Hypoxia-inducible angiopoietin-2 expression is mimicked by iodonium compounds and occurs in the rat brain and skin in response to systemic hypoxia and tissue ischemia

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Abstract

Angiopoietins are ligands for the endothelial cell tyrosine kinase receptor Tie-2. Ang-1, the major physiological activator of Tie-2, promotes blood vessel maturation and stability. Ang-2 counteracts this effect by competitively inhibiting the binding of Ang-1 to Tie-2. Using a combined RNase protection/semiquantitative reverse transcriptase-polymerase chain reaction approach, we demonstrate that hypoxia up-regulates Ang-2 mRNA levels by up to 3.3-fold in two human endothelial cell lines. In bovine microvascular endothelial (BME) cells, the flavoprotein oxidoreductase inhibitor diphenylene iodonium (DPI) and the related compound iodonium diphenyl mimic induction of Ang-2 but not vascular endothelial growth factor (VEGF) by hypoxia; in combination with hypoxia, DPI further increases Ang-2 expression but has no effect on the induction of VEGF by hypoxia. Neither Ang-2 nor VEGF was increased by cyanide or rotenone, suggesting that failure in mitochondrial electron transport is not involved in the oxygen-sensing system that controls their expression. In ischemic rat dorsal skin flaps or in the brain of rats maintained for 12 [...]
Hypoxia-Inducible Angiopoietin-2 Expression Is Mimicked by Iodonium Compounds and Occurs in the Rat Brain and Skin in Response to Systemic Hypoxia and Tissue Ischemia

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Angiopoietins are ligands for the endothelial cell tyrosine kinase receptor Tie-2. Ang-1, the major physiological activator of Tie-2, promotes blood vessel maturation and stability. Ang-2 counteracts this effect by competitively inhibiting the binding of Ang-1 to Tie-2. Using a combined RNase protection/semiquantitative reverse transcriptase-polymerase chain reaction approach, we demonstrate that hypoxia up-regulates Ang-2 mRNA levels by up to 3.3-fold in two human endothelial cell lines. In bovine microvascular endothelial (BME) cells, the flavoprotein oxidoreductase inhibitor diphenylene iodonium (DPI) and the related compound iodonium diphenyl mimic induction of Ang-2 but not vascular endothelial growth factor (VEGF) by hypoxia; in combination with hypoxia, DPI further increases Ang-2 expression but has no effect on the induction of VEGF by hypoxia. Neither Ang-2 or VEGF was increased by cyanide or rotenone, suggesting that failure in mitochondrial electron transport is not involved in the oxygen-sensing system that controls their expression. In ischemic rat dorsal skin flaps or in the brain of rats maintained for 12 hours under conditions of hypoxia, Ang-2 mRNA was up-regulated 7.5- or 17.6-fold, respectively. VEGF was concomitantly increased, whereas expression of Ang-1, Tie-2, and the related receptor Tie-1 was unaltered. In situ hybridization localized Ang-2 mRNA to endothelial cells in hypoxic skin. These findings 1) show that up-regulation of Ang-2 by hypoxia occurs widely in endothelial cells in vitro and in vivo; 2) suggest that induction of Ang-2, but not VEGF, by hypoxia in BME cells is controlled by a flavoprotein oxidoreductase that is sensitive to iodonium compounds; and 3) point to Ang-2 and VEGF as independently regulated and selective effectors of hypoxia-induced vascular sprouting. (Am J Pathol 2000, 156:2077–2089)
two with target specificity for endothelial cells. VEGF, the prototype member of an expanding family of angiogenic polypeptides, binds to the endothelial cell receptors VEGFR-1/flk1 and VEGFR-2/KDR/flk-1, the expression of which is restricted to endothelial cells and progenitors of the hematopoietic/endothelial cell lineage.6 The relevance of the VEGF/VEGF receptor system to both developmental and pathological angiogenesis has been clearly demonstrated.6 In cultured endothelial cells, VEGF stimulates proliferation, extracellular proteolytic enzyme production, as well as the formation of capillary-like structures in reconstituted collagen matrices.7–12 Taken together, these results suggest that VEGF plays a key role in the sprouting phase of angiogenesis.

The discovery of the angiopoietin family has contributed significantly to our understanding of the process of blood vessel maturation. The first angiopoietin described, angiopoietin-1 (Ang-1), is an agonist of the endothelial cell tyrosine kinase receptor Tie-2, which is prominently expressed during development in the myocardium, and later throughout the embryo in the mesenchyme surrounding the developing vasculature.13,14 Analysis of Ang-1 function in vivo through targeted inactivation of the Ang-1 locus in the mouse resulted in embryonic lethality at E12.5.14 This was due to impaired development of myocardium, defective remodeling of the primitive vascular plexus into large and small vessels, as well as the complete lack of perivascular cells.14 Similar vascular defects had previously been reported in mice lacking Tie-2.15 Taken together, these findings demonstrated that, when compared to the VEGF/VEGF receptor system, the Ang-1/Tie-2 system plays a role in the later stages of angiogenesis.

Angiopoietin-2 (Ang-2) shares ~60% amino acid identity with Ang-1 and binds to Tie-2 with similar affinity. However, Ang-2 does not induce Tie-2 autophosphorylation, but instead blocks Tie-2 autophosphorylation induced by Ang-1, thus behaving like an Ang-1 antagonist.16 Thus overexpression of Ang-2 in the developing mouse embryo mimicked the phenotypes of Ang-1 or Tie-2 inactivation.16 During embryogenesis and adult life, Ang-2 expression occurs almost exclusively at sites of vascular remodeling, where it begins in endothelial cells of the sprouting microvasculature and where VEGF is concomitantly expressed.16–18 However, Ang-2 expression is also pronounced at sites of vascular regression, where VEGF mRNA is almost undetectable.16,18 Taken together, these findings have led to the proposal that by virtue of its capacity to counteract blood vessel maturation/stability, the function of Ang-2 may be context-dependent. When acting in the absence of angiogenic inducers (such as VEGF), Ang-2 induces endothelial cell apoptosis with consequent vascular regression. When acting in concert with VEGF, Ang-2 may facilitate endothelial cell migration and proliferation, thus serving as a permissive angiogenic signal.19,16

Hypoxia is a fundamental angiogenic stimulus that is believed to play a key role in a number of settings in which angiogenesis is an important component, including embryonic development and tumor growth. It is believed that the effects of hypoxia are initiated at the cellular level by an as yet uncharacterized heme protein oxygen sensor that in turn activates hypoxia inducible factor-1, a heterodimeric basic helix-loop-helix transcription factor composed of a constitutive β subunit (HIF-1β/ARNT) and a regulatory α subunit (HIF-1α) (reviewed in refs. 20–22). Functional HIF-1 binding sites have been described in the majority of genes regulated by hypoxia, including VEGF, erythropoietin (Epo), tyrosine hydroxylase, glucose transporter-1 (GLUT-1), most glycolytic enzymes, and inducible nitric oxide synthase (iNOS),20,22 suggesting that HIF-1 is a central component of the organism’s response to hypoxia. However, the recent finding that both HIF subunits exist as gene families suggests that a precise tailoring of the hypoxic response might be achieved through differences in HIFα subunit activation and/or target gene specificity.

We23 and others24 have previously reported that hypoxia increases Ang-2 expression in bovine endothelial cells but not in smooth muscle cells,23 which is consistent with the pattern of Ang-2 expression observed in vivo. This finding strongly suggested that, in addition to VEGF, Ang-2 might be a crucial effector of hypoxia-induced neovascularization. In the present study, we wished to assess whether hypoxia also induces Ang-2 expression in human endothelial cells and whether induction of Ang-2 by hypoxia occurs in vivo. Finally, we wished to compare the pharmacological features of hypoxia-inducible Ang-2 expression with those of VEGF.

Materials and Methods

Cell Culture

Simian virus 40 large T-antigen-transformed human dermal microvascular endothelial (HMEC-1) cells25 (kindly provided by Drs. E. W. Ades and T. Lawley) were cultured in endothelial cell basal medium (EBM) 131 medium (Clonetics) supplemented with 10% fetal calf serum (FCS) (Life Technologies), 10 ng/ml of recombinant human epidermal growth factor (EGF) (Boehringer Mannheim), and 1 μg/ml of hydrocortisone. Human umbilical vein endothelial (HUVE) cells (kindly provided by Dr. N. Maggiano) and bovine microvascular endothelial (BME) cells from adrenal cortex26 (kindly provided by Drs. M. B. Furie and S. C. Silverstein) were cultured as described.12

Twenty-four to 72 hours after the last medium change, confluent monolayers of HUVE, HMEC-1, or BME cells were incubated for the times indicated in an airtight Plexiglas container in which atmospheric air was replaced with a 95% N2/5% CO2 gas mixture. In experiments aimed at characterizing Ang-2 and VEGF regulation in BME cells, confluent monolayers were incubated for 15 hours in the presence of the above-mentioned hypoxic conditions, or in the presence of the following treatments: 100 μmol/L cobalt chloride hexahydrate (catalog no. C-8661; Sigma); 130 μmol/L desferrioxamine mesylate (DFO) (catalog no. D-9533; Sigma); 1 mmol/L potassium cyanide (KCN) (catalog no. 60180; Fluka); 5 μg/ml of cycloheximide (CHX) (catalog no. C-4218; Sigma), alone or in combination with hypoxia (CHX was added to the
cells 30 minutes before the hypoxic incubation was started; 5 μmol/L diphenyle iodonium chloride (DPI) (catalog no. D-2926; Sigma), alone or in combination with hypoxia; 50 μmol/L diphenyle iodonium diphenyl (IDP) (catalog no. 43088; Fluka); or 100 nmol/L rotenone (catalog no. R-8875; Sigma).

Stock solutions were prepared in H2O, except for DPI (EtOH 50% in H2O), IDP (EtOH 50% in H2O), and rotenone (dimethyl sulfoxide, DMSO), and diluted to the indicated concentrations in cell culture medium. Equivalent volumes of EtOH or DMSO were tested alone in parallel cultures for possible effects on Ang-2 mRNA levels. Where indicated, cells were washed twice with serum-free medium and incubated for 15 hours in serum-free medium under normoxic or hypoxic conditions.

Ischemic Dorsal Skin Flap Model

Female Wistar rats weighing 250–300 g were anesthetized with intraperitoneal sodium pentobarbital, 35 mg/kg. Caudally-based 9 × 3 cm dorsal skin/panniculus carnosus flaps were raised with the two constant sacral axial vessels systematically cut. The caudal border of the flaps was marked 1 cm below the posterior iliac crests. The flaps were resutured back onto their original bed without further manipulation. Postoperatively the rats were fed ad libitum and housed in individual cages. Flaps were examined daily after surgery. Rats were sacrificed using an intracardiac pentobarbital overdose of 200 mg/kg. Immediately after sacrifice, sections II and III of the flaps (Figure 6) were divided into longitudinal strips; edges of the flap were excluded. Flap segments were snap frozen in liquid nitrogen-cooled isopentane. RNA was extracted from frozen tissues.

Systemic Hypoxia in Rats

Three- to six-month-old male Sprague-Dawley rats were kept for 12 hours in an airtight Plexiglas container in which atmospheric air was replaced with a continuous flow of a 6% oxygen/94% nitrogen gas mixture. Under these conditions, animals appeared to feed normally. Age-matched animals were used as controls. At the end of the incubation, rats were killed by cervical dislocation, and the experiments mentioned were carried out. For each RT product, 1/20 of the final reaction volume, or an equivalent volume of H2O, was amplified in parallel PCR reactions, using, as indicated in each case, the above-mentioned primers for human Ang-2, a pair of specific primers for human VEGF (forward: 5′-GGAGCC TCGCCTTGCTGCTTACCC; reverse: 5′-CCGAAACCCT GAGGGAGGQCTC; located outside of the alternatively spliced region), a pair of specific primers for bovine VEGF (forward: 5′-CGCGAATTCCAGGATCCACAGTG AG; reverse: 5′-CGCGAGTCGCTACCCGCTGQCTG TC, containing an artificial EcoRI or BamHI site at the 5′ end, respectively, and located outside of the alternatively spliced region), a pair of partially degenerate primers for Ang-1 or Ang-2 previously described, a pair of partially degenerate primers for Tie-1 (forward: 5′-GATGTAGACAGGCC(A/G/T/C)GA(A/G)GA(A/G); reverse: 5′-CTCAAAAGTG(A/G/T)AT(A/GT)(C/T)TCCTCA), a pair of partially degenerate primers for Tie-2 (forward: 5′-GGCA(A/G)ATGAAAG(C/T)CA(A/G)CA(C/T); reverse: 5′-TCCTGAAAAC(T/C)TGAT(A/G)TTC(CTAG)(T)TCCTCA), a pair of specific primers for mouse and rat VEGF, or, finally, a pair of partially degenerate primers for the acidic ribosomal phosphoprotein P0 that efficiently amplify the corresponding cDNA of bovine, human, and rat origin. For all of the reactions, preliminary experiments were performed to determine the number of PCR cycles at which saturation occurred, and the experiments mentioned were carried out with a number of cycles that precedes saturation. The specificity of the PCR reactions using degenerate primers for

Semi-quantitative RT-PCR

Two micrograms (5 μg in the case of experiments using rat brains) of total RNA was reverse transcribed using oligo-dT15 (Boehringer Mannheim) and Superscript II RT (Life Technologies). Where indicated, RT was omitted. For each RT product, 1/20 of the final reaction volume, or an equivalent volume of H2O, was amplified in parallel PCR reactions, using, as indicated in each case, the above-mentioned primers for human Ang-2, a pair of specific primers for human VEGF (forward: 5′-GGAGCC TCGCCTTGCTGCTTACCC; reverse: 5′-CCGAAACCCT GAGGGAGGQCTC; located outside of the alternatively spliced region), a pair of specific primers for bovine VEGF (forward: 5′-CGCGAATTCCAGGATCCACAGTG AG; reverse: 5′-CGCGAGTCGCTACCCGCTGQCTG TC, containing an artificial EcoRI or BamHI site at the 5′ end, respectively, and located outside of the alternatively spliced region), a pair of partially degenerate primers for Ang-1 or Ang-2 previously described, a pair of partially degenerate primers for Tie-1 (forward: 5′-GATGTAGACAGGCC(A/G/T/C)GA(A/G)GA(A/G); reverse: 5′-CTCAAAAGTG(A/G/T)AT(A/GT)(C/T)TCCTCA), a pair of partially degenerate primers for Tie-2 (forward: 5′-GGCA(A/G)ATGAAAG(C/T)CA(A/G)CA(C/T); reverse: 5′-TCCTGAAAAC(T/C)TGAT(A/G)TTC(CTAG)(T)TCCTCA), a pair of specific primers for mouse and rat VEGF, or, finally, a pair of partially degenerate primers for the acidic ribosomal phosphoprotein P0 that efficiently amplify the corresponding cDNA of bovine, human, and rat origin. For all of the reactions, preliminary experiments were performed to determine the number of PCR cycles at which saturation occurred, and the experiments mentioned were carried out with a number of cycles that precedes saturation. The specificity of the PCR reactions using degenerate primers for

RNA Purification

Total cellular RNA was purified from cultured cells or rat tissues using Trizol reagent (Life Technologies).

RNase Protection Assay

Ten micrograms of total RNA from cultured endothelial cells or rat skin was analyzed. In the case of hypoxic brain, 30 μg of total RNA was used. Rat and bovine Ang-2 cRNA probes were synthesized from the partial cDNA fragments previously described, with the modification that the rat Ang-2 fragment was digested at the internal PvuII site, thus resulting in a shorter cRNA probe (~320 bases). The human Ang-2 probe was generated by reverse transcriptase-polymerase chain reaction (RT-PCR) from total RNA from HUVE cells exposed to the above-mentioned hypoxic conditions for 15 hours. Briefly, total cellular RNA was reverse transcribed using oligo-dT15 (Boehringer Mannheim) and Superscript II reverse transcriptase (RT) (Life Technologies), according to the manufacturer’s instructions. The oligonucleotide primer sequences used were as follows: forward: 5′-GGTGTAGATTTCAGAGGACTGG; reverse: 5′-GGCGAA- TAGCCTGAGCCCTCCA. PCR cycles were as follows: 94°C, 2 minutes (1×); 94°C, 1 minute, 60°C, 1 minute, 72°C, 1 minute (30×); 72°C, 3 minutes. The unique 450-bp product was cloned into pGEM-TE (Promega) and sequenced in its entirety. The 393-bp rat VEGF cDNA probe, spanning the common and alternatively spliced regions of rat VEGF mRNA, was kindly provided by Dr. B. Berse. Equal amounts of acidic ribosomal phosphoprotein P0 cRNA probe (obtained by RT-PCR from the corresponding species as previously described) were included in all samples as an internal control. RNase protection was performed as described.

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Ang-1, Ang-2, and P0 has previously been confirmed by sequencing. For reactions with degenerate Tie-1 and Tie-2 primers, human Ang-2 primers, and bovine VEGF primers (only for the isoform corresponding in size to the 164 aa isoform), identity of the RT-PCR product was confirmed by cloning into pGEM-TE (Promega) or pBlue-scriptKS (Stratagene), entire sequencing, and RNase protection analysis using the appropriate total cellular RNA from the corresponding species. Sequences of rat Tie-1 and Tie-2 partial cDNAs were unreported and have been deposited in GenBank with accession numbers AF030377 and AF030423, respectively.

PCR cycles were as follows: Human Ang-2 primers (HUVE and HMEC-1 cells): 94°C, 2 minutes (1 minute); 94°C, 1 minute, 60°C, 1 minute, 72°C, 45 sec (25x); 72°C, 3 minutes. Human VEGF primers (HUVE and HMEC-1 cells): 94°C, 2 minutes (1x); 94°C, 1 minute, 65°C, 1 minute, 72°C, 1 minute (23x); 72°C, 3 minutes. Bovine VEGF primers (BME cells): 94°C, 2 minutes (1x); 94°C, 1 minute, 62°C, 1 minute, 72°C, 1 minute (23x); 72°C, 3 minutes. Degenerate Ang-1, Ang-2, tie-1, and Tie-2 primers (rat brain): 94°C, 2 minutes (1x); 94°C, 30 seconds, 55°C, 1 minute, 72°C, 45 seconds (25x; 28x in the case of Ang-2); 72°C, 3 minutes. Mouse/rat VEGF primers (rat brain): 94°C, 2 minutes (1x); 94°C, 30 seconds, 60°C, 1 minute, 72°C, 45 seconds (23x); 72°C, 3 minutes. Degenerate P0 primers (HUVE, HMEC-1, and BME cells; rat brain): 94°C, 2 minutes (1x); 94°C, 30 seconds, 60°C, 1 minute, 72°C, 45 seconds (16x; 20x in the case of rat brain); 72°C, 3 minutes. Five microcuries of 32P-labeled dCTP were added to each sample to visualize PCR products by autoradiography. Equal volumes of PCR products were electrophoresed in 6% acrylamide gels.

In Situ Hybridization

A 35S-UTP-labeled antisense rat Ang-2 cRNA probe of ~320 bases was generated by transcription of a PvuII-digested rat Ang-2 cDNA fragment, whereas 32P-UTP-labeled antisense rat VEGF cRNA probe was synthesized from the above-mentioned rat VEGF cDNA fragment. Both probes were used for in situ hybridization on paraffin sections of 4% parafomaldehyde perfusion-fixed rat skin flap fragments, using a previously published procedure.

Results

We and others have previously reported that hypoxia increases Ang-2 mRNA and protein in bovine endothelial cells. This phenomenon was apparently endothelial cell-specific, in that it was not observed in smooth muscle cells (SMCs). To investigate whether expression of Ang-2 is inducible by hypoxia in other endothelial cell lines, confluent cultures of HUVE or transformed HMEC-1 cells were incubated for 15 hours under hypoxic conditions. Total RNA was reverse transcribed with oligo-dT, and Ang-2 mRNA levels were studied by semiquantitative RT-PCR, using the same partially degenerated oligonucleotide primers that we previously designed to clone bovine and rat Ang-2 partial cDNAs. Alternatively, specific primers corresponding to the same regions of human Ang-2 were used. Parallel reactions on the same RT products assessed 1) the levels of VEGF mRNAs as an internal control for the hypoxic response and 2) the levels of the acidic ribosomal phosphoprotein P0 mRNA as an internal control of the amount of total cDNA used in each amplification reaction. After 25 PCR cycles using specific primers for human Ang-2, a unique band of ~450 bp, thus corresponding in size to the RT-PCR product expected from human Ang-2 cDNA (456 bp), was detectable in normoxic HUVE cells, and to a much lesser extent in normoxic HMEC-1 cells (Figure 1). When normalized with respect to the P0 signal, the intensity of the putative Ang-2 band was increased by 3.3- or 2.5-fold by hypoxia in HUVE or HMEC-1 cells, respectively. Identical results were obtained using the partially degenerate primers for Ang-2 mentioned above (data not shown). Massive induction of VEGF expression occurred in both cell lines in response to hypoxia (Figure 1), which is consistent with previous results. The Ang-2, VEGF, and P0 bands were undetectable when RT was omitted (Figure 1), demonstrating that they were not amplified from contaminating genomic DNA.

To confirm that the ~450-bp RT-PCR product from HUVE and HMEC-1 cells was in fact Ang-2, the RT-PCR product amplified from hypoxic HUVE cells was cloned and sequenced in its entirety. Nucleotide sequence analysis revealed 100% identity with the human Ang-2 cDNA. This fragment was therefore used in RNase protection assays to more precisely characterize the in-
Ang-2 Induction by Hypoxia and DPI

Hypoxia-inducible expression of typical hypoxia-responsive genes such as VEGF and erythropoietin (Epo) has well-known pharmacological features. For instance, it is mimicked in normoxic cells by transition metals such as cobalt, nickel, and manganese, and by the iron chelator DFO, but not by poisons of mitochondrial electron transport, such as cyanide or azide (reviewed in ref. 20). DPI, a potent inhibitor of flavoproteins, blocks hypoxia-induced VEGF and Epo expression in HepG2 and Hep3B cells, whereas it has no effect on normoxic levels of either gene.23 A similar effect has been reported for the DFI-related molecule IDP.24 Because cultured endothelial cells express both VEGF and Ang-2, we wished to compare the pharmacological features of hypoxia-inducible Ang-2 and VEGF expression in the same cell type.

To this end, confluent monolayers of BME cells cultured in serum-containing medium were incubated for 15 hours in the presence of hypoxia, 100 μmol/L cobalt chloride, 130 μmol/L DFO, 1 mmol/L potassium cyanide (KCN), 5 μg/ml of CHX alone or in combination with hypoxia, 5 μmol/L DFI alone or in combination with hypoxia, or were exposed to hypoxia in the absence of serum. Ang-2 mRNA levels were studied by RNase protection, using the bovine Ang-2 cDNA fragment previously described,23 whereas VEGF mRNA levels were studied in the same samples by means of semiquantitative RT-PCR, because basal levels of VEGF mRNA were barely detectable by RNase protection in BME cells (data not shown). PCR primers for bovine VEGF were chosen outside of the alternatively spliced region, thus allowing us to identify VEGF164 and VEGF120 as the two VEGF mRNA isoforms expressed in equal proportion by BME cells (Figure 4); an additional band corresponding in size to VEGF188 was detectable in bovine adult lung (Figure 4). The specificity of the PCR reaction was confirmed by cloning and sequencing of the VEGF164 isoform (data not shown). In both RNase protection and semiquantitative RT-PCR analysis, modulations of Ang-2 or VEGF expression were normalized with respect to the P0 signal. When compared to controls, hypoxia induced both Ang-2 and VEGF in BME cells (Figures 3 and 4). However, whereas the amplitude of VEGF induction was dramatic (20.6- or 14.7-fold for VEGF164 or VEGF120, respectively), that of Ang-2 was relatively modest (2.9-fold), which is consistent with previous results.23,24 Cobalt chloride and DFO induced a weak and similar increase in VEGF164 and VEGF120 (1.9- and 1.8-fold, respectively), in the case of cobalt chloride, and 3.0- or 2.6-fold, respectively, in the case of DFO) (Figure 4), but did not alter Ang-2 mRNA levels (Figure 3), perhaps because of the low amplitude of Ang-2 inducibility by hypoxia. KCN had no effect on VEGF or Ang-2 expression (Figures 3 and 4), suggesting that hypoxia-inducible Ang-2 expression, similar to that of VEGF, is not a consequence of failure in mitochondrial electron transport.

Experiments using CHX, DPI, and serum-free medium revealed the most interesting differences between Ang-2 and VEGF. CHX very strongly increased VEGF164 and VEGF120 expression when tested alone (53.3- or 11.0-
fold, respectively) or in combination with hypoxia (212.3- or 88.8-fold, respectively) (Figure 4). Because CHX inhibits activity of hypoxia-inducible factor (HIF) (reviewed in ref. 20), the effect of CHX on VEGF expression most probably reflects increased stability of VEGF mRNA, which, interestingly, is more marked in the case of the 164-aa isoform (Figure 4). In contrast, CHX reduced the basal level of Ang-2 mRNA by 50% and did not alter the extent of Ang-2 inducibility by hypoxia (3.4-fold) (Figure 3), showing that ongoing protein synthesis is required for the maintenance of basal Ang-2 mRNA levels but not for hypoxia-inducible expression of Ang-2.

DPI is an inhibitor of flavoproteins that does not alter Epo or VEGF mRNA levels under normoxic conditions and that blocks hypoxia-inducible VEGF and Epo expression in HepG2 and Hep3B cells.35 When assessed in BME cells, DPI increased Ang-2 mRNA levels in normoxic conditions to an extent similar to that of hypoxia (3.3-fold) (Figure 3) and further slightly increased Ang-2 mRNA levels when tested in combination with hypoxia (3.8-fold) (Figure 3). Intriguingly, DPI, which did not alter the basal levels of VEGF mRNA in BME cells (Figure 4), did not affect hypoxia-inducible VEGF expression (Figure 4), suggesting that the response to DPI might vary, depending on the cell type considered.

Because most hypoxia-inducible genes need to integrate diverse regulatory signals to efficiently responding to hypoxia, we wished to assess whether serum had any effect on hypoxia-inducible Ang-2 expression. When cultured in the absence of serum for 15 hours, normoxic BME cells showed a basal level of Ang-2 mRNA that was ~2.0-fold higher than that observed in the same cells cultured in the presence of serum (Figure 3). Under hypoxic conditions, the level of Ang-2 mRNA was equivalent in the presence or absence of serum (Figure 3). Similar results were obtained by semiquantitative RT-PCR in independent experiments (data not shown). Thus, in the absence of serum, induction of Ang-2 by hypoxia was reduced by 40%, and this was apparently due to the fact that the absence of serum per se increases the levels of Ang-2 mRNA, rather than to the fact that hypoxic-inducible expression of Ang-2 is serum dependent. In contrast, the absence of serum decreased the expression of VEGF164 and VEGF120 by 30% and 50%, respectively (Figure 4 and data not shown). Serum-free medium did not affect hypoxic inducibility of either VEGF isoform (Figure 4).

Finally, prompted by the positive effect of DPI on Ang-2 mRNA levels, we wished to assess the role of two additional flavoprotein inhibitors in the regulation of Ang-2 expression in normoxic BME cells. When analyzed by
RNase protection and normalized with respect to the internal control P0, IDP also stimulated BME cell Ang-2 mRNA levels by 4.8- or 3.1- fold, respectively, in two independent experiments (Figure 5). In the same experiments, DPI induced Ang-2 mRNA levels by 4.5- or 3.9- fold, respectively (Figure 5). In contrast, rotenone, a specific inhibitor of complex I in the mitochondrial respiratory chain, had no effect (Figure 5).

Taken together, these findings show both similarities and differences between Ang-2 and VEGF regulation. First, experiments using KCN and rotenone suggested that, similar to VEGF and Epo, hypoxic induction of Ang-2 is not mediated by sensing of failure in electron mitochondrial transport. However, a DPI- and IDP-sensitive flavoprotein oxidoreductase seems to be involved in the mechanism of oxygen sensing that controls Ang-2 but not VEGF expression in BME cells. Second, hypoxia-inducible expression of either VEGF or Ang-2 in BME cells does not require ongoing protein synthesis. However, opposing effects were observed with CHX alone, in that it decreased basal levels of Ang-2 expression and strongly increased basal levels of VEGF expression. Third, hypoxia-inducible expression of both Ang-2 and VEGF in BME cells occurred independently of the presence of serum. When considered alone, serum had opposite effects on the regulation of VEGF or Ang-2 in BME cells, in that it was required to maintain the expression of VEGF, and, intriguingly, repressed the expression of Ang-2.

We next wished to assess Ang-2 expression in response to tissue hypoxia in vivo. The first model analyzed was a caudally based rat dorsal skin/panniculus carnosus flap (Figure 6) that predictably develops acute tissue hypoxia and massive necrosis in its distal (cranially localized) portion.27,28 Ang-2 mRNA levels were studied by RNase protection over a time period of 7 days after intervention, using a rat Ang-2 partial cDNA23 as a probe and a rat P0 cDNA fragment as an internal control. Interestingly, a low but detectable level of Ang-2 mRNA could be observed in the skin of control rats (Figure 7). When assessed in the distal part of the skin flap, where tissue hypoxia is pronounced and tissue necrosis reaches a maximum after 3 days (refs. 27, 28, and data not shown), Ang-2 mRNA levels were increased by 3.7- fold after 12 hours, further increased by up to 7.5- fold after 2 days, and gradually returned to control levels after 1 week (Figures 7 and 8). In the middle part of the flap, where tissue hypoxia is insufficient to promote necrosis, Ang-2 mRNA was induced to a lesser extent (Figure 8 and data not shown). Analysis of VEGF expression revealed an expression profile that was similar in terms of kinetics, but heterogeneous in nature, in that it occurred in endo-
thelial cells, stromal cells (most likely macrophages), basal keratinocytes, hair follicles, and brown adipocytes (Figure 9, B and B'), and our unpublished results). This was most likely due to the strong and persistent tissue ischemia that occurs in the distal part of the skin flap. Both Ang2 and VEGF were undetectable by in situ hybridization in the skin of control animals, or when the corresponding sense probes were used (Figure 9, C and C', and data not shown). Thus, in the setting of tissue ischemia, in contrast to VEGF, the expression of which is widespread, Ang-2 is specifically induced in vascular endothelial cells. In accord with previous reports, these results suggest that, in ischemic tissues, Ang-2 might contribute to hypoxia-induced neovascularization and/or to tissue necrosis through induction of vascular regression. However, in the specific case of the model presented here, it should be pointed out that although neovascularization accompanied the formation of granulation tissue at the edges of the flap (data not shown), over the time period of 7–10 days neovascularization in the skin flap in itself was limited. This is most likely due to the extensive necrosis observed in this model.

Ang-2 mRNA levels were next assessed in the brains of rats exposed to systemic hypoxia, a model in which angiogenesis is known to occur as part of the adaptive response to hypoxia. As a first approach, Ang-2 mRNA levels were studied by semiquantitative RT-PCR, using the partially degenerate oligonucleotide primers mentioned above and previously described in conjunction with a parallel reaction for P0 as an internal control. After 12 hours of hypoxia (6% oxygen), in two independent experiments, Ang-2 mRNA levels were increased in the brain by 7.4- or 7.9-fold, respectively (Figure 10). A strong increase in VEGF expression was also observed (4.7- or 2.9-fold for VEGF164, and 15.5- or 5.6-fold for VEGF120) (Figure 10), which is consistent with previous results. A semiquantitative RT-PCR screening of various organs of mice subjected to the same hypoxic conditions revealed a similar increase in Ang-2 and VEGF in the brain and to some extent of Ang-2 in the liver, but no significant up-regulation of either gene in the lung, spleen, or kidney, despite strong and selective induction of Epo mRNA in the latter organ (data not shown). In contrast to Ang-2 and VEGF, the levels of Ang-1, Tie-1, and Tie-2 mRNAs were unaffected or slightly decreased in the brain of hypoxic rats (Figure 10), which is consistent with our previous findings in cultured cells. The specificity of the PCR reactions for Ang-1, Ang-2, Tie-1, and Tie-2 was confirmed by cloning and sequencing of the respective RT-PCR products (ref. 23; see also Materials and Methods).

Up-regulation of Ang-2 and VEGF mRNAs in the hypoxic rat brain was confirmed by RNase protection analysis. When analyzed by this technique and normalized with respect to the P0 signal, the same RNA samples of Figure 10 revealed an increase in Ang-2 mRNA of 17.6- or 17.0-fold, respectively, and an increase in VEGF164 of 3.0- or 5.3- fold, respectively (Figure 11).

Thus selective up-regulation of VEGF and Ang-2 occurs in the brain of rats and mice exposed to systemic hypoxia at a time point that precedes the formation of new capillaries, suggesting a crucial role for these two molecules in the promotion of vascular sprouting in this model.
Genetic studies have shown that Ang-1, the major physiological agonist of the endothelial cell tyrosine kinase receptor Tie-2, serves important functions during embryonic angiogenesis. Although not required for vasculogenesis, Ang-1 is essential for remodeling of the primary vascular plexus, branching of new blood vessels from the latter, and maturation of the vessel wall through the recruitment of perivascular cells. The finding that Ang-1 is

**Figure 9.** *In situ* hybridization for Ang-2 and VEGF in rat ischemic skin flaps. Animals were perfusion-fixed with 4% paraformaldehyde, and skin flap tissue was paraffin-embedded. Consecutive sections of the distal third (region III in Figure 6), harvested 2 days after surgical intervention, were analyzed by *in situ* hybridization using ^35^S-UTP labeled antisense (**A**, **A’**) or sense (**C**, **C’**) rat Ang-2 or antisense VEGF (**B**, **B’**) cRNA probes. **A’**, **B’**, and **C’** are dark-field views of **A**, **B**, and **C**, respectively. Ang-2 mRNA was confined to vascular endothelial cells of small and medium vessels (**A**, **A’**), whereas VEGF mRNA was expressed by both stromal cells (**arrowheads**) and endothelial cells (**arrows**).

**Discussion**

Genetic studies have shown that Ang-1, the major physiological agonist of the endothelial cell tyrosine kinase receptor Tie-2, serves important functions during embryonic angiogenesis. Although not required for vasculogenesis, Ang-1 is essential for remodeling of the primary vascular plexus, branching of new blood vessels from the latter, and maturation of the vessel wall through the recruitment of perivascular cells. The finding that Ang-1 is
constitutively expressed in most adult organs, where Tie-2 is also constitutively phosphorylated, has led to the hypothesis that, besides being essential for blood vessel maturation during embryonic development, Ang-1 could also be a key molecule for the maintenance of blood vessel stability in the adult. It has been proposed that Ang-2, the Ang-1 antagonist that binds to Tie-2 and blocks Ang-1-induced Tie-2 phosphorylation, could be an important pro-angiogenic factor, in that by interrupting the stabilizing Ang-1 signal it might promote a process of vessel wall disassembly that would allow endothelial cells to respond to angiogenic inducers. Consistent with this view, an increasing number of reports have shown that expression of Ang-2 occurs almost exclusively at physiological and pathological sites of vascular remodeling, where it appears to begin at the tip of the developing vessel sprouts. In addition, we have previously reported that expression of Ang-2 is increased in bovine microvascular endothelial cells by a number of angiogenic inducers, including VEGF and bFGF, thus providing a possible mechanism for the induction of Ang-2 in vivo and suggesting that induction of an autocrine Ang-2 loop resulting in Ang-1 inactivation may represent part of the mechanism by which angiogenic inducers act.

Perhaps most importantly, we and others also found that endothelial cell expression of Ang-2 is increased by hypoxia. Hypoxia is a fundamental angiogenic stimulus that plays a key role during a variety of physiological and pathological settings, including embryonic development and tumor growth. Although intensely studied, the mechanisms by which hypoxia induces angiogenesis are only partially known. Hypoxia induces up-regulation of VEGF expression in a variety of in vivo and in vitro systems, a phenomenon that is initiated at the level of a yet uncharacterized cellular oxygen sensor and effected intracellularly through the activation of the heterodimeric basic helix-loop-helix PAS transcription factor HIF-1 (reviewed in refs. 6, 21, and 22). However, although VEGF induction is certainly a relevant mechanism in hypoxia’s angiogenic effect, it is certainly not the only one, because a large body of experimental evidence clearly shows that VEGF is necessary but not sufficient for angiogenesis to occur, and that the angiogenic effects of VEGF or other inducers are largely determined by a local, complex, and probably tissue-specific balance between different positive and negative angiogenic regulators. A search for other hypoxia-inducible angiogenic molecules has revealed a role for PDGF-B and VEGF receptor-1/flt-4. However, neither of these molecules appears to play a role in the promotion of vascular sprouting, because PDGF-B is involved in blood vessel maturation and VEGFR-1 seems to act as a dominant negative regulator of VEGF’s effects on the vascular endothelium and progenitors of the hematopoietic/endothelial lineage. Regulation of other angiogenic molecules by hypoxia has led to conflicting and unclear results: although Tie-1 has been reported to be induced by hypoxia in one line of

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**Figure 10.** Semiquantitative RT-PCR analysis of Ang-1, Ang-2, Tie-1, Tie-2, and VEGF mRNA levels in the brains of rats maintained for 12 hours under hypoxic conditions. Five micrograms of total RNA from 12.5-day rat embryo + placenta (E/P12.5) or from the brains of rats maintained for 12 hours under normoxic (Control) or hypoxic (Hypoxia) conditions (6% oxygen) in two independent experiments was analyzed for Ang-1, Ang-2, Tie-1, Tie-2, or VEGF mRNA expression (using P0 mRNA as an internal control) by semiquantitative RT-PCR. Where indicated, RT was omitted.

**Figure 11.** RNase protection analysis of Ang-2 and VEGF mRNA levels in the brains of hypoxic rats. Purified 32P-labeled rat Ang-2 or VEGF164 cRNA probes (pr.) or rat P0 cRNA probe (P0 pr.) was hybridized to hybridization mix (pr. + h.m., P0 pr. + h.m.), yeast tRNA (tRNA), 30 μg of total RNA from 12.5-day rat embryo + placenta (E/P12.5), or 50-μg aliquots of the brain RNA samples shown in Figure 10.

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**AJP** June 2000, Vol. 156, No. 6
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nium compounds; second, that the mechanism of oxygen sensing in endothelial cells may have different properties when compared to other cell types. It is tempting to speculate that different cell types have different mechanisms of oxygen sensing, perhaps due to the existence of a family of oxygen sensors (in association with cell-specific components), whose precise distribution and configuration would depend on the cell type considered, its localization in the body with respect to blood, its metabolic requirements and specific pattern of gene expression in response to hypoxia.

Is hypoxia a physiological stimulator of Ang-2 expression in the microvasculature in vivo? Although the constant presence of blood in vessels may argue against this idea, four pieces of evidence suggest that this may indeed be the case. First, endothelial cells of the microvasculature are likely to be subjected to hypoxic conditions in situations of exceptionally high tissue metabolism and/or reduced blood flow, as would result, for example, from the formation of microthrombi or in situations of vascular stasis that may occur in tumors and other pathological settings. Second, HIF-1α protein has been detected in the microvascular endothelium of hypoxic ferret lungs.\(^5\)\(^1\) HIF-1α is continuously synthesized within cells and degraded by the proteasome, and protein stabilization is a hallmark of the cellular response to hypoxia.\(^2\)\(^2\) Third, expression of VEGFR-1, which has a functional HIF-1-binding site within its promoter region,\(^4\)\(^3\) is induced in the microvasculature of mice exposed to systemic hypoxia.\(^3\)\(^8\) Fourth, the HIF-1α-related transcription factor, HIF-2α, the expression of which is restricted almost exclusively to endothelial cells, is activated by conditions of intermediate hypoxia, whereas HIF-1α is not.\(^5\)\(^2\) This is interesting in view of the fact that when compared to other cell types, endothelial cells, by being constantly exposed to the circulating blood, may need to sense and to respond to conditions of less severe hypoxia. Taken together, these findings suggest that endothelial cells do sense hypoxia in vivo and respond to hypoxic conditions with an appropriate and specific program of gene expression, in which induction of the Ang-2 gene is prominent.

### Acknowledgments

We thank Dr. P. Maisonpierre for helpful discussions, Dr. N. Maggiano for the HUVE cells; Dr. E.W. Ades and T. Lawley for the HMEC-1 cells; Drs. M.B. Furie and S.C. Silverstein for the BME cells; Dr. B. Berse for the rat VEGF probe; M. Quayzin, Joao Oliveira, J. Mandelbaum, and B. Richter for excellent technical assistance; and P.-A. Ruttimann and J.-P. Gerber for photographic work.

### References