multiple comparisons using Dunn’s method were used for statistical analysis. Results were considered to be statistically different at $P < 0.05$.

RESULTS

Hypoxia-induced endothelial cell monolayer permeability is mediated by autocrine VEGF production. BMEC cultures showing resistance values of more than 100 Ω·cm² were used for permeability studies. The permeability of the BMEC monolayer to inulin increased significantly after 6 h of hypoxia compared with normoxic control cultures. These permeability changes were completely prevented in the presence of a polyclonal neutralizing Ab to VEGF, whereas the anti-IgG Ab showed no effect (Fig. 1A). Hypoxia recently has been shown to significantly increase the amount of VEGF protein released from BMEC into the culture medium (18a). These results suggest that VEGF released during hypoxia is responsible for the enhanced permeability. Although in hypoxia the permeability was further increased in the presence of additional VEGF ($10^4$ ng/ml; Fig. 1A), under normoxia the same and even higher concentrations of VEGF (up to 100 ng/ml) did not significantly increase the permeability of the BMEC monolayer (Fig. 1B). Even after 24 h of VEGF treatment (5 ng/ml), no significant change of the endothelial barrier function was observed.

Hypoxia and VEGF treatment induced specific binding sites for VEGF. To explain the unexpected finding that VEGF induces permeability under hypoxic but not normoxic conditions, changes in VEGF receptor expression were assessed by radioligand binding studies.

Therefore, the specific binding of $^{125I}$VEGF to BMEC cultures after 6 h of normoxia or hypoxia with or without VEGF (5 ng/ml) was determined. After 6 h of VEGF treatment (5 ng/ml) of normoxic BMEC cultures or after 6 h of hypoxia, the specific binding of $^{125I}$VEGF to BMEC cultures increased significantly (Fig. 2). When cells were incubated during hypoxia in the presence of additional VEGF (5 ng/ml), the specific binding of $^{125I}$VEGF increased even further. The addition of neutralizing antibodies to VEGF inhibited the elevated binding levels induced by hypoxia, both in the absence
and presence of additional VEGF (Fig. 2). This suggests that the hypoxia-induced upregulation of VEGF binding sites is mediated by an autocrine action of VEGF.

mRNA expression of the two VEGF receptors after hypoxia and VEGF treatment. To evaluate which VEGF receptor is upregulated after VEGF treatment and hypoxia of BMEC cultures, Northern blot analysis for Flt-1 and KDR expression was performed. During normoxia, the expression of both receptors was very weak, but VEGF treatment (5 ng/ml) of normoxic cultures as well as hypoxia led to a twofold increase of the Flt-1 mRNA expression (Fig. 3). Addition of VEGF (5 ng/ml) during hypoxia elevated the mRNA expression even further. The upregulation of the Flt-1 mRNA expression during hypoxia was abolished by a polyclonal neutralizing Ab to VEGF.

In contrast to the results with Flt-1, mRNA expression of the KDR receptor remained unchanged either after VEGF treatment (5 ng/ml) or hypoxia alone. However, the addition of VEGF (5 ng/ml) during hypoxia induced the KDR mRNA expression about two times (Fig. 4). Again, this induction was completely abolished by a polyclonal neutralizing Ab to VEGF.

Role of NO in hypoxia-induced permeability changes. Previous studies demonstrated that VEGF induces microvascular permeability via a signaling cascade involving NO synthesis (61). To evaluate whether VEGF elevates the permeability of the BMEC monolayer during hypoxia by inducing the synthesis of NO, endothelial barrier function during hypoxia was assessed in the presence of l-NMMA, a specific inhibitor of NO synthesis. The presence of 100 µM l-NMMA completely abolished hypoxia-induced hyperpermeability, suggesting that these permeability changes are mediated by VEGF-induced NO synthesis. During normoxia, l-NMMA did not affect endothelial barrier function. However, the NO donor SNP increased the permeability to the same extent as hypoxia, which was not changed by l-NMMA (Fig. 5).

Because NO is a known inducer of guanylate cyclase, which leads to elevated cGMP levels, we also examined, by adding 8-BrcGMP, whether increased cGMP levels might increase the permeability of the BMEC monolayer. As shown in Fig. 5, 8-BrcGMP increased the permeability significantly. The permeability changes...
Activity of guanylate cyclase during hypoxia and VEGF treatment. To confirm the release of NO during hypoxia, the activity of guanylate cyclase was determined by measuring the amount of cGMP. The hypoxia-induced increase of the cGMP level was abolished in the presence of a neutralizing Ab to VEGF and by L-NMMA. Neither VEGF nor L-NMMA showed any effect on the cGMP level during normoxia. VEGF itself did not activate guanylate cyclase during normoxia. However, during hypoxia, VEGF significantly increased the cGMP levels. As a control, we demonstrated that the NO donor SNP increased the amount of cGMP to a similar level as did hypoxia, which was not changed in the presence of L-NMMA (Fig. 6).

Stabilization of NO by antioxidants mediates VEGF activity. Because of the short half-life of NO, we tested the hypothesis that the inability of VEGF to increase permeability in normoxic BMEC cultures was caused by reactive superoxide. This free radical has been
shown to be a potent scavenger of NO (2, 13). Therefore, we assessed the effect of VEGF on endothelial barrier function during normoxia in the presence of antioxidants. Addition of α-lipoic acid or vitamin C in combination with vitamin E in the presence of VEGF (5 ng/ml) significantly induced the permeability under normoxia (Fig. 7). These permeability changes were completely abolished by l-NMMA. None of the antioxidants used increased the permeability of the BMEC monolayer in the absence of VEGF. Also, hypoxia-induced permeability changes were enhanced in the presence of the antioxidants (data not shown). Thus the increased permeability induced by VEGF under hypoxia is likely to be regulated by the redox status of the cell. In agreement with the permeability changes, the cGMP level was increased during VEGF treatment in the presence of 2 µM α-lipoic acid to 205 ± 15% in comparison with normoxic control cultures; this increase was abolished by l-NMMA (109 ± 14%).

**DISCUSSION**

This study shows that, although hypoxia-induced hyperpermeability of BMEC monolayers is mediated by VEGF in an autocrine manner, VEGF added to normoxic BMEC cultures is ineffective. Therefore, we investigated the mechanism of the contrasting effects of VEGF on permeability induction in BMEC monolayers by either normoxia or hypoxia. One explanation might be that the VEGF receptors are upregulated by hypoxia but not by normoxia. Hypoxia has been proposed to play an important role in the regulation of VEGF receptor gene expression. Both VEGF receptors are upregulated during hypoxia in the lung vasculature (56), during myocardial infarction in the rat (35), and in ischemic regions of tumors (41, 54). However, in vitro studies are less conclusive. Hypoxia upregulates Flt-1 mRNA in dermal endothelial cell monolayer cultures and in microvessels in skin explants (14), but in human umbilical vein and microvascular endothelial cells no effect on 125I-VEGF binding was observed (7). Our studies on VEGF receptor expression during hypoxia and normoxia in the absence and presence of VEGF demonstrate that both hypoxia and treatment of normoxic cultures with VEGF increase the specific binding of 125I-VEGF to BMEC. Both conditions upregulate the expression of Flt-1 mRNA but not the KDR mRNA. This is in accordance with reports showing that hypoxia regulates Flt-1 expression at the transcriptional level via a hypoxia-inducible enhancer element (21), whereas hypoxia-induced KDR upregulation has been reported to occur by a posttranscriptional mechanism (59). However, we observed KDR mRNA expression in the presence of both VEGF and hypoxia. This could be of relevance for ischemic in vivo situations, such as necrotic areas of glioblastoma, in which additional production of VEGF by sources other than endothelial cells have been reported (33, 41, 54).

Because VEGF treatment of normoxic cultures also upregulates receptor expression, which does not lead to increased permeability, VEGF receptor upregulation is not sufficient to explain the increase in permeability during hypoxia. One explanation may be that hypoxia alters the intracellular environment and/or signal transduction pathways. Recently, it was reported that VEGF is able to augment the NO release from quiescent rabbit and human vascular endothelium (57). NO is synthesized from the amino acid L-arginine by either the constitutive endothelial NOS (eNOS) or the inducible NOS (iNOS) (37). The exact mechanisms by which NO modulates permeability are not known, but NO activates cell guanylate cyclase, resulting in elevation of cytosolic cGMP levels (32, 37). In agreement with these data, Wu et al. (61) demonstrated that VEGF induces NO-dependent permeability in coronary vessels via a signaling cascade involving NO synthesis and activation of guanylate cyclase and the cGMP-dependent protein kinase. Accordingly, agents known to elevate intracellular cGMP levels have been shown to increase macromolecular permeability of capillaries both in vivo (63) and in vitro (29), which is in agreement with our own results showing that 8-BrcGMP increases the permeability of the BMEC monolayer. Furthermore, elevated cGMP concentrations appear to oppose the effects of raised cAMP levels on the electrical resistance of primary brain endothelial cells (47). Therefore, we investigated whether NO produced during hypoxia is involved in the effect of VEGF on the permeability properties of the BMEC monolayer. NO increased the permeability of BMEC, as shown by the addition of SNP, an exogenous source of NO, and hypoxia-induced permeability was prevented in the presence of l-NMMA, which selectively inhibits NOS. Hypoxia-induced NO release was confirmed by the finding that hypoxia increased the activity of guanylate

![Fig. 7. Permeability of BMEC monolayer to [3H]inulin during 6 h of normoxia in absence of added compounds (solid bar) and in presence of α-lipoic acid (αLA) or vitamin (Vit) C and vitamin E, without VEGF, with VEGF, or with VEGF and l-NMMA. α-Lipoic acid (2 µM), combination of vitamin C and vitamin E (each 100 µM), VEGF (5 ng/ml), and l-NMMA (100 µM) were added to culture medium at start of experiment, and cultures were incubated under normoxic conditions at 37°C for 6 h. Cr/C0 as per value of experiment, and cultures were incubated under normoxic conditions for 6 h. (Mean ± SE, n = 6). *P < 0.05 vs. control.)](http://ajpcell.physiology.org/)

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cylase, which was abolished in the presence of a neutralizing Ab to VEGF as well as by L-NMMA. These results suggest that VEGF released from hypoxic BMEC cultures induces NO release as the mediator of permeability changes.

However, these results cannot explain why addition of VEGF alone does not change the permeability during normoxia. The half-life of NO during normoxia is very short, because NO reacts with molecular oxygen and oxygen radicals in aqueous solutions, yielding nitrite, nitrate, and peroxynitrite (30, 46). The major pathway of NO reactivity is the rapid bimolecular reaction of NO with superoxide to peroxynitrite, which is more than three times faster than the enzymatic dismutation of superoxide catalyzed by superoxide dismutase (31). The kinetics of NO autoxidation reactions are dependent on oxygen concentration and on rates of tissue superoxide production. Therefore, we hypothesize that autoxidation reactions of NO during normoxia are faster than the activation of second messenger pathways followed by the increase in permeability. To decrease the concentration of oxygen radicals in the culture medium, naturally occurring antioxidants such as α-lipoic acid (4) as well as the combination of vitamin C and E were added to normoxic, VEGF-treated BMEC cultures. Under these conditions, VEGF increased the monolayer permeability as well as the cGMP level significantly. Accordingly, Morbidelli et al. (38) reported that NO production in response to VEGF was augmented by prolonging NO half-life in the presence of superoxide dismutase. These results suggest that VEGF increases the permeability under conditions that prevent the rapid autoxidation of the second messenger NO, namely during hypoxia or in the presence of antioxidants. Cellular antioxidant status was previously suggested to preserve the potency of nitrergic transmission and to prevent NO from reacting with other compounds to produce cytotoxic metabolites (22).

Although the increase of BMEC permeability evoked by VEGF released during hypoxia does not appear to be dramatic, it is in the range of values observed by others using the same in vitro system as in this study (60, 64). Higher permeability changes with VEGF can be observed when a special system employing hydrostatic pressures is applied (26), indicating that this effect may be more prominent under dynamic conditions in vivo. However, to evaluate whether the increase of BMEC permeability evoked by VEGF released during hypoxia is of significance in vivo, further studies such as intracerebral injections of VEGF in hypoxic vs. normoxic mice are warranted (45).

In conclusion, hypoxia-induced permeability in BMEC is mediated by the upregulation of the VEGF/VEGF receptor system, leading to NO synthesis, in an autocrine manner (Fig. 8). NO is synthesized from the amino acid L-arginine by either constitutive calcium/calcmodulin-dependent NOS (eNOS) or iNOS. In BMEC, only eNOS, and not iNOS, is expressed (data not shown), which corresponds to other results showing that eNOS is the predominant isoform expressed in endothelial cells (40). The release of calcium from the endoplasmic reticulum, necessary to activate eNOS, could occur by VEGF receptor-mediated activation of phospholipase C-γ (PLCγ). Increased Ca2+ levels together with calmodulin (CaM) activate NO synthetase [endothelial NO synthase (eNOS)] and increase NO concentration. NO, released either by activated eNOS or chemically from SNP, increases cGMP level via activated cyclic guanylate cyclase (cGC), which may result in increased permeability via activated protein kinase G (PKG). In this system of BMEC, release of NO alone, however, is not sufficient to induce hyperpermeability and requires reducing conditions stabilizing second messenger NO. DAG, diacylglycerol; INP3, inositol trisphosphate; P, phosphorus.

Fig. 8. Schematic drawing depicting relation between hypoxia, VEGF, and NO. Hypoxia upregulates expression of VEGF, which activates the VEGF and VEGF receptor (VEGF-R) system in an autocrine manner, followed by release of Ca2+ from endoplasmic reticulum (ER). This may occur by VEGF receptor-mediated activation of phospholipase C-γ (PLCγ). Increased Ca2+ levels together with calmodulin (CaM) activate NO synthetase [endothelial NO synthase (eNOS)] and increase NO concentration. NO, released either by activated eNOS or chemically from SNP, increases cGMP level via activated cyclic guanylate cyclase (cGC), which may result in increased permeability via activated protein kinase G (PKG). In this system of BMEC, release of NO alone, however, is not sufficient to induce hyperpermeability and requires reducing conditions stabilizing second messenger NO. DAG, diacylglycerol; INP3, inositol trisphosphate; P, phosphorus.
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