

Angiogenic Actions of Angiopoietin-1 Require Endothelium-Derived Nitric Oxide

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Angiopoietin1 (Ang1) is a novel angiogenic factor with important actions on endothelial cell (EC) differentiation and vascular maturation. Ang1 has been shown to prevent EC apoptosis through activation of PI3-kinase/Akt, a pathway that is also known to activate endothelium nitric oxide synthase (eNOS). Therefore, we hypothesized that the angiogenic effects of Ang1 would also be dependent on the PI3-kinase/Akt pathway, possibly mediated by increased eNOS activity and NO release. Treatment of human umbilical vein endothelial cells with recombinant Ang1* (300 ng/ml) for 15 minutes resulted in PI3-kinase-dependent Akt phosphorylation, comparable to that observed with vascular endothelial growth factor (VEGF) (50 ng/ml), and increased NO production in a PI3-kinase/Akt-dependent manner. Capillary-like tube formation induced by Ang1* in fibrin matrix at 24 hours (differentiation index, DI: 13.74 ± 0.76 versus control 1.71 ± 0.31) was abolished in the presence of the selective PI3-kinase inhibitor, LY294002 (50 $\mu\text{mol/L}$) (DI: 0.31 ± 0.31 , $P < 0.01$) or the NOS inhibitor, L-NAME (3 mmol/L) (DI: 4.10 ± 0.59 , $P < 0.01$). In subcutaneous Matrigel implants *in vivo*, addition of recombinant Ang1* or wild-type Ang1 from conditioned media of COS-1 cells transfected with a pFLAG Ang1 expression vector, induced significant neovascularization to a degree similar to VEGF. Finally, angiogenesis *in vivo* in response to both Ang1 and VEGF was significantly reduced in eNOS-deficient compared with wild-type mice. In summary, our results demonstrate for the first time that endothelium-derived NO is required for Ang1-induced angiogenesis, and that the PI3-kinase signaling mediates the activation of eNOS and NO release in response to Ang1. (Am J Pathol 2003, 162:1927–1936)

Angiopoietin-1 (Ang1) has recently been identified as a ligand of the endothelial selective receptor tyrosine kinase (RTK), Tie2.¹ Tie2 signaling has been shown to be

required for later stages of embryonic blood vessel development,^{2–4} including vascular remodeling, vessel integrity, and maturation.^{1,5} *In vitro* experiments have shown that Ang1 induces endothelial cell (EC) sprouting *in vitro*,^{6,7} and stimulates EC migration⁸ and the formation and stabilization of capillary-like networks in culture systems.^{9–11}

One of the signaling cascades initiated by Tie2 phosphorylation is the recruitment of Dok-R,¹² a novel docking molecule that on phosphorylation binds to proteins containing Src homology 2 (SH2) domains.¹³ The recruitment of a Nck-p21 activated kinase (Pak) complex to Dok-R,¹⁴ mediates EC migration.¹⁵ In addition, the p85 subunit of phosphatidylinositol (PI) 3-kinase can interact specifically with the phosphorylated Tie2 through its SH2 domain. Although an initial report suggested that activated Tie2 does not associate with PI3-kinase,¹⁶ subsequent studies have confirmed that activation of Tie2 leads to PI3-kinase activation,¹⁷ the lipid products of which participate in the regulation of cell survival by activation of the serine/threonine kinase, Akt.¹⁸ Indeed, the PI3-kinase/Akt pathway has been shown to transduce the antiapoptotic effect of both vascular endothelial growth factor (VEGF)¹⁹ and Ang1.^{17,20}

Recently, Akt has also been demonstrated to directly phosphorylate endothelial nitric oxide synthase (eNOS), leading to calcium/calmodulin-independent enzyme activation.^{21,22} Endothelium-derived nitric oxide (NO) is a crucial mediator in the regulation of EC growth, survival, and angiogenesis.^{23,24} Importantly, eNOS knockout (KO) mice have also been shown to exhibit marked impairment in postnatal angiogenesis in response to growth factors delivery and ischemia.^{25,26}

The goal of this study was to define the role of endothelium-derived NO in Ang1-mediated angiogenesis both *in vitro* and *in vivo*. We now report that Ang1 induced the activation of Akt and increased NO release in cultured ECs by a PI3-kinase-dependent mechanism. Moreover, Ang1-induced capillary-like tube formation in three-dimensional fibrin matrices *in vitro* and neovascularization

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of Matrigel implants in response to Ang1 *in vivo* were dependent on endothelium-derived NO.

Materials and Methods

Materials

Human Ang1* was kindly provided by Regeneron Pharmaceuticals, Inc. (Tarrytown, NY). Ang1* is a genetically engineered variant of naturally occurring Ang1 that retains similar properties in all assays. In Ang1*, the non-conserved cysteine at residue 245 has been mutated to the corresponding serine residue of Ang2, and the first 77 amino acids of human Ang1 have been replaced with the first 73 residues of Ang2.¹ The recombinant Ang1* protein was prepared in buffer containing 0.05 mol/L Tris-HCl pH 7.5, 150 mmol/L NaCl and 0.05% CHAPS. Native human Ang1 and VEGF₁₆₅ were obtained from R&D Systems (Minneapolis, MN). Other sources of materials are indicated as mentioned.

Cell Lines and Culture

Human umbilical vein endothelial cells (HUVEC) and monkey kidney (COS-1) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). HUVECs were maintained in culture in Ham's 12 medium (Invitrogen/Gibco, Burlington, ON) supplemented with 15% fetal bovine serum (FBS), penicillin (500 U/ml), streptomycin (50 μ g/ml) and heparin (100 μ g/ml), (all from Invitrogen/Gibco), and EC growth factor (ECGF 20 μ g/ml; Roche Diagnostics, Mannheim, Germany) and equilibrated with 95% air and 5% CO₂ at 37°C. Cells between passages 13 and 18 were used in these experiments. COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics as indicated above.

Plasmid Transfection

Plasmid (pFLAG-Ang1 kindly provided by Dr. Injune Kim, University of South Korea), encoding the human Ang1, fused with a c-Myc tag at the C terminus, was expressed in COS-1 cell line. Transient transfection was performed using Superfect reagent (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Twenty hours after transfection, cells were incubated in serum-free DMEM for another 24 hours. The conditioned medium (CM) was harvested and concentrated 100 \times using Amicon Centricon 10-kd cutoff columns (Millipore Corp., Bedford, MA).

Animals

Male C57 (WT) and eNOS KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in filter-topped cages, maintained with a day/night cycle of 12 hours under pathogen-free conditions, fed a standard diet of rodent chow, and given water *ad*

libitum until they reached 6 to 8 weeks of age. All animal use was approved by and adhered closely to the guidelines set out by the Animal Care and Use Committee, St. Michael's Hospital.

Preparation of Fibrin Gels

Endotoxin- and plasminogen-free human fibrinogen (10 mg/ml, Calbiochem-Novabiochem Corp., La Jolla, CA) was prepared as previously described.²⁷ After polymerization, gels were soaked in cultured medium containing 15% FBS for 2 hours at 37°C to inactivate the thrombin. EC were plated on the surface of the three-dimensional matrix and culture for 24 hours in the presence or absence of study agents as described above.

In Vitro Angiogenesis

HUVECs were cultured on fibrin-matrix, pretreated with N^G-nitro-L-arginine methyl ester (L-NAME, 3 mmol/L; 1 hour) or with LY294002 (50 μ mol/L; 2 hours) before exposure to recombinant Ang1* (300 ng/ml). After 24 hours, total length of capillary-like structures >30 μ m was derived using an Olympus BX50 inverted microscope (100 \times) for each of six randomly preselected fields. At the same time, the total area of residual EC monolayer was determined for the same fields, and differentiation index (DI) was calculated as the ratio of total tube length over cell area for each field. Images were taken using a digitized Sony CCD-IRIS/RGB camera (Cohu Inc., Japan) and analyzed by a computer-assisted morphometric analysis system (C-Imaging, Compix Inc., Cranberry Township, PA) by observers blinded to the experimental conditions.

Western Blot Analysis and Tie2 Phosphorylation

CM was collected from pFLAG (mock)-transfected or pFLAG-Ang1 transfected COS-1 cells. Ten microliters of 100 \times concentrated CM was subjected to SDS-PAGE, and proteins were transferred onto nitrocellulose membrane and probed with an anti c-Myc monoclonal antibody (1:5000 dilution according to the supplier's instruction; Invitrogen, Carlsbad, CA).

For Tie2 phosphorylation studies, HUVECs were maintained overnight in F12 medium with 1% FBS, and stimulated for 10 minutes in serum-free medium with Ang1* protein (300 ng/ml), mock-CM or concentrated Ang1-CM (200 μ l CM/ml). Cells were solubilized with radio immunoprecipitation assay (RIPA) lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mmol/L Tris, pH 7.6, 50 mmol/L sodium fluoride, 150 mmol/L sodium chloride, 1 mmol/L EDTA, 5 mmol/L benzamidine, 1 mmol/L sodium orthovanadate, 10 μ g/ml aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 1 μ g/ml pepstatin). The lysates were immunoprecipitated with anti-Tie2 antibodies (C-20, Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were recovered on Protein G-Sepharose and separated by SDS-PAGE. Proteins were transferred onto nitrocellulose

membrane, blocked with 5% bovine serum albumen, 1X TBS, 0.1% Tween-20 for 2 hours, and probed with anti-phosphotyrosine antibodies 4G10 (1:2500, Upstate Biotechnology, Inc., Lake Placid, NY), stripped and re-probed with anti-Tie2 (1:1000). Specific bands were visualized using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). Densitometry was performed by scanning the immunoblots (imaging densitometer; BioRad, Hercules, CA) and the intensity of each band was analyzed using the Molecular Analyst software (BioRad).

Akt and eNOS Phosphorylation

HUVEC were grown until confluence, serum-starved for 12 hours in F12 medium containing 1% FBS, and then the medium was changed to serum-free F12 containing the inhibitors of PI3-kinase, wortmannin (100 nmol/L) or LY294002 (50 μ mol/L) (both from Sigma, St. Louis, MO), or the selective Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (20 to 75 μ mol/L; Calbiochem-Novabiochem). After a 2-hour pretreatment with inhibitors, the cells were stimulated with Ang1* (100 to 600 ng/ml, and in some cases native Ang1 for comparison) for 15 to 30 minutes before lysis with 200 μ l of SDS sample buffer (10 mmol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.4% dithiothreitol, 1 mmol/L orthovanadate). Proteins (50 μ g per lane) were separated on SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blotted using antibodies specific to phospho-Akt (Ser⁴⁷³, 1:1000; New England Biolabs, Beverly, MA), or phospho-eNOS (Ser¹¹⁷⁷, 1:1000; Cell Signaling Technology, New England Biolabs) and developed by ECL as described above. The blots were then stripped and re-probed with Akt (1:1000, New England Biolabs), eNOS (1:2500, Transduction Laboratories, Lexington, KY), or Tie2 antibodies (1:1000, Santa Cruz) and the bands were scanned and analyzed by densitometry as described above.

Measurement of NOS Activity by Quantification of NO Release

The measurement of NO production in ECs were performed using the amino-700 NO sensor (Innovative Instruments, Inc., Tampa, FL) which is 100 times more sensitive than the Griess reagent (detection limit below 1 nmol/L). Cells were treated as described above and 50 μ l of cell culture media was injected into an acid/iodide solution to reduce NO₂ stoichiometrically to NO, detected by sensor. The exchange of electrons between NO and the electrode surface resulted in an electrical current, which was monitored and recorded. The analyzer was calibrated on the day of the experiment with nitrite (NO₂) standards, and the results were normalized to the cell number in the plate. NO production was confirmed using the Griess reaction as previously described.²⁷

The Matrigel Plug Assay to Assess in Vivo Angiogenesis

Matrigel (0.5 ml, Collaborative Biomedical Products) containing Ang1* (300 ng/ml), VEGF (100 or 300 ng/ml, as indicated), or PBS was injected subcutaneously at either side of the abdominal midline of WT or eNOS KO mice ($n = 12$ /group). For the CM experiments, 100 μ l of concentrated CM (100 \times) were mixed with 400 μ l of Matrigel and injected subcutaneously into the animals. On day 10, Matrigel plugs were harvested and subjected to histological analysis or determination of blood content (see below).

For histology, the Matrigel plugs were fixed in 10% buffered formalin for 24 hours and paraffin blocks were cut to 5- μ m sections and stained with hematoxylin and eosin (H&E). Adjacent sections from each group of animals were also collected for immunohistochemistry using vWF polyclonal rabbit anti-human antibody (1:250 dilution, Dako Corp., Santa Barbara, CA) for 1 hour at 37°C and subsequently treated with goat anti-rabbit antibody (1:300 dilution; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature and then treated with streptavidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories) for 30 minutes at room temperature. Diaminobenzadine was used as the peroxidase substrate and hematoxylin as the nuclear counterstain. Negative controls were prepared by substituting preimmune rabbit serum for the primary antibody. The sections were stained for vWF and the images were captured under 20 \times objective with the use of a Cool SNAP camera (Photometrics, Tucson, AZ) connected to an inverted microscope. On average, 6 to 8 fields were taken from each section. Results were expressed as mean number of vWF-positive vessels per mm².

Fluorescein isothiocyanate-dextran (FITC-dextran; molecular weight, 200-kd) was used as an index of functionality of the Matrigel neovessel.²⁸ FITC-dextran was prepared fresh (25 mg/ml in PBS) and 0.02 ml was injected into the lateral tail vein of each mouse 10 days after the Matrigel injection, and then the mice were sacrificed by cervical dislocation after 20 minutes, blood samples were collected by cardiac puncture into heparinized tubes, centrifuged immediately, and plasma was separated and protected from light at 4°C. The matrix plugs were then promptly removed and placed into tubes containing 1 ml of dispase (1:10 dilution in PBS), and incubated overnight, in the dark, in a 37°C shaker. The following day, the plugs were homogenized using a polytron and centrifuged at 2000 \times g for 15 minutes, and the supernatant was saved and kept in the dark until fluorescence analysis. Fluorescence readings were obtained on FL600 fluorescence plate reader using a standard curve created by serial dilution of FITC-dextran used for injection. Angiogenic response was calculated as a ratio of Matrigel plug/plasma fluorescence and presented as fluorescent unit (FU).

Statistics

One-way analysis of variance was used to determine the significance of differences between groups, where ap-

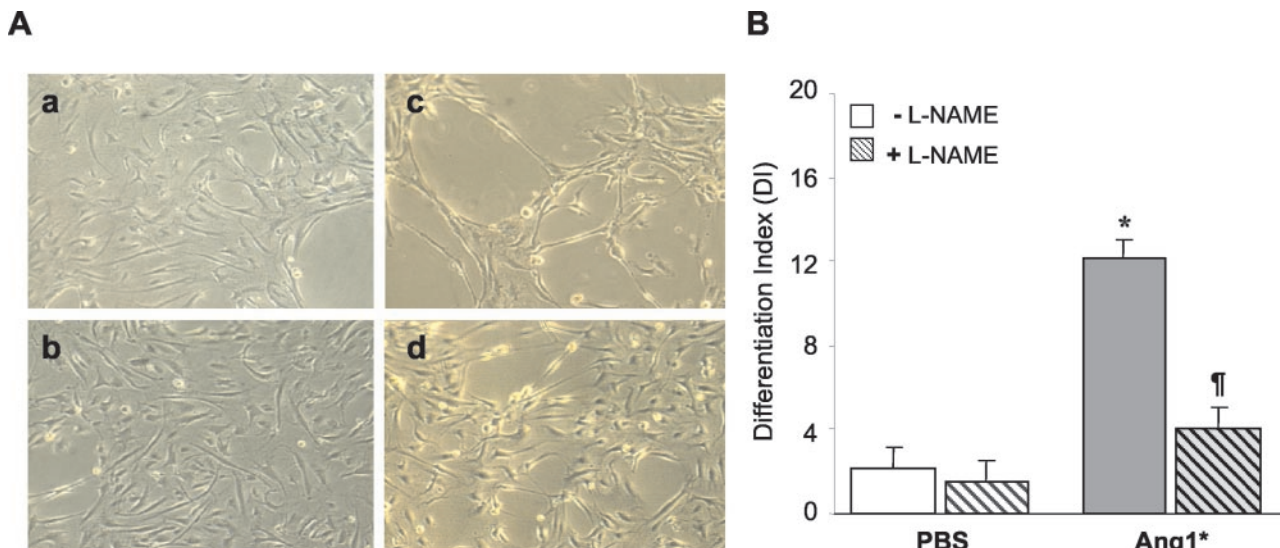


Figure 1. Nitric oxide-synthase activity required for angiogenic effects of Ang1. **A:** Ang1*-induced capillary-like tube formation in the three-dimensional fibrin matrices is blocked by inhibition of endogenous eNOS activity. HUVECs were cultured on fibrin matrices in the presence (**c** and **d**) or absence (**a** and **b**) of recombinant Ang1*, with L-NAME (**b** and **d**) or without L-NAME (**a** and **c**) for 24 hours. A significant increase in tube length was observed in the presence of Ang1* which was inhibited by L-NAME (original magnification, $\times 100$). **B:** Quantitative analysis of capillary-like network formation calculated as the ratio of total tube length over cell area for each field (DI, differentiation index). Scale bars represent a mean \pm SEM of DI value obtained for each culture well from six independent experiments. The difference among groups were analyzed by analysis of variance followed by Student's *t*-test. * $P < 0.05$ versus control, # $P < 0.05$ versus Ang1*. Ang1* (300 ng/ml), L-NAME (3 mmol/L).

appropriate, with post hoc Student's *t*-test for unpaired observations and Bonferroni correction for multiple comparisons. Summary data are presented as means \pm SEM or as percentage of control of the indicated numbers of observations. Statistical significance is defined as $P < 0.05$.

Results

Ang1-Induced Angiogenesis and NO

The addition of Ang1* to HUVEC cultured on 3-D fibrin matrices resulted in the formation of extensive capillary-like networks at 24 hours (Figure 1A, panel c), as has previously been reported.⁹ Addition of the nonselective inhibitor of NOS, L-NAME, nearly completely inhibited this effect of Ang1* in cultured ECs (Figure 1A, panel d). Figure 1B shows summary data for six separate experiments. The addition of L-NAME markedly reduced differentiation index (DI) in Ang1*-treated wells to values not significantly different from control.

Tie2 and Akt Phosphorylation Studies

Stimulation of HUVECs with Ang1* (300 ng/ml) or Ang1-CM for 10 minutes induced a marked increase in Tie2 phosphorylation (Figure 2A, left). This was associated with Akt phosphorylation at Ser⁴⁷³ (Figure 2B), which was nearly completely blocked by two specific inhibitors of PI3-kinase, wortmannin and LY294002. A 70-kd single band was observed in Ang1-CM but not in mock-CM subjected to Western blot analysis using anti-c-Myc antibody, consistent with the expression of the Ang1 c-Myc fusion protein (Figure 2A, right).

eNOS Phosphorylation Studies

Densitometric analysis of the Western blot bands showed that Ang1*-stimulated eNOS phosphorylation in a dose-dependent manner (100 ng/ml: 2.96 ± 0.18 , 300 ng/ml:

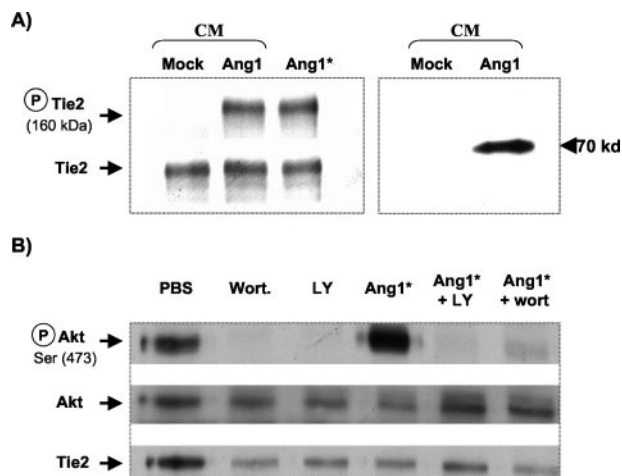


Figure 2. Ang1 stimulates Tie2 and Akt phosphorylation. **A:** Tie2 receptor tyrosine phosphorylation by Ang1-CM or Ang1* in HUVECs visualized by immunoblot analysis with mouse monoclonal anti-phosphotyrosine antibodies (**left panel, top**), and anti-Tie2 antibody to normalize equal loading (**bottom**). Note that the similar level of phosphorylation of Tie2 could be detected following stimulation with Ang1-CM or Ang1* recombinant protein, whereas no phosphorylation was seen following stimulation of cells with mock-CM. Western blot analysis of the concentrated CM obtained from pFLAG (mock)-transfected COS-1 cells or cells expressing pFLAG-Ang1, shows a single 70-kd band, confirming the expression of Ang1 as a c-Myc fused protein (**right**). Data shown are representative of two independent experiments. **B:** Stimulation of HUVEC with Ang1* (300 ng/ml), increased Akt phosphorylation, which was blocked by the PI3-kinase inhibitors, LY294002 (50 μ mol/L) or wortmannin (100 nmol/L). All figures are representative of three separate experiments. Akt phosphorylation (P-Akt, Ser 473, **top**); total Akt expression (**center**) and total Tie2 expression (**bottom**).

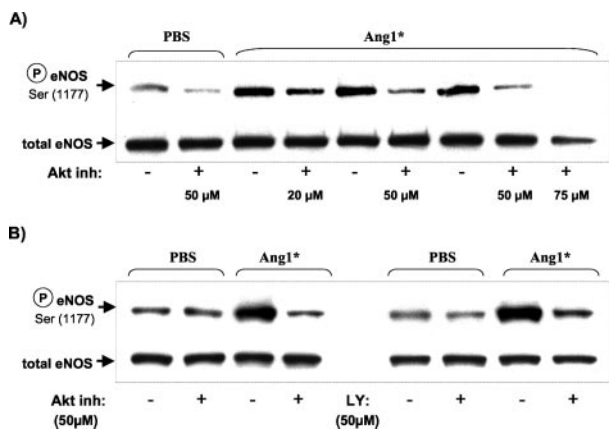


Figure 3. Ang1*-dependent phosphorylation of eNOS is blocked by PI3-kinase and Akt inhibitors. **A:** HUVEC were maintained overnight in F12 medium with 1% FBS, then pretreated for 2 hours in serum-free medium with an Akt inhibitor at various doses (20 to 75 $\mu\text{mol/L}$) to evaluate optimal conditions as indicated. **B:** HUVECs were cultured as above and pretreated either with an Akt inhibitor (50 $\mu\text{mol/L}$) or a PI3-kinase inhibitor LY294002 (50 $\mu\text{mol/L}$). In both panels the cells were either stimulated with Ang1* (300 ng/ml, 30 minutes) or buffer solution. After the stimulation, Western analysis was performed using antibodies specific to phosphorylated eNOS and total eNOS.

$3.27 \pm 0.24^*$ and 600 ng/ml: $3.74 \pm 0.37^{**}$; $*P < 0.05$, $**P < 0.01$ versus PBS: 1.63 ± 0.27) with a maximal response similar to that of native Ang1 (600 ng/ml: 4.39 ± 0.60 , $P < 0.01$). Based on these experiments the 300 ng/ml concentration was used for the *in vivo* studies.

Next we tested the upstream pathways that were involved in eNOS phosphorylation by using selective inhibitors of Akt and PI3-kinase (Figure 3). Addition of Akt inhibitor (20 to 75 $\mu\text{mol/L}$) dose-dependently inhibited eNOS phosphorylation in response to Ang1* (Figure 3A); however, at 75 $\mu\text{mol/L}$ total eNOS expression was also reduced, possibly due to cytotoxicity. Therefore, in further experiments a concentration of 50 $\mu\text{mol/L}$ was used. Inhibition of Akt (50 $\mu\text{mol/L}$) reduced eNOS phosphorylation in response to Ang1* to a similar degree as the inhibition of PI3-kinase (Figure 3B).

PI3-Kinase Inhibitors Significantly Reduce Ang1*-Dependent Tube Formation and NO Release

Capillary-like tube formation in the fibrin matrix model in response to Ang1* was completely inhibited by the PI3-kinase inhibitor, LY 294002 (50 $\mu\text{mol/L}$) (Figure 4C). To determine whether the effect of Ang1* was associated with an increase in the release of NO, HUVECs were stimulated with Ang1* for up to 24 hours and the medium was collected for measurement of NO. As shown in Figure 5, treatment of HUVECs with Ang1* resulted in an increase in NO production, which was apparent after only 30 minutes of exposure and was sustained for up to 24 hours. Increase in NO release after 24 hours of incubation with Ang1* was confirmed using the Griess reagent (data not shown). The ability of Ang1* to stimulate NO release was abrogated by pretreatment with the PI3-kinase inhib-

itor LY294002 (Figure 5), which itself had no effect on basal NO levels (data not shown).

Effect of Ang1 on *in Vivo* Neovascularization

To assess the role of Ang1 on angiogenesis *in vivo*, we studied neovascularization of Matrigel implants supplemented with Ang1* (300 ng/ml) or VEGF (100 ng/ml). Both of the angiogenic factors induced neovascularization of the Matrigel plugs within 10 days (Figure 6,b and c), while only minimal angiogenesis was observed in the control plugs (Figure 6a). To determine the effect of wild-type Ang1, we added 100 μl of either concentrated CM from pFLAG-Ang1 transfected cells (Ang1-CM) or CM from mock-vector transfected cells (mock-CM) to the Matrigel (PBS served as a negative control). In WT mice, Ang1-CM resulted in greater neovascularization of the Matrigel implants compared to plugs containing mock-CM or PBS alone (Figure 7A). Moreover, the response to Ang1-CM was not different from that observed with the purified recombinant Ang1* or VEGF protein (both 300 ng/ml). Similar results were seen when FITC-dextran perfusion was used as an index of Matrigel blood content (Figure 7B); however, in this case VEGF produced substantially greater increases in fluorescent signals than Ang1* or Ang1-CM, possibly due to differential effects on vascular permeability which may have resulted in greater extravasations of labeled dextran in the VEGF-treated inserts. To test whether the combination of these two factors would have additive effects, we measured the blood volume in the Matrigel plugs using FITC-dextran content as a marker. The results of this experiment suggested that the combination of these two agents was slightly less than additive (Ang1*: 17.27 ± 1.05 , VEGF: 28.47 ± 1.47 and Ang1* plus VEGF: 33.82 ± 3.23 FU; all = $P < 0.05$ vs. PBS: 5.19 ± 0.61).

eNOS Plays a Critical Role in Ang1-Induced Angiogenesis *in Vivo*

The role of endothelium-derived NO in Ang1-induced neovascularization *in vivo* was tested in the Matrigel model. Ang1-CM resulted in significantly less neovascularization in eNOS KO (Figure 6f) compared to the WT (Figure 6e) mice as determined by the number of vWF positive neovessels. Summary data of the vessels numbers are shown in Figure 7A. These results were consistent with a significant decrease in FITC-dextran content of the Ang1* or Ang1-CM treated Matrigel plugs obtained from the eNOS KO mice compared to those collected from the WT mice (Figure 7B).

Discussion

This is the first report to explore the importance of NO as a downstream mediator of the biological effects of Tie2 activation. We now show that the formation of a capillary-like network *in vitro* in response to Ang1 required a PI3-kinase/Akt-dependent increase in NO release and that

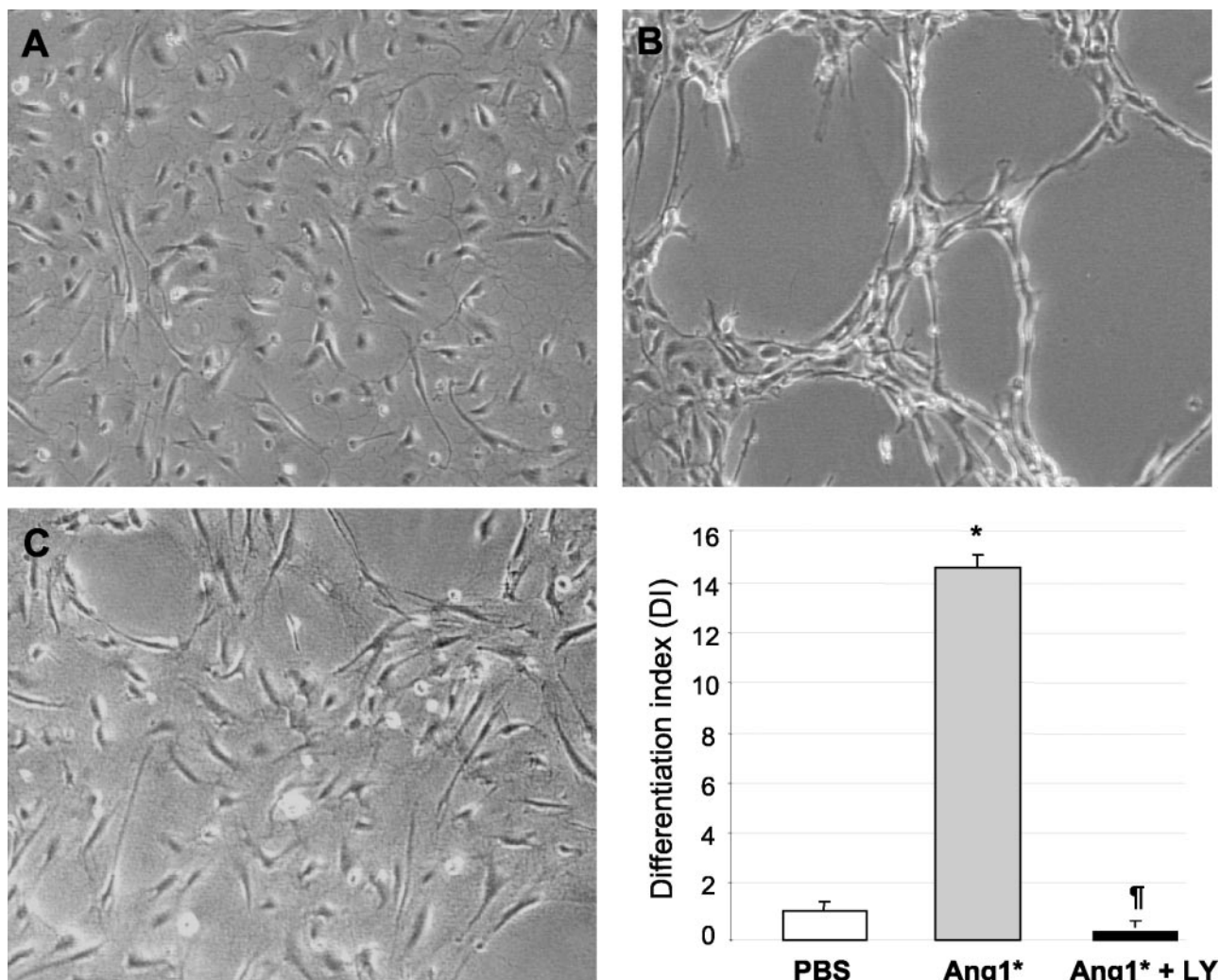


Figure 4. Ang1*-induced activation of PI3-kinase/Akt pathway is required for endothelial cell tube formation. **A:** HUVEC plated on fibrin matrices were pretreated with 50 μ mol/L of LY294002 for 2 hours before addition of Ang1* (300 ng/ml) or PBS, and then incubated for 24 hours. HUVEC grown on fibrin gel in the presence of PBS (control) showed no significant angiogenic response (**A**). In contrast, cells treated with Ang1* showed an extensive network of capillary-like tube formation (**B**), which was prevented by addition of LY294002 (**C**). Original magnification, $\times 100$. Bars represent the mean \pm SEM of four independent experiments. Statistical comparisons were performed using one-way analysis of variance followed by Student's *t*-test. * $P < 0.05$ versus control, [†] $P < 0.05$ versus Ang1*.

Ang1-induced neovascularization *in vivo* was dependent on eNOS expression.

Ang1 and its receptor, Tie2, play a critical role in embryonic vascular development. Targeted disruption of these genes has been reported to produce embryonic lethality due to major abnormalities in vascular development. However, unlike VEGF, which is necessary for the differentiation of EC from the primitive mesoderm,²⁹ defects in Ang1/Tie2 KO mice involve a later stage of vascular development.⁵ These embryos are characterized by large and poorly organized vessels, which lack mature basement membrane and exhibit partial failure of pericytes recruitment.⁵ Ang1 has also been shown to induce postnatal angiogenesis in a variety of experimental models. In cultured cells, Ang1 alone induced EC chemotaxis,^{8,14,15,30} capillary-like tube formation⁹⁻¹¹ and sprouting angiogenesis,³⁰ and enhanced EC survival.^{11,20} *In vivo* reports have shown that Ang1 is unable to induce angiogenesis alone, but potentiated the angiogenic response to VEGF.³¹⁻³³ In contrast, in the present report, Ang1

resulted in robust neovascularization of Matrigel implants directly, and the combination with VEGF was at best additive. These results are consistent with previous reports showing Ang1-induced angiogenesis in the absence of exogenous VEGF.^{34,35} The reason for the variable requirement for VEGF in these models is unclear, but may relate to differences in its endogenous expression of growth factors. Since Ang1 does not directly induce EC proliferation,^{6,9,31} the action of an additional EC mitogen such as VEGF may be crucial in facilitating the angiogenic effects of Ang1. The cornea is normally avascular and thus unlikely to express angiogenic factors, whereas in the ischemic hindlimb model, tissue hypoxia results in up-regulation of angiogenic factors and their receptors.³⁶ Matrigel is derived from tumor basement membrane and contains low concentrations of multiple growth factors, possibly providing a suitable background to support the angiogenic actions of Ang1.

In a recent report, overexpression of Ang1 targeted to the skin was shown to result in a network of large venular-

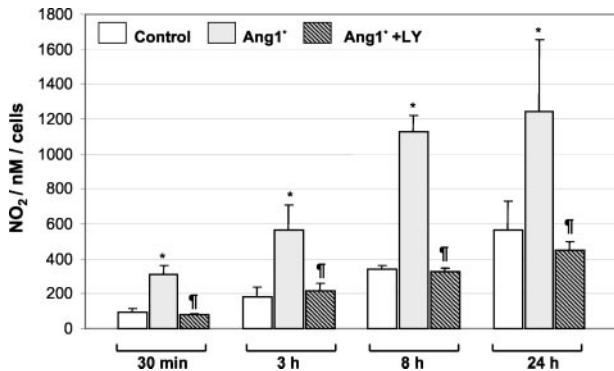


Figure 5. Ang1* activates NO production and release. HUVEC CM was collected after incubation with Ang1* (300 ng/ml) for the periods indicated, and assayed for NO production, as described in the Methods section. Results are expressed as the mean \pm SEM of three independent experiments (five experiments for the 24-hour time point). * $P < 0.05$ versus PBS, † $P < 0.05$ versus Ang1*.

like dermal vessels³⁷ that were resistant to increases in permeability caused by inflammatory agents or concomitant VEGF overexpression.³⁸ This is consistent with the stabilizing effect of Ang1 on vascular endothelium and its underlying basement membrane. In the Matrigel model, the induction of angiogenesis in response to Ang1 also appeared to produce relatively non-leaky neovessels, because although VEGF and Ang1 resulted in similar increases in vessel number, VEGF-treated Matrigel implants contained significantly greater amounts of FITC-labeled dextran. This finding is consistent with differences in permeability characteristics between vessels produced by Ang1 or VEGF; the former resulted in more competent vascular structures, whereas VEGF-induced neovessels may have led to greater extravasation of dex-

tran into the Matrigel. Thus, the apparent increase in blood content of the VEGF-containing Matrigel plugs may have been exaggerated by the hypermeability effects of this factor. This finding may also have relevance for therapeutic angiogenesis, since patients treated with VEGF for lower limb ischemia exhibited limb edema, presumably due to increased capillary leakiness caused by VEGF.³⁹ In our experiments, although both Ang1 and VEGF induced robust neovascularization in the Matrigel implant model, the combination of Ang1 and VEGF appeared to be less than additive. Of interest, Visconti et al⁴⁰ have recently reported that overexpression of Ang1 in a nonischemic transgenic model reduced rather than enhanced VEGF-induced angiogenesis in the heart, suggesting that the interactions between Ang1 and VEGF *in vivo* are complex and depend critically on the model and experimental conditions.

Tie2 Signal Transduction

Recently it has been recognized that the PI3-kinase/Akt pathway plays a pivotal role in signal transduction by a number of tyrosine kinase receptors including VEGF receptors (Flk/KDR) and Tie2. Enhanced EC survival in response to Ang1 has been shown to be dependent on activation of Akt by PI3-kinase (also known as PKB).^{10,17,20,41} Akt promotes cell survival through its ability to phosphorylate Bad and procaspase-9.^{42,43} In addition, a recent report has demonstrated that Ang1 increased EC survival by the induction of the apoptosis inhibitor, survivin, again through the PI3-kinase/Akt pathway.⁴¹ Similarly, the angiogenic response to Ang1 has been shown to be dependent on PI3-kinase activity¹⁵ and

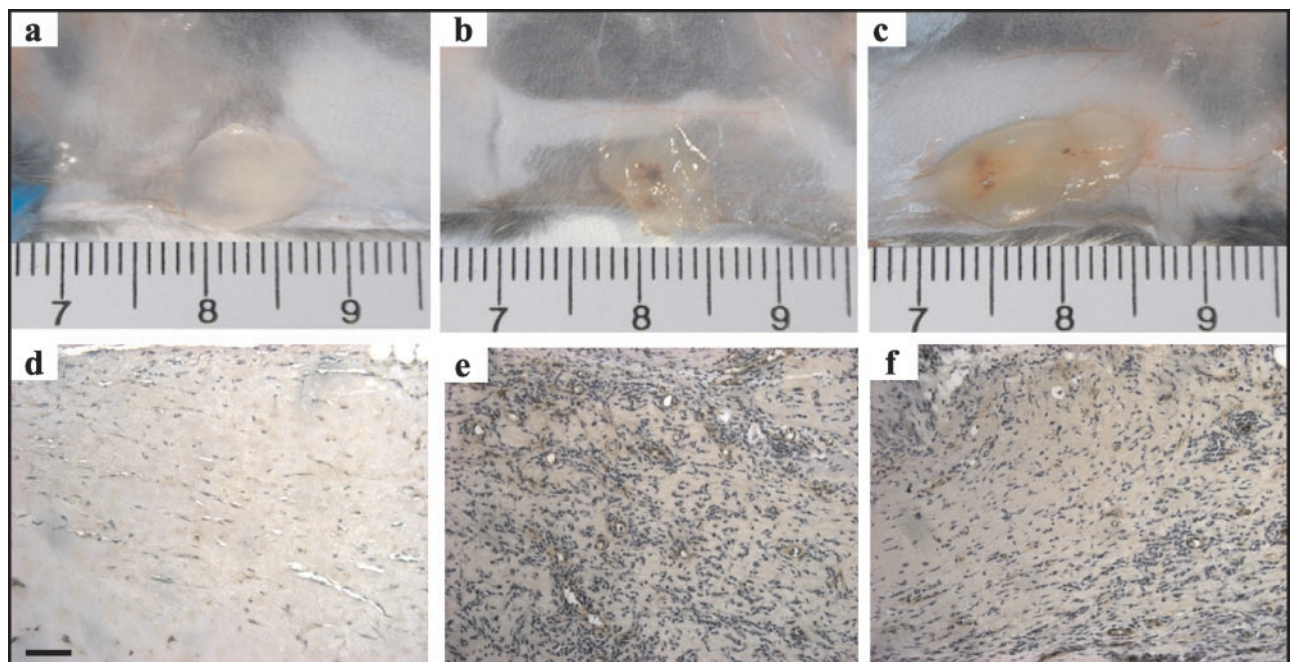


Figure 6. Ang1-induced neovascularization of Matrigel plugs *in vivo*. Gross appearance of Matrigel plugs containing PBS (**a**), Ang1* (300 ng/ml, **b**) or VEGF (100 ng/ml, **c**) implanted for 10 days in WT mice. Histological sections of the Matrigel plugs retrieved from WT (**d** and **e**: mock-CM and Ang1-CM, respectively) or eNOS KO (**f**: Ang1-CM) mice and immunostained with vWF to identify neovessels.

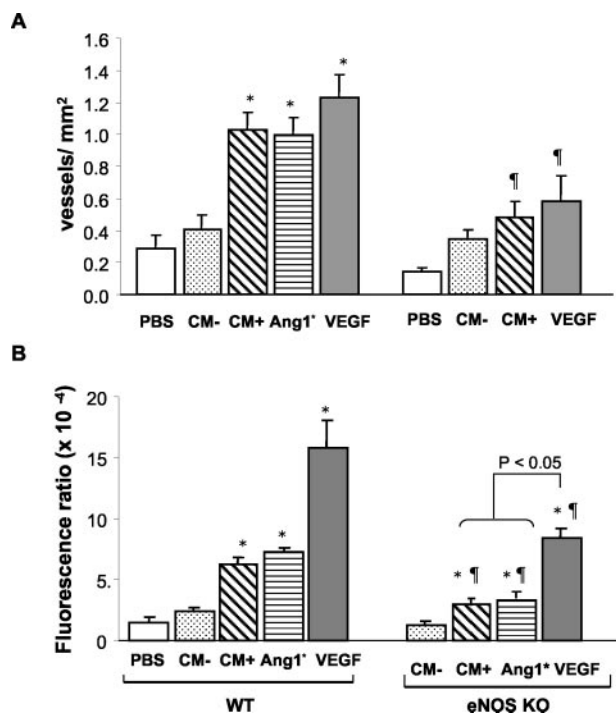


Figure 7. Quantitative analysis of neovascularization of the Matrigel plugs at day 10. **A:** Number of vWF positive vessels per mm² of Matrigel plugs implanted into WT or eNOS KO mice. **B:** Blood content in the Matrigel explants assessed by FITC-dextran perfusion in both WT and eNOS KO mice. Data are presented as mean \pm SEM (6 animals per group). mock-CM (CM-), Ang1-CM (CM+), Ang1* and VEGF (both 300 ng/ml). **P* < 0.05 versus PBS or CM-, [¶]*P* < 0.05 versus WT mice.

downstream activation of p125FAK.³⁰ In addition, it has been reported that phosphorylation of Y1106 on the Tie2 receptor recruits the Tie2-associated docking molecule Dok-R.¹² Recruitment of Dok-R to activated Tie2 leads to its phosphorylation and subsequent recruitment of Nck-p21 activated kinase (Pak) complex to the membrane.¹⁴

Release of endothelium-derived NO requires the activation of eNOS, which until recently has been thought to be largely regulated by a Ca²⁺ and calmodulin. However, several reports have demonstrated that the activity of eNOS can be regulated by phosphorylation. The serine/threonine protein kinase Akt has been shown to increase human eNOS activity in ECs by Akt phosphorylation at Ser¹¹⁷⁹,²¹ which leads to increase NO production even at low intracellular Ca²⁺ concentrations, whereas mutant eNOS (Ser^{1179A} and Ser¹¹⁷⁷) is resistant to phosphorylation and Akt-dependent activation.^{21,22}

Role of NO in Angiogenesis

The mechanisms by which NO mediates angiogenesis have not been fully elucidated. Both clinical and experimental studies support an association between NO production and tumor progression, wherein the level of NOS protein and/or activity in the tumor has been positively correlated with the degree of malignancy for tumors.⁴⁴ The hemodynamic effects of this potent vasodilator may contribute to its angiogenic effects. Increased shear stress in the microcirculation may initiate angiogenesis

by perturbing the luminal side of ECs, or by releasing growth factors or other vasoactive agents.⁴⁵ Increased NO production has also been shown to directly result in EC proliferation²³ and migration,⁴⁶ and to promote angiogenesis both *in vitro* and *in vivo*^{27,47} by a cGMP-dependent mechanism.⁴⁸ Thus, NO has been implicated in nearly all levels of the angiogenic response. Moreover, a number of reports suggest that NO mediates the angiogenic activity of several angiogenic growth factors including TGF- β , bFGF, and VEGF.^{24,27,49} EC proliferation,^{24,50,51} migration,^{50,52} and tube formation²⁴ in response to VEGF *in vitro* could be inhibited by the addition of NOS inhibitors to the cultured EC. Also, experiments using systemic inhibition of NOS⁵³ or eNOS KO animals²⁶ have provided evidence supporting an important role for NO in VEGF-induced angiogenesis *in vivo*. However, the possible role of NO in the angiogenic actions of the angiopoietins remains largely unexplored. An earlier study suggested that the NO pathway was not involved in the response to Ang1,¹¹ but this conclusion was based only on the measurement of cellular cGMP levels. In contrast, the results of the present study support an important role for NO release via the PI3-kinase/Akt pathway in the angiogenic response to Ang1. Pharmacological inhibitors of PI3-kinase and NOS blocked Ang1-induced NO release and prevented EC capillary-like network formation; therefore, signaling between Tie2 and PI3-kinase appears to be essential for Ang1-induced angiogenesis *in vitro*. This is in apparent conflict with the effects of Ang1 in a rodent model of diabetic retinopathy,⁵⁴ in which the diabetes-induced increases in retinal eNOS and NO levels were reduced by Ang1. However, this may have been a result of indirect anti-inflammatory effects of Ang1 in this model, outweighing the direct actions of Ang-1 on these pathways.⁵⁴

The role of endothelium-derived NO in Ang1-induced neovascularization was confirmed *in vivo* using eNOS KO mice. Although the *in vitro* angiogenesis models are useful in exploring the mechanisms underlying this complex process, they may not reproduce faithfully all of the events of the angiogenic cascade required for new blood vessel formation *in vivo*. The Matrigel implant model allows reliable estimation of the angiogenic response since these plugs are completely avascular on implantation and therefore any blood vessels penetrating into the Matrigel arise *de novo*. The present results from the eNOS KO model implicate the endothelial isoform directly in Ang1-induced postnatal angiogenesis, and provide the first data *in vivo* supporting a role for endothelium-derived NO in the angiogenic response to this factor. These data also raise a question concerning the possible role of NO in vasculogenesis during embryonic development since, by necessity, this must occur normally for viable offspring to be produced. However, the litter sizes of eNOS KO mice are much smaller than WT,⁵⁵ consistent with a high degree of embryonic lethality (unpublished data). Although the cause of embryonic wastage has not been fully elucidated, several reports point to abnormalities in vascular and cardiac development.^{55,56}

In summary, our results support an important role for NO in mediating the angiogenic response to Ang1, in a

manner analogous to VEGF and other angiogenic growth factors. Specifically, Ang1 induced increased NO production and capillary-like network formation via activation of PI3-kinase/Akt, and the angiogenic response *in vitro* could be abrogated by inhibition of NOS or PI3-kinase activity. Also, we have confirmed that Ang1 alone induced robust neovascularization *in vivo*, which was largely absent in eNOS KO animals. These data represent the first demonstration of the NO-dependent nature of Ang1-induced angiogenesis and suggest that endothelial function and endothelium-derived NO release will be of critical importance for the full biological response to Ang1.

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