Hypoxia and Vascular Endothelial Growth Factor Selectively Up-regulate Angiopoietin-2 in Bovine Microvascular Endothelial Cells*

(Received for publication, November 4, 1998, and in revised form, January 15, 1999)

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The formation of blood vessels requires a series of events, including differentiation of endothelial cells, tube formation, and vascular maturation (1). Two processes termed vasculogenesis and angiogenesis take place during the formation of a mature vascular network (2–4). Previous studies have revealed some of the molecular mechanisms involved, and two families of largely endothelial cell-specific receptor tyrosine kinases are known to play crucial roles in these processes. The vascular endothelial growth factor receptor (VEGFR) family is composed of Flt-1 (fms-like tyrosine kinase-1; VEGFR-1) (5), Flk-1/KDR (fetal liver kinase/kinase domain-containing receptor; VEGFR-2) (6), and Flt-4 (fms-like tyrosine kinase-4; VEGFR-3) (7). Targeted gene disruption for Flk-1 in embryonic mice leads to loss of endothelial cells and embryonic death at embryonic day 8.5 and thus indicates the requisite role of this receptor in differentiation of hemangioblasts into endothelial cells (8). Mice lacking Flt-1, despite the presence of normal hematopoietic precursors and endothelial cells, also die at embryonic day 8.5, and the absence of tube formation strongly suggests a major role for this receptor in endothelial cell-cell or cell-matrix interactions (9). VEGF, a potent angiogenic factor and the common ligand for these two receptors, is distinctive in that its mitogenic effect is highly specific for endothelial cells (10) and its expression is up-regulated by hypoglycemia (11) and by hypoxia (12, 13). It has been suggested that VEGF is implicated not only in embryonic vascular development, but also in both physiologic angiogenesis, such as in female reproductive tissues (14), and pathologic angiogenesis, including proliferative diabetic retinopathy (15) and solid tumor growth (16). This is further substantiated by experiments in which inhibition of either VEGF or Flk-1/KDR resulted in suppression of pathologic angiogenesis (17, 18), which validates the hypothesis that the VEGF signal transduction system is a viable target for antiangiogenic therapeutic intervention.

Another family of receptor tyrosine kinases, designated the Tie (tyrosine kinase that contains immunoglobulin-like loops and epidermal growth factor-like domains) family, has also been studied and found to be expressed primarily on cells of endothelial lineage (19, 20). Recent studies revealed that mice lacking either of these receptors, Tie1 or Tie2, die later than do those lacking VEGF or VEGFRs, indicating that this family exerts its effect in the later stages of embryonic blood vessel formation (21, 22). The Tie1 signal has been implicated in the control of fluid exchange and hemodynamic stress resistance (22, 23), even though its ligand remains unidentified. In contrast, Tie2 appears to regulate the capability of endothelial cells to recruit stromal cells around the endothelial tubes and stabilizes vascular integrity (24). The phenotype of Tie2 knockout mice is distinct from that of mice lacking VEGFRs. Endothelial cells are detected in normal numbers, and tube formation occurs; but the distinction between large and small vessels is obscure, and encapsulation by periendothelial cells is absent (22). Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are newly identified ligands for the Tie2 receptor, and both bind to Tie2 receptors with similar affinity (25–27). Ang1 induces autophosphorylation of Tie2 and has a remarkable chemotactic effect on endothelial cells, whereas Ang2 competitively inhibits this effect (26, 28). Moreover, Ang2-overexpressing transgenic mice mimic the phenotype of knockout mice of Ang1 and Tie2, suggesting that Ang2 is a natural antagonist for Tie2 (26). Ang1 has been reported to be down-regulated by treatment with serum or several cytokines in human lung fibroblasts (29, 30) and also by hypoxia in rat glioma cells (30). Regulation of
Ang2 and Tie2 expression, however, has not yet been well characterized. This study addresses that both hypoxia and VEGF selectively up-regulate Ang2 expression in bovine endothelial cells despite the stable expression of Ang1 and Tie2 and that Ang2 expression is up-regulated in vivo in a mouse model of ischemia-induced retinal angiogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture and VEGF Treatment—Primary cultures of bovine retinal endothelial cells (BRECs) were isolated by homogenization and a series of filtration steps as described previously (31). Cells were grown on fibronectin (Sigma)-coated dishes (Iwaki Glass, Tokyo, Japan) containing Dulbecco’s modified Eagle’s medium with 5.5 mM glucose, 10% platelet-derived horse serum (Wheaton, Pipersville, PA), 50 mg/ml heparin, and 50 units/ml endothelial cell growth factor (Roche Molecular Biochemicals). Bovine aortic endothelial cells (BAECs) were also isolated from bovine aorta and cultured in Dulbecco’s modified Eagle’s medium containing 5% calf serum and 10% platelet-derived horse serum. Cells were cultured in 5% CO2 at 37 °C, and media were changed every 3 days. Cells were characterized for their endothelial homogeneity by immunoreactivity for factor VIII antigen and remained morphologically unchanged under these conditions, as confirmed by light microscopy. For the kinetic studies of VEGF treatment, cells were incubated with VEGF (0–125 ng/ml; Genzyme, Cambridge, MA) for the indicated time points. To determine the roles of tyrosine kinase, protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) in VEGF-induced angiogenesis, the tyrosine kinase inhibitor genistein (40 μM; LC Laboratories, Boston, MA), GF 109203X (5 μM; LC Laboratories), and PD 98059 (10 μM; Upstate Biotechnology, Lake Placid, NY), respectively, followed by stimulation with 25 ng/ml VEGF. These drug levels of the inhibitors have been shown to block each target selectively and effectively in endothelial cells (32, 33).

Amplification of Human Ang1, Ang2, and Tie2 cDNAs Using Reverse Transcriptase-Polymerase Chain Reaction (PCR)—cDNA templates for PCR were synthesized by reverse transcriptase (first strand kit, Invitrogen, Carlsbad, CA) from human umbilical vein endothelial cells (Kuraray, Osaka, Japan) according to the method recommended by the manufacturer. For Ang1, Ang2, and Tie2 cDNAs, a standard PCR was performed (PCR optimizer kit, Invitrogen) using 5′-AGA ACC ACA CGG GTA CCA CTA TGC T-3′ (Ang1 sense primer corresponding to nucleotides +671 to +692), 5′-TGT GAT CTC CAG CTC TCG C-3′ (Ang2 sense primer corresponding to nucleotides +225 to +244), and 5′-GCC TTA ATG AAC CAG CAC G-3′ (Tie2 sense primer corresponding to nucleotides +335 to +356), and 5′-ACT TCT GGG CCT CAT ACC TCC G-3′ (Tie2 antisense primer) for PCR amplification by using one cycle of 92 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min followed by 30 cycles of 92 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The PCR products were then subcloned into a vector (pCRII, Invitrogen) and sequenced in their entirety, and comparison with the published human sequences revealed complete identity by means of 36B4 control cDNA (generously provided by Dr. Lloyd P. Aiello).

Nuclear Run-on Transcription Analysis—BRECs were treated with vehicle or VEGF (25 ng/ml) for 2 h. The cells were lysed in a solubilization buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl2, and 0.5% Nonidet P-40), and the nuclei were isolated. ATF, CTP, and GTP (50 μM each) and 3.7 MBq of [3H]UTP (Amersham International) were added to the nuclear suspension (100 μl) and incubated for 30 min. The samples were extracted with phenol/chloroform and precipitated. cDNA probes (Ang2 and 36B4, 10 μg each) were then slot-blotted onto nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) and hybridized with the precipitated samples of equal counts/ml in hybridization buffer (5× SSPE, 0.1% sodium dodecyl sulfate) at 48 °C for 1 h. The binding activity was measured using the BAS-2000II densitometer. The level of Ang2 mRNA was normalized to that of 36B4 mRNA.

Analysis of Ang2 mRNA Half-life—BRECs were treated with 25 ng/ml VEGF for 2 h prior to mRNA stability experiments. Thereafter, half of the plates were returned to Dulbecco’s modified Eagle’s medium containing 50 μg/ml actinomycin D (Wako, Osaka, Japan) was added to all plates. Total RNA was isolated at 0, 3, and 6 h after the addition of actinomycin D, and Northern blot analysis was performed.

Hypoxic Treatment—BRECs or BAECs were exposed to hypoxic conditions of 1% oxygen using an advanced computer-controlled infrared water-jacketed multigas incubator (Model BL-M10, Jukigakyo, Tokyo). All cells were maintained at 37 °C in a constant 5% CO2 atmosphere with oxygen deficit induced by nitrogen replacement. Cells maintained under these conditions for periods exceeding 24 h showed no morphologic changes by light microscopy and could subsequently be passaged normally. Cells incubated under standard normoxic conditions (95% air and 5% CO2) from the same batch and passage were used as controls. To study the effect of hypoxia-induced VEGF on Ang2 expression, BRECs were incubated under hypoxic or normoxic conditions for 2 h with or without anti-VEGF neutralizing antibody (10 μg/ml; R&D Systems, Minneapolis, MN).

Immunoprecipitation Analysis of Ang2—BRECs were treated with 25 ng/ml VEGF for the VEGF study or subjected to hypoxic conditions for the hypoxic study for 12 h in serum-free, mentine-free medium with 35S (100 μCi/ml; Amersham International). Cells were washed three times with cold phosphate-buffered saline and lysed in a solubilization buffer (50 mM Hepes, pH 7.4, 10 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1% Triton X-100, 10 mM NaVO3, 20 μM leupeptin, 1.5 μM aprotinin, and 2 mM phenylmethylsulfonyl fluoride) at 4 °C for 1 h. To clear the protein extract, protein A-Sepharose (20 μl of a 50% suspension; Pharmacia Biotech, Uppsala, Sweden) was added to the cell lysates, after which they were incubated for 1 h, followed by centrifugation and collection of the supernatant. Protein concentrations were measured by a protein assay (BCA protein assay, Pierce). A specific goat anti-human Ang2 antibody (5 μg; Santa Cruz Biotechnology, Santa Cruz, CA) was added and rocked with the protein sample (500 μg) at 4 °C for 1.5 h; 10 μg of protein A-Sepharose was then added, and the sample was rocked for another 1.5 h at 4 °C. For denaturation, protein in the supernatants was denatured by the addition of 0.5% sodium dodecyl sulfate, washed five times, and boiled for 3 min in Laemmli sample buffer. The samples were separated on 7.5% SDS-polyacrylamide gel (Bio-Rad), and the gel was vacuum-dried. Results were visualized and analyzed by densitometric scanning (BAS-2000II).

Mouse Model of Ischemia-induced Retinal Neovascularization—The well established mouse model of ischemia-induced retinal neovascularization created as described previously (36). Briefly, litters of 7-day-old (postnatal day 7) C57BL/6J mice were exposed to 75% O2 for 5 days and then returned to room air at postnatal day 12 to produce retinal neovascularization. Mice of the same age maintained in room air served as controls. For in situ hybridization studies, mice at different time points during the induction of neovascularization were deeply and intraperitoneally anesthetized with sodium pentobarbital and killed by perfusion through the left ventricle with 4% paraformaldehyde in phosphate-buffered saline. Eyes were enucleated, fixed in 4% paraformaldehyde at 4 °C overnight, and embedded in paraffin. Serial 5-μm sections of the whole eyes were placed on microscope slides.

In Situ Hybridization of Ang2 mRNA Expression—Slides were treated with 0.2× SSC for 20 min, followed by washing in phosphate-buffered saline containing 0.01% diethyl pyrocarbonate, digestion with 20 μg/ml proteinase K at 37 °C for 10 min, and fixation in 4% paraformaldehyde for 5 min. Blocking was performed in phosphate-buffered saline containing 50% formamide and 2× SSC at room temperature for 1 h. Sense and antisense Ang2 cRNA probes were generated from the same plasmid used for Northern hybridization and labeled with digoxigenin-dUTP (DIG RNA labeling kit, Roche Molecular Biochemicals) as recommended by the manufacturer. The probe was confirmed by agarose gel electrophoresis. The probe was used at a concentration of 50 ng/ml. Hybridization was performed at 45 °C for 16 h. After extensive sequential washings in 2×, 1×, and 0.5× SSC, the unhybridized probe was digested with ribonuclease (Promega, Madison, WI) in 0.5× SSC. The hybridization product was detected after incubation with an alkaline phosphatase-conjugated anti-digoxigenin anti-
VGFR Stimulates Ang2 (but Not Ang1 or Tie2) mRNA Expression—To investigate the effect of VEGF treatment on Ang1, Ang2, and Tie2 expression, BRECs were exposed to VEGF (25 ng/ml) and Northern blot analysis was performed. Increased Ang2 mRNA expression was observed after 1 h of stimulation and was time-dependent, with a maximal response of a 4.6 ± 0.7-fold (p = 0.0031) increase after 2 h of stimulation (Fig. 1A). In contrast, both Ang1 and Tie2 mRNA expression remained stable. To analyze dose dependence, cells were treated with various concentrations of VEGF for 2 h. A dose-dependent increase in Ang2 mRNA was observed with an EC50 of ~12.5 ng/ml and peaked at 25 ng/ml (p < 0.0001) (Fig. 1B). These data suggest that VEGF increases Ang2 mRNA expression in both a time- and dose-dependent manner. Since expression of angiopoietins has been reported to be cell-type dependent (28) and to confirm a similar effect of VEGF on macrovascular endothelial cells, we also tested the effect of VEGF on BAECs. The resultant response revealed a 2.6 ± 0.3-fold (p = 0.0026) increase in Ang2 mRNA expression after 2 h of VEGF stimulation (25 ng/ml) (Fig. 1C). As in BRECs, VEGF had no significant effects on Ang1 or Tie2 mRNA expression in BAECs.

VGFR Increases the Rate of Ang2 mRNA Transcription—We investigated whether the VEGF-induced increase in Ang2 mRNA is derived from up-regulation of transcription or from increased mRNA stability. Nuclear run-on transcription analysis was employed to determine whether VEGF leads to an increase in the transcription initiation rate. Nuclei prepared from cells treated with VEGF (25 ng/ml) or vehicle were evaluated. VEGF treatment increased the rate of Ang2 gene transcription by 3.8-fold compared with that of controls (Fig. 2).

VGFR Does Not Increase the Half-life of Ang2 mRNA—To determine whether VEGF affects the stability of Ang2 mRNA, we evaluated the half-life of Ang2 mRNA with the aid of actinomycin D to inhibit de novo gene transcription. The half-life of Ang2 mRNA was 4.2 h when treated with VEGF and 3.8 h in unstimulated controls (Fig. 3). No significant difference was observed. These findings, together with the data from nuclear run-on transcription analysis, clearly demonstrate that the VEGF-induced increase in Ang2 mRNA was derived mainly from an increase in the transcription rate.

Effects of Tyrosine Kinase, PKC, and MAPK Inhibition on VGFR-induced Ang2 mRNA Expression—Since previous reports have shown that tyrosine kinase and PKC (32) and MAPK (37) have significant roles in VGFR-induced intracellular signaling pathways, we determined whether these molecules could have effects on VGFR-induced Ang2 mRNA expression. BRECs were treated with 25 ng/ml VEGF for 2 h after pretreatment with a protein kinase inhibitor: genistein, a tyrosine kinase inhibitor (40 μM); GF 109203X, a PKC-specific inhibitor (5 μM); or PD 098059, a MAPK kinase inhibitor (25 μM) (Fig. 4). The addition of these agents abolished the VEGF-induced increase in Ang2 mRNA expression by 84.6 ± 13.1% (p = 0.0009), 65.4 ± 16.8% (p = 0.0029), and 92.4 ± 8.5% (p = 0.0005), respectively. The 0.1% Me2SO carrier used to solubilize these inhibitors did not significantly alter Ang2 mRNA expression (data not shown). These data indicate that MAPK and tyrosine phosphorylation have a predominant role in...
Ang2 mRNA expression and that the PKC-dependent pathway makes a more minor contribution.

**Hypoxia Increases Ang2 mRNA Expression**—To investigate the effects of hypoxia on the angiopoietin-Tie2 system, BRECs were exposed to hypoxic conditions. Ang2 mRNA expression revealed a time-dependent increase that peaked at 2 h (3.6 ± 0.09-fold, p < 0.0001) and returned to the basal level after 4 h of stimulation (Fig. 5A). In contrast, Ang1 and Tie2 mRNA expression remained stable despite hypoxia. As in the VEGF stimulation study, we found a similar response in BAECs. Two hours of hypoxia induced a 2.1 ± 0.1-fold (p = 0.0327) increase in Ang2 mRNA expression, whereas no significant effect on Ang1 or Tie2 mRNA expression was observed (Fig. 5B). Since hypoxia is the major stimulus for VEGF induction and our result showed that VEGF increases Ang2 expression, we investigated whether hypoxia-induced VEGF is involved in the observed hypoxic regulation of Ang2 in BRECs. The anti-VEGF neutralizing antibody (10 μg/ml) exhibited no significant effect on Ang2 mRNA under not only normoxic, but also hypoxic conditions (Fig. 6). These results suggest that the increase in Ang2 expression under hypoxic conditions is the direct effect of hypoxia and is not mediated by VEGF induction.

**Both VEGF and Hypoxia Increase Ang2 Protein Synthesis**—To determine whether the increase in Ang2 mRNA was accompanied by an increase in new protein synthesis, we precipitated the 35S-labeled cell lysates with a specific goat anti-human Ang2 antibody. The molecular mass of Ang2 protein has been reported to range from 55 to 70 kDa, due to glycosylation (27). The detected size of Ang2 protein was ~55 kDa, and the expression level increased 5.3-fold after VEGF treatment (25 ng/ml) and 4.3-fold after hypoxic exposure (Fig. 7).

**Ang2 mRNA Expression Is Increased in the Retina of the Mouse Model of Ischemia-induced Retinal Neovascularization**—To determine in vivo whether Ang2 expression is up-regulated in response to hypoxia, the well-established mouse model of ischemia-induced retinal neovascularization was employed. In this model, proliferative retinal neovascularization peaks at 5 days after hypoxia (postnatal day 17) and regresses by postnatal day 26. Hybridization with an antisense probe showed a basal level of signal located in the ganglion cell layer and the inner nuclear layer at postnatal day 12, just prior to removal of the animal from oxygen, in both hypoxic retinas and age-matched nonhypoxic control retinas (Fig. 8, A and B). After 12 h of relative hypoxia at postnatal day 12, when the expression of VEGF has been shown to peak (38), a mild elevation of signal level was detected in the ganglion cell layer, and an intense signal was observed in the inner nuclear layer (Fig. 8C). Of note, the signal of vascular cells in the inner nuclear layer was also up-regulated. In contrast, control retinas did not show a marked change (Fig. 8D). At postnatal day 17, the time at which retinal neovascularization has been shown to be most prominent (38), an intense signal was detected in neovascular tufts (Fig. 8E), which have been reported to develop preferentially in the mid-peripheral retina at the junction of perfused and nonperfused retinas. Control retinas of the same age did not show significant change (Fig. 8F). At postnatal day 21, when neovascular vessels began to regress, the difference between hypoxic and control retinas had diminished, although stronger signals were still detected in both the ganglion cell layer and the inner nuclear layer of the hypoxic retinas (Fig. 8, G and H).

**DISCUSSION**

Ischemia or hypoxia is well known to be a primary inducer of neovascularization in a variety of conditions, such as tumor angiogenesis (12), collateral vessel formation in cardiovascular diseases (39), and proliferative retinal neovascularization (40, 41), and VEGF has been shown to be a potent mediator of these ischemia-induced neovascularizations (18, 42, 43). This study is the first demonstration that both hypoxia and VEGF selectively enhance Ang2 expression, with Ang1 and Tie2 remaining stable in retinal microvascular endothelial cells, and that Ang2 expression—

**FIG. 2. Effect of VEGF on the transcription rate of Ang2.** BRECs were treated with 25 ng/ml VEGF or vehicle (control (CTR)) for 2 h. Nuclei were isolated and incubated with ATP, CTP, and GTP, and 32P-labeled RNA probes were hybridized to nitrocellulose filters on which Ang2 and 36B4 CDNA had been blotted. To normalize the difference of the loading RNA, the radioactivity of Ang2 was normalized to that of 36B4. Data are shown as a percentage of the control. Representative blots of three independent experiments are shown.

**FIG. 3. Decay of Ang2 mRNA in the presence of actinomycin D in BRECs.** Cells were preincubated with VEGF (25 ng/ml) for 2 h. Half of the plates were returned to Dulbecco’s modified Eagle’s medium without VEGF, and 10 μg/ml actinomycin D (ACD) was added to all plates. Total RNA was isolated at the indicated time points after administration of actinomycin D, and Northern blot analysis was performed. Ang2 mRNA levels were normalized to those of 36B4 mRNA for correction of the loading differences. ○, control cells; □, cells in the presence of VEGF. The values shown represent the percentage of initial Ang2 mRNA signal remaining under the specified conditions and are plotted in logarithmic scale. Representative data of three independent experiments are shown.

**FIG. 4. Role of tyrosine kinase, PKC, and MAPK.** BRECs were pretreated with genistein (40 μM), GF 109203X (5 μM), or PD 98059 (25 μM), followed by stimulation with VEGF (25 ng/ml) for 2 h. Total RNA was isolated, and Northern blot analysis was performed. Ang2 mRNA levels were normalized to those of 36B4 mRNA. Representative blots of three independent experiments are shown (upper panel). Results are presented as a percentage of the VEGF-induced increase in Ang2 mRNA levels obtained without inhibitors and are expressed as means ± S.D. (lower panel). kb, kilobases.
is up-regulated in hypoxic retinas and neovascular vessels in vivo. Since Ang2 has been verified to be a natural antagonist for Tie2, the angiogenic stimuli of hypoxia and VEGF might well deteriorate the integrity of the vasculature by suppressing Ang1 activation of Tie2.

In this study, we used cells of endothelial lineage to delineate regulation of the angiopoietin-Tie2 system in response to VEGF stimulation and hypoxic exposure. Among the receptor and its two relevant ligands, Ang2 was up-regulated selectively in both a time- and concentration-dependent manner by VEGF treatment. The increased Ang2 mRNA expression peaked at 2 h after stimulation by hypoxia or normoxia with or without 10 μg/ml anti-VEGF antibody, and Northern blot analysis was performed. Three triplicate experiments were performed, and the representative blots are shown (upper panels). Results were quantified by densitometric analysis of the autoradiograms derived from the upper panels after normalization to the 36B4 control cDNA signals. Values are presented as a percentage of the control and are expressed as means ± S.D. (lower panels). Black, hatched, and white bars indicate Ang1, Ang2, and Tie2 mRNA levels, respectively.

As shown in Fig. 5, hypoxic regulation of Ang1, Ang2, and Tie2 gene expression. Shown is the time course of Ang1, Ang2, and Tie2 mRNA expression in BRECs (A) and BAECs (B) subjected to hypoxia. Total RNA was isolated at the indicated time points of hypoxia, and Northern blot analysis was performed. Three triplicate experiments were performed, and the representative blots are shown (upper panels). Results were quantified by densitometric analysis of the autoradiograms derived from the upper panels after normalization to the 36B4 control cDNA signals. Values are presented as a percentage of the control and are expressed as means ± S.D. (lower panels). Black, hatched, and white bars indicate Ang1, Ang2, and Tie2 mRNA levels, respectively. kb, kilobases.

As depicted in Fig. 6, Effect of anti-VEGF neutralizing antibody on hypoxia-induced Ang2 mRNA expression in BRECs. Total RNA was isolated at 2 h after stimulation by hypoxia or normoxia with or without 10 μg/ml anti-VEGF antibody, and Northern blot analysis was performed. Three triplicate experiments were performed, and the representative blots are shown (upper panel). Results were quantified by densitometric analysis of the autoradiogram derived from the upper panel after normalization to the 36B4 control cDNA signals. Values are presented as a percentage of the control and are expressed as means ± S.D. (lower panel). kb, kilobases.

As illustrated in Fig. 7, Immunoprecipitation analysis of de novo Ang2 protein synthesis. Confluent BRECs were either treated with VEGF (25 ng/ml) or shifted to hypoxic conditions for 12 h and then labeled with [35S]methionine. The cell lysates were incubated with a specific goat anti-human Ang2 antibody and then immunoprecipitated with protein A-Sepharose. The conjugates were removed by centrifugation and washing, denatured by boiling, and size-fractionated on 7.5% SDS-polyacrylamide gel. Labeled protein signals were analyzed by densitometric scanning. Representative results of three independent experiments are shown. Lane 1, control; lane 2, VEGF treatment; lane 3, hypoxic exposure.

Previous studies have revealed that VEGF promotes activation of several signaling molecules, including phospholipase Cγ, phospholipase D, PKC, phosphatidylinositol 3-kinase, GTPase-activating protein, Nck, and MAPK (32, 37, 44, 45). We performed further studies to explore the signaling pathway underlying the VEGF-induced Ang2 mRNA expression. Blockage of tyrosine kinase by genistein abrogated ~90% of the increase in Ang2 mRNA stimulated by VEGF. Recent studies revealed the critical roles of VEGF-induced MAPK activation in diverse biological responses of endothelial cells, including mitogenesis (46, 47), and actin reorganization and cell migration (33). We further tested the role of the MAPK-dependent signaling pathway using PD 098059, a specific MAPK kinase inhibitor, in the VEGF-induced increase in Ang2 mRNA expression. Our results revealed that PD 098059 abrogated the increase in Ang2 mRNA expression by >90%, which suggests a predominant role of this signaling molecule in VEGF-induced Ang2 expression. PKC has also been reported to mediate both.
the mitogenic effect on endothelial cells and increased vascular permeability in response to VEGF (32, 48). The PKC inhibitor GF 109203X caused a significant decrease (by 65%) in Ang2 expression, suggesting also a crucial role of PKC-dependent signaling. The observed inhibition by GF 109203X was less than that induced by the MAPK kinase inhibitor. The difference in the inhibitory effect of PD 098059 and GF 109203X on VEGF-stimulated Ang2 expression suggests a contribution of the PKC-independent pathway to MAPK activation following VEGF stimulation, such as the Ras-dependent pathway (49) and the nitric oxide-dependent cascade (46).

Hypoxia is the major stimulus that leads to ischemia-induced angiogenesis and has been reported to up-regulate various genes that encode erythropoietin (50), the platelet-derived growth factor B chain (50), fibroblast growth factor (51), and VEGF (12, 16). In this study, we demonstrated that hypoxia induces both mRNA expression and protein synthesis of Ang2. Since the response peaked at as soon as 2 h after hypoxic exposure, it is probably not mediated by induction of other growth factors, such as VEGF; rather, it may be regulated directly by hypoxia. This hypothesis is further confirmed by the experiment showing that anti-VEGF neutralizing antibody had no significant effect on hypoxic Ang2 induction. Transcriptional regulation by hypoxia-inducible factor (52) and stabilization of mRNA by the AUUUA motif of the 3'-untranslated region (53) have been reported to underlie hypoxic gene regulation of VEGF and erythropoietin. Since the observed response of Ang2 to hypoxia is much more rapid and transient in comparison with these genes (54, 55), it is possible that a different molecular mechanism underlies this response.
Ang1 expression has generally been detected in nonendothelial cells surrounding blood vessels in vivo (27), which suggests a paracrine role of this ligand. Our results from Northern blot analysis, however, demonstrated that Ang1 mRNA was also detectable in both BRECs and BAECs and thus indicate that, in addition to its paracrine role, Ang1 can act in an autocrine manner, as recently evidenced in a leukemia cell line and cutaneous fat pad endothelial cells (28, 56). Ang1 has been reported to be down-regulated not only by hypoxia in rat glioma cells, but also by treatment with serum or several cytokines, such as platelet-derived growth factor and tumor growth factor-β, in human lung fibroblasts (30). In contrast, this study demonstrated that Ang1 mRNA expression in BRECs remained essentially unchanged in response to both VEGF stimulation and hypoxia. The observed response is consistent with the informative histologic study showing that Ang1 exhibits uniform expression, irrespective of both developmental stage and VEGF expression, in ovaries undergoing the reproductive cycle (26).

Unlike VEGFR-1 and VEGF-2, whose regulation has been well documented by recent findings that stimuli such as hypoxia (37, 57, 58) and several cytokines, including VEGF, tumor necrosis factor-α, and interleukin-1β, can exert significant effects on their expression (29, 59), little is known about the regulation of Tie2. A recent report suggests that Tie2 is present in both quiescent and angiogenic adult tissue and that up-regulation of Tie2 is observed in skin wounds (60); however, the possibility that increased numbers of blood vessels per se during wound healing lead to the increased amount of Tie2 protein expression was not excluded in that study. Our data demonstrating that Tie2 remains stable in response to both VEGF stimulation and hypoxic exposure, together with a recent report showing that proinflammatory stimuli such as interleukin-1β and tumor necrosis factor-α also have no effect on the expression of this receptor (29), might suggest that the potential role of the angiopoietin-Tie2 system in pathologic angiogenesis or under proinflammatory conditions is derived mainly from altered ligand expression rather than from changes in Tie2 receptor expression.

As in retinal microvascular endothelial cells, we have demonstrated that Ang2 is up-regulated by hypoxia and VEGF stimulation in bovine aortic endothelial cells as well, with Ang1 and Tie2 expression remaining stable. In the macrovascular milieu, VEGF has been suggested to play a role in vascular injury such as atherosclerosis (61). Although the significance of the angiopoietin-Tie2 system remains unknown in such lesions, VEGF might decrease vascular integrity by up-regulating Ang2, thereby facilitating endothelial damage and intima formation. Further studies are required to address this point.

Our in situ hybridization studies using the mouse model of retinal neovascularization demonstrated that Ang2 mRNA is produced in the ganglion cell layer and inner nuclear layer of the retina. These portions of retina are consistent with the locations where transcripts of VEGF have been detected (38). As in the case of VEGF, the up-regulation of Ang2 mRNA expression precedes the development of neovascularization and parallels the temporal and spatial changes of neovascularization development, which suggests that Ang2 plays a critical role in retinal neovascularization. In addition to these layers, we also detected a substantial signal in intraretinal vessels and neovascular tufts that grew into the vitreous cavity through the inner limiting membrane of the retina. Since pericytes have not been shown to be associated with neovascular tufts or neovascular vessels (36), the prominent up-regulation of Ang2 message in these retinal components might be attributed to endothelial cells and thus further supports the results of our in vitro study. Interestingly, in ovaries undergoing the reproductive cycle, Ang2 expression appears to increase from the stage of the developing corpus luteum, when VEGF shows remarkable expression (26), thus suggesting a critical role of VEGF in in vivo Ang2 induction. Although our in vitro data suggested that autocrine VEGF production has little effect on hypoxic induction of Ang2, it is possible that paracrine VEGF production in vivo under hypoxic conditions might have a role in the observed hypoxia-induced Ang2 expression. These findings, together with our results of in situ hybridization, suggest that hypoxia per se, hypoxic induction of VEGF, or both in concert might play a major role in at least the early stage of Ang2 induction in vivo.

Recent in vitro findings regarding the bioactivity of Ang1 have demonstrated that this ligand can induce potent chemotaxis, weak but positive mitogenesis, and capillary sprouts in endothelial cells, which confirms its critical role in angiogenesis (28, 62). When coadministered with VEGF in vivo, Ang1 can potentiate vascular network maturation, whereas Ang2 can contribute substantially to initiation of neovascularization (63). In light of the latter finding, the observed up-regulation of Ang2 in this study might imply this ligand in the initial step of pathologic angiogenesis, where VEGF expression is abundant. Several studies have shown that blocking the Tie2 signal using soluble Tie2 receptor can reduce tumorigenic vascular growth (64, 65). Since neither Ang1 nor Ang2 alone, although contradictory to in vitro studies (28, 62), can significantly promote in vivo neovascularization (63), the decreased vascular growth might be attributed at least partly to suppression of Ang2 induced by angiogenic stimuli such as hypoxia and VEGF, as is evidenced in this study. The angiogenic effect of Ang2 might possibly be explained by destabilization of the preexisting vascular tube structure to set up a favorable environment for endothelial cells to migrate to or contact with additional angiogenic cytokines. Alternatively, since communication between endothelial cells and surrounding mesenchymal cells has been reported to have an inhibitory effect on endothelial cell growth by modulating the bioactivity of tumor growth factor-β (66, 67), destabilization of blood vessels by Ang2 may diminish this inhibition and facilitate subsequent neovascularization. In light of recent studies showing the differential effects of Ang2 on endothelial cells and nonendothelial cells (26, 28) and by virtue of the fact that endothelial progenitor cells, for which the resultant effects of angiopoietin-Tie2 signaling still remain unknown, also contribute to angiogenesis (68), further investigations are required to fully elucidate the detailed roles of the increased Ang2 expression in angiogenesis.

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