Long-Term and Sustained COMP-Ang1 Induces Long-Lasting Vascular Enlargement and Enhanced Blood Flow

Chung-Hyun Cho, Kyung Eun Kim, Jonghoe Byun, Hyung-Suk Jang, Duk-Kyung Kim, Peter Baluk, Fabienne Baffert, Gyun Min Lee, Naoki Mochizuki, Jin Kim, Byeong Hwa Jeon, Donald M. McDonald, Gou Young Koh

Abstract—Vascular enlargement is a characteristic feature of angiopoietin-1 (Ang1)-induced changes in adult blood vessels. However, it is unknown whether tissues having Ang1-mediated vascular enlargement have more blood flow or whether the enlargement is reversible. We have recently created a soluble, stable and potent Ang1 variant, COMP-Ang1. In the present study, we investigated the effects of varied dose and duration of COMP-Ang1 on vascular enlargement and blood flow in the tracheal microvasculature of adult mice and explored a possible mechanism of long-lasting vascular enlargement. We found that COMP-Ang1 administered by adenoviral vector induced long-lasting vascular enlargement and increased tracheal blood flow. In contrast, short-term administration of COMP-Ang1 recombinant protein induced transient vascular enlargement that spontaneously reversed within a month. In both cases, the vascular enlargement resulted from endothelial proliferation. The COMP-Ang1–induced vascular remodeling is mediated mainly through Tie2 activation. Sustained overexpression of Tie2 could participate in the maintenance of vascular changes. Together, our findings indicate that sustained treatment with COMP-Ang1 can produce long-lasting vascular enlargement and increased blood flow. (Circ Res. 2005;97:86-94.)

Key Words: angiopoietin-1 ■ COMP-Ang1 ■ vascular enlargement ■ blood flow

Angiopoietin-1 (Ang1) is known to be a ligand to Tie2 tyrosine kinase receptor expressed on endothelial cells.1 Ang1/Tie2 signaling is thought to be involved in branching and remodeling of the primitive vascular network and in the recruitment of mural cells during development.2,3 Transgenic overexpression of Ang1 using the skin-specific keratin-14 promoter produces leakage-resistant and enlarged vessels with an increased number of endothelial cells in skin.4,5 Gene transfer of Ang1 into ischemic tissues produces notably enlarged blood vessels.6,7 Baffert et al recently identified that Ang1-induced vascular enlargement could be the result of endothelial proliferation in trachea mucosa.8 Thus, a cardinal feature of Ang1-induced vascular remodeling is vascular enlargement resulting from endothelial cell proliferation in adult animals.4–8

Given that Ang1-induced therapeutic benefits correlated with vascular enlargement in the ischemic tissues,6,7,9 enhanced blood flow through blood vessels enlarged by Ang1 treatment could provide a great therapeutic benefit to ischemic peripheral tissues. However, it is not known whether the tissues having Ang1-mediated enlarged vessels have more blood flow. In addition, the effective dose and treatment period of Ang1 for inducing effective vascular enlargement is not known. Moreover, it is not known whether Ang1-mediated vascular enlargement regresses when Ang1 stimulation is withdrawn.

We have recently developed a soluble, stable, and potent Ang1 variant, COMP-Ang1.10 To create this protein, we replaced the amino-terminal portion of Ang1 with the short coiled-coil domain of cartilage oligomeric matrix protein (COMP). COMP-Ang1 is more potent than native Ang1 in phosphorylating the Tie2 receptor and signaling via Akt in primary cultured endothelial cells.10

In the present study, we investigated effects of period and dose of COMP-Ang1 on vascular enlargement and tissue blood flow in adult mice and investigated a possible mechanism for long-lasting vascular enlargement induced by long-term and sustained COMP-Ang1. To determine the underly-
ing mechanism of COMP-Ang1–stimulated vascular remodeling in adult mice, we focused on the microvasculature of the trachea, which is distinguished by its simplicity and monolayer structure. Our results indicate that long-term and sustained COMP-Ang1 produced by adenoviral delivery of COMP-Ang1 induces a long-lasting vascular enlargement and enhanced blood flow without enhanced pericyte recruitment in adult mice. Long-lasting Tie2 expression could be involved in the long-lasting vascular enlargement and enhanced blood flow.

Materials and Methods

Generation of COMP-Ang1 Recombinant Protein and Ade-COMP-Ang1
Recombinant Chinese hamster ovary cells expressing COMP-Ang1 (CA1–2; production rate, ~30 mg/L) were established as previously described. Recombinant adenovirus expressing COMP-Ang1 or LacZ was constructed using the pAdEasy vector system (Qbiogene). For additional Materials and Methods, see online data supplement at http://circres.ahajournals.org.

Animals, Treatment, and Measurement of Blood Pressure and Heart Rate
Specific pathogen-free FVB/N mice and Tie2-GFP transgenic mice (FVB/N) were purchased from Jackson Laboratory and bred in our pathogen-free animal facility. Male mice 8 to 10 weeks old were used for this study. Animal care and experimental procedures were performed under approval from the Animal Care Committees of the Korea Advanced Institute of Science and Technology. For protein treatment, 200 μg of COMP-Ang1 recombinant protein or BSA dissolved in 50 μL of sterile 0.9% NaCl was injected directly through the tail vein for 2 weeks. For adenoviral treatment, the indicated amount of Ade-COMP-Ang1, Ade-LacZ, or Ade-sTie2-Fc (generous gift from Drs Gavin Thurston and Ella Ioffe at Regeneron Pharmaceuticals, Tarrytown, NY) diluted in 50 μL of sterile 0.9% NaCl was injected intravenously through the tail vein. Systemic blood pressure and heart rate were measured under anesthesia.

Enzyme-Linked Immunosorbent Assay
Approximately 50 μL of blood was obtained from the tail vein into a heparinized capillary tube at the indicated times. ELISA was adopted for precise detection of COMP-Ang1 in plasma.

Immunohistochemical Staining
Mice were anesthetized, perfused with 1% paraformaldehyde in PBS, and several organs including tracheas were removed. Tracheas and ear skins were immunostained as whole mounts, whereas other organs were immunostained as sections. Signals were visualized, and digital images were obtained with a Zeiss LSM 510 confocal microscope.

Measurement of Tracheal Tissue Blood Flow
After the mice were anesthetized, a type N flowprobe (Transonic Systems Inc, Ithaca, NY) was placed on tracheal wall along second, third, and forth cartilage rings without applying pressure, as this would occlude the vessels and reduce perfusion in the area of interest. The flowprobe was kept in place on the position of the highest sensitivity by a micromanipulator and connected to a laser-Doppler flowmeter (model BLF21; Transonic Systems Inc), which can measure microcirculation in 1 mm³ of tissue for real-time assessment of perfusion (mL/min per 100 g of tissue).

Morphometric Measurements and Statistics
Morphometric measurements of the vessel diameters and area densities in mouse trachea were made as previously described. For each trachea, the numbers of PH3-immunopositive endothelial cells, platelet/endothelial cell adhesion molecule (PECAM)-1-immunopositive blood vessels, and desmin/NG2-immunopositive pericytes were measured in 5 regions, each 0.21 mm² in area. Values were expressed per millimeter squared. Values presented are mean±SD. Significance of differences between mean was tested by analysis of variance followed by the Student–Newman–Keuls test. Statistical significance was set at \( P<0.05 \).

Results

Systemic Adenoviral COMP-Ang1 Produces Differential Enlargements of Blood Vessels in Mouse Tracheal Mucosa
For in vivo treatments with COMP-Ang1, we developed a stable Chinese hamster ovary cell line (CA1–2) which produces COMP-Ang1 at ~30 mg/L. The potency, solubility, oligomerization status, and stability of the COMP-Ang1 produced from CA1–2 are similar to those of COMP-Ang1 produced from COS-7 cells transiently transfected with plasmid vector containing the COMP-Ang1 gene (data not shown). Adult mice were treated with a daily intravenous injection of 200 μg of COMP-Ang1 recombinant protein or BSA through the tail vein for 2 weeks, then blood vessels in the tracheal mucosa were visualized with PECAM-1 immunostaining (Figure 1). Six segments of the microvasculature were distinguished by their position in the vascular hierarchy and differences in endothelial cell morphology. Enhancement of tracheal blood vessels was found in mice that received COMP-Ang1 in the following descending order of effect: postcapillary venules > capillaries > collecting venules > venules > terminal arterioles (Figure 1B). No significant change was noted in segmental arterioles. These phenomena were observed in all individuals of several mouse strains studied (FVB/N, C57BL/6, BALB/c, BALB/c- nu, C3H/HeJ). No changes in the sizes or shape of tracheal blood vessels were found in mice that received BSA.

Short-Term and Intermittent Circulating COMP-Ang1 Induces Reversible Enlargement of Postcapillary Venules and Arterioles in Tracheal Vessels
When 200 μg of COMP-Ang1 recombinant protein was injected intravenously into adult male mice, circulating COMP-Ang1 level peaked immediately after injection (~3.75 minutes), then declined, and returned almost to the control level 3 to 4 hours after treatment (Figure 2A, left). The half-life (\( t_{1/2} \)) of circulating COMP-Ang1 was 11.8 minutes. Daily intravenous injection of 200 μg of COMP-Ang1 for 1 week in mice produced an ~2.0-fold enlargement of postcapillary venules and a 1.4-fold enlargement of terminal arterioles in the trachea (Figure 2). The COMP-Ang1–induced enlargement of postcapillary venules, collecting venules, venous end of capillaries, venules, and terminal arterioles were further increased up to 2 weeks on continuation of daily injection of COMP-Ang1 for up to 2 weeks. However, COMP-Ang1–induced enlarged blood vessels returned gradually to normal after discontinuation of the COMP-Ang1 treatment (Figure 2). One month after discontinuation of the COMP-Ang1 treatment, a second round of treatment with a daily intravenous injection of 200 μg of COMP-Ang1 for 2 weeks induced similar enlargements.
of tracheal vessels, again in a reversible manner (data not shown). In comparison, the diameters of tracheal vessels were indistinguishable between the control and experimental periods in tracheal vessels of mice treated with BSA (data not shown). These results indicate that short-term spikes of circulating COMP-Ang1 induce reversible enlargement of some tracheal vessels.

Long-Term and Sustained Circulating COMP-Ang1 Induces Long-Lasting Enlargement of Postcapillary Venules and Terminal Arterioles in Tracheal Vessels

As an alternative method for systemic treatment with COMP-Ang1, an adenoviral vector encoding the COMP-Ang1 gene (Ade-COMP-Ang1) was developed. As a control, an adenoviral vector encoding the LacZ gene (Ade-LacZ) was developed. As a control, an adenoviral vector encoding the LacZ gene (Ade-LacZ) was developed. The potency, solubility, oligomerization status, and stability of the COMP-Ang1 produced from HEK293 cells transduced with Ade-COMP-Ang1 are similar to that of COMP-Ang1 produced from COS-7 cells transiently transfected with plasmid vector containing the COMP-Ang1 gene (data not shown). Adult mice were treated with 1×10⁹ pfu Ade-COMP-Ang1 or Ade-LacZ. At multiple times more than a period of 16 weeks, circulating plasma levels of COMP-Ang1 were measured by ELISA after a single injection of COMP-Ang1 recombinant protein (200 μg/mouse) (A, left). Diameters of 35 to 40 postcapillary venules (PV)/5 fields (brown curve) and 10 to 12 terminal arterioles (TA)/10 fields (blue curve) were measured at the edge of cartilage rings in each mouse. Values are mean±SD from 4 to 5 mice. *P<0.05 vs control period. COMP-Ang1 induced enlargement of postcapillary venules, collecting venules, venous ends of capillaries, venules, and terminal arterioles for up to 2 weeks, and then the enlarged blood vessels returned gradually to normal after discontinuation of the COMP-Ang1 treatment. Scale bar=50 μm.
Figure 3. Effect of adenoviral COMP-Ang1 on postcapillary venules and terminal arterioles and blood flow. A through D, FVB/N mice were treated with 1×10⁶ pfu Ade-COMP-Ang1 (n=6). At the indicated times, circulating plasma levels of COMP-Ang1 were measured by ELISA (A, black circle), and tracheal vessels were visualized with PECAM-1 immunostaining (B, red). The diameters of postcapillary venules (PV, brown curve) and terminal arterioles (TA, blue curve) are shown. Diameters of 35 to 40 PV/5 fields and 10 to 12 TA/10 fields were measured at the edge of cartilage rings in each mouse. Values are mean±SD from 4 to 5 mice. *P<0.05 vs control period. Scale bar=50 μm. C, Laser-Doppler flowmetric analyses for tracheal tissue blood flows of the mice treated with 1×10⁸ pfu Ade-LacZ (Con) or 1×10⁸ pfu Ade-COMP-Ang1 (CA1). Quantification of tracheal blood flows at 2 and 16 weeks after treatment with Con or CA1. Bars represent mean±SD from 4 to 5 mice. *P<0.05 vs Con. D and E, FVB/N mice were pretreated with 1×10⁶ (1+T2) or 5×10⁷ (5+T2) pfu Ade-sTie2-Fc (n=5 each), or 5×10⁸ pfu Ade-LacZ (LacZ, n=5) at 24 hours before 1×10⁸ pfu Ade-COMP-Ang1 treatment. Two weeks later, tracheal vessels were visualized by PECAM-1 immunostaining (D, red). Scale bar=50 μm. Diameters of 35 to 40 PV/5 fields and 10 to 12 TA/10 fields were measured at the edge of cartilage rings in each mouse. E, Bars represent the mean±SD from 5 experiments as percentage of inhibition of vascular remodeling induced by the pretreatment. Vascular remodeling induced by pretreatment of the Ade-LacZ is arbitrarily given as 100%. *P<0.05 vs LacZ, #P<0.05 vs 1+T2.

Ade-COMP-Ang1 treatment induces long-lasting enlargement of tracheal blood vessels with long-lasting enhancement of tissue blood flow in the adult mice.

**Tie2 Activation Is Involved in COMP-Ang1-Induced Vascular Remodeling**

To determine the involvement of Tie2 activation in COMP-Ang1–induced vascular remodeling, the mice were pretreated with 1×10⁶ pfu or 5×10⁷ pfu Ade-sTie2-Fc at 24 hours before 1×10⁸ pfu Ade-COMP-Ang1 treatment. Two weeks later, the diameters of postcapillary venules and terminal arterioles were measured. Pretreatment with 1×10⁶ pfu or 5×10⁷ pfu Ade-sTie2-Fc suppressed COMP-Ang1–induced vascular remodeling to the following extent: 46.5±7.7% or 93.5±6.4% in postcapillary venules and 59.7±6.6% or 95.1±5.7% in terminal arterioles, respectively (Figure 3E and 3F). These data indicate that COMP-Ang1–induced vascular remodeling is mainly mediated through Tie2 activation in adult tracheal vessels.

**Long-Term and Sustained Circulating COMP-Ang1 Induces Various Vascular Remodeling in Different Organ**

Both mice treated with Ade-LacZ (1×10⁶ pfu) and those treated with Ade-COMP-Ang1 (1×10⁶ pfu) appeared generally healthy, as they gained weight normally. However, the skin of mice treated with Ade-COMP-Ang1 appeared strikingly redder than the skin of mice treated with Ade-LacZ, beginning 10 to 14 days after the treatment. The Ade-COMP-
Ang1–induced skin redness persisted for as long as 16 weeks after the treatment (Figure 4). Sixteen weeks after the treatment, skin color in hair-sparse portions such as the face, hands, soles, penis, and tail were distinctly redder than those of mice treated with Ade-LacZ. Blood vessels of the ear and capillaries of the heart, adrenal cortex, and liver of the mice treated with Ade-COMP-Ang1 were enlarged (Figures 4 and 5). More PECAM-1–positive endothelial cells were present in the lung, heart, liver, and renal medulla of mice treated with Ade-COMP-Ang1 compared with the mice treated with Ade-LacZ (Figures 4 and 5 and online Figure I in the data supplement). However, blood vessels of the renal cortex, including glomeruli, and intestinal villi of the mice treated with Ade-COMP-Ang1 and the mice treated with Ade-LacZ were indistinguishable. In addition, the body weights, systemic blood pressures, and heart rates of the 2 groups of mice were indistinguishable. These results indicate that long-term and sustained circulating COMP-Ang1 treatment induces long-lasting tissue-specific vascular remodeling in different blood vessels without notable changes in systemic blood pressure and heart rate (online Table I).

Induction of Tie2 Could Be Involved in Permanent Changes of COMP-Ang1–Induced Vascular Remodeling

Based on these observations, we asked whether Tie2 expression was more abundant in postcapillary venules than termi-
nal arterioles in mouse trachea. Therefore, we examined the extent of Tie2 expression using transgenic mice with Tie2 promoter–driven green fluorescent protein (GFP).12 In the tracheal mucosa of adult mice, Tie2 expression was not detectable in most endothelial cells of postcapillary venules, whereas it was moderately expressed in endothelial cells of terminal and precapillary arterioles of tracheal vessels (Figure 6). Thus, differential enlargement of tracheal vessels on COMP-Ang1 stimulation is not dependent on the extent of Tie2 expression. However, Tie2 expression was markedly increased in endothelial cells of collecting venules, venules, postcapillary venules, and capillaries at 2 weeks after the Ade-COMP-Ang1 treatment (Figure 6), which is somewhat consistent with a recent report with Ade-Ang1.8 Tie2 expression was further increased in endothelial cells of the same vessels at 16 weeks after the Ade-COMP-Ang1 treatment (Figure 6). In contrast, Tie2 expression was not changed in any endothelial cells of enlarged tracheal vessels at 2 weeks after the recombinant COMP-Ang1 protein treatment (Figure 6). Area densities of Tie2 expression in a given microscopic field area (0.22 mm²) for arterioles, capillaries, and venules in tracheal mucosa were 8.2±1.7, 2.8±0.4, and 3.3±0.6% (mean±SD from 4 mice), respectively, after Ade-LacZ treatment (at 2 weeks); 7.6±1.9, 3.1±0.5, and 3.7±0.6% after COMP-Ang1 protein treatment (at 2 weeks); 11.3±2.2, 10.3±1.7, and 28.1±5.4% after Ade-COMP treatment (at 2 weeks); and 13.3±2.7, 18.2±3.5, and 47.7±7.2 after Ade-COMP treatment (at 16 weeks). In addition, Tie2 expression was notably higher in the enlarged veins of abdominal skin and the sinusoidal capillaries in liver of the mice treated with Ade-COMP-Ang1 than the mice treated with Ade-LacZ at 16 weeks after the treatment (online Figure II). Thus, Tie2 expression in venular and capillary endothelial cells could be induced with long-term and sustained Tie2 stimulation induced by Ade-COMP-Ang1 but not with short-term spiked Tie2 stimulation induced by recombinant COMP-Ang1 protein.

COMP-Ang1–Induced Vascular Enlargement Could Result From Circumferential Endothelial Cell Proliferation

COMP-Ang1–induced enlargement of blood venules appears to result from endothelial cell proliferation rather than vaso-dilation or endothelial cell hypertrophy because the endothelial cells were normal in size (Figure 7A and 7B). To test this possibility, we examined by immunostaining the number of endothelial cells of enlarged venules at 2 weeks after the recombinant COMP-Ang1 protein treatment (Figure 6). In contrast, Tie2 expression was not changed in any endothelial cells of enlarged tracheal vessels at 2 weeks after the recombinant COMP-Ang1 protein treatment (Figure 6). Area densities of Tie2 expression in a given microscopic field area (0.22 mm²) for arterioles, capillaries, and venules in tracheal mucosa were 8.2±1.7, 2.8±0.4, and 3.3±0.6% (mean±SD from 4 mice), respectively, after Ade-LacZ treatment (at 2 weeks); 7.6±1.9, 3.1±0.5, and 3.7±0.6% after COMP-Ang1 protein treatment (at 2 weeks); 11.3±2.2, 10.3±1.7, and 28.1±5.4% after Ade-COMP treatment (at 2 weeks); and 13.3±2.7, 18.2±3.5, and 47.7±7.2 after Ade-COMP treatment (at 16 weeks). In addition, Tie2 expression was notably higher in the enlarged veins of abdominal skin and the sinusoidal capillaries in liver of the mice treated with Ade-COMP-Ang1 than the mice treated with Ade-LacZ at 16 weeks after the treatment (online Figure II). Thus, Tie2 expression in venular and capillary endothelial cells could be induced with long-term and sustained Tie2 stimulation induced by Ade-COMP-Ang1 but not with short-term spiked Tie2 stimulation induced by recombinant COMP-Ang1 protein.

COMP-Ang1–Induced Vascular Enlargement Could Result From Circumferential Endothelial Cell Proliferation

COMP-Ang1–induced enlargement of blood venules appears to result from endothelial cell proliferation rather than vaso-dilation or endothelial cell hypertrophy because the endothelial cells were normal in size (Figure 7A and 7B). To test this possibility, we examined by immunostaining the number of endothelial cells of enlarged venules at 2 weeks after the recombinant COMP-Ang1 protein treatment (Figure 6). In contrast, Tie2 expression was not changed in any endothelial cells of enlarged tracheal vessels at 2 weeks after the recombinant COMP-Ang1 protein treatment (Figure 6). Area densities of Tie2 expression in a given microscopic field area (0.22 mm²) for arterioles, capillaries, and venules in tracheal mucosa were 8.2±1.7, 2.8±0.4, and 3.3±0.6% (mean±SD from 4 mice), respectively, after Ade-LacZ treatment (at 2 weeks); 7.6±1.9, 3.1±0.5, and 3.7±0.6% after COMP-Ang1 protein treatment (at 2 weeks); 11.3±2.2, 10.3±1.7, and 28.1±5.4% after Ade-COMP treatment (at 2 weeks); and 13.3±2.7, 18.2±3.5, and 47.7±7.2 after Ade-COMP treatment (at 16 weeks). In addition, Tie2 expression was notably higher in the enlarged veins of abdominal skin and the sinusoidal capillaries in liver of the mice treated with Ade-COMP-Ang1 than the mice treated with Ade-LacZ at 16 weeks after the treatment (online Figure II). Thus, Tie2 expression in venular and capillary endothelial cells could be induced with long-term and sustained Tie2 stimulation induced by Ade-COMP-Ang1 but not with short-term spiked Tie2 stimulation induced by recombinant COMP-Ang1 protein.

COMP-Ang1–Induced Vascular Enlargement Could Result From Circumferential Endothelial Cell Proliferation

COMP-Ang1–induced enlargement of blood venules appears to result from endothelial cell proliferation rather than vaso-dilation or endothelial cell hypertrophy because the endothelial cells were normal in size (Figure 7A and 7B). To test this possibility, we examined by immunostaining the number of endothelial cells of enlarged venules at 2 weeks after the recombinant COMP-Ang1 protein treatment (Figure 6). In contrast, Tie2 expression was not changed in any endothelial cells of enlarged tracheal vessels at 2 weeks after the recombinant COMP-Ang1 protein treatment (Figure 6). Area densities of Tie2 expression in a given microscopic field area (0.22 mm²) for arterioles, capillaries, and venules in tracheal mucosa were 8.2±1.7, 2.8±0.4, and 3.3±0.6% (mean±SD from 4 mice), respectively, after Ade-LacZ treatment (at 2 weeks); 7.6±1.9, 3.1±0.5, and 3.7±0.6% after COMP-Ang1 protein treatment (at 2 weeks); 11.3±2.2, 10.3±1.7, and 28.1±5.4% after Ade-COMP treatment (at 2 weeks); and 13.3±2.7, 18.2±3.5, and 47.7±7.2 after Ade-COMP treatment (at 16 weeks). In addition, Tie2 expression was notably higher in the enlarged veins of abdominal skin and the sinusoidal capillaries in liver of the mice treated with Ade-COMP-Ang1 than the mice treated with Ade-LacZ at 16 weeks after the treatment (online Figure II). Thus, Tie2 expression in venular and capillary endothelial cells could be induced with long-term and sustained Tie2 stimulation induced by Ade-COMP-Ang1 but not with short-term spiked Tie2 stimulation induced by recombinant COMP-Ang1 protein.

COMP-Ang1–Induced Vascular Enlargement Could Result From Circumferential Endothelial Cell Proliferation

COMP-Ang1–induced enlargement of blood venules appears to result from endothelial cell proliferation rather than vaso-dilation or endothelial cell hypertrophy because the endothelial cells were normal in size (Figure 7A and 7B). To test this possibility, we examined by immunostaining the number of endothelial cells of enlarged venules at 2 weeks after the recombinant COMP-Ang1 protein treatment (Figure 6). In contrast, Tie2 expression was not changed in any endothelial cells of enlarged tracheal vessels at 2 weeks after the recombinant COMP-Ang1 protein treatment (Figure 6). Area densities of Tie2 expression in a given microscopic field area (0.22 mm²) for arterioles, capillaries, and venules in tracheal mucosa were 8.2±1.7, 2.8±0.4, and 3.3±0.6% (mean±SD from 4 mice), respectively, after Ade-LacZ treatment (at 2 weeks); 7.6±1.9, 3.1±0.5, and 3.7±0.6% after COMP-Ang1 protein treatment (at 2 weeks); 11.3±2.2, 10.3±1.7, and 28.1±5.4% after Ade-COMP treatment (at 2 weeks); and 13.3±2.7, 18.2±3.5, and 47.7±7.2 after Ade-COMP treatment (at 16 weeks). In addition, Tie2 expression was notably higher in the enlarged veins of abdominal skin and the sinusoidal capillaries in liver of the mice treated with Ade-COMP-Ang1 than the mice treated with Ade-LacZ at 16 weeks after the treatment (online Figure II). Thus, Tie2 expression in venular and capillary endothelial cells could be induced with long-term and sustained Tie2 stimulation induced by Ade-COMP-Ang1 but not with short-term spiked Tie2 stimulation induced by recombinant COMP-Ang1 protein.
ing venules, venules, and terminal arterioles of tracheal vessels at 4 days and 2 weeks after the Ade-COMP-Ang1 treatment (Figure 7D, 7F, and 7I) or after recombinant COMP-Ang1 protein treatment (data not shown). However, almost no phosphohistone H3–positive endothelial cells were detected in any portion of tracheal vessels at 4 days or 2 and 16 weeks after the Ade-LacZ treatment and at 16 weeks after the Ade-COMP-Ang1 treatment (Figure 7C, 7E, 7G, and 7I). These findings indicate that vascular enlargement induced by COMP-Ang1 is more likely to result from endothelial cell proliferation depending on concentration of circulating COMP-Ang1 than from vasodilation or endothelial cell hypertrophy.

COMP-Ang1–Induced Postcapillary Venule Enlargement Is Not Accompanied by Pericyte Recruitment

Ang1 is known to be a strong growth factor for pericyte recruitment to nascent endothelial cells during vasculogenesis in physiological and pathological conditions.3–5 Therefore, we examined the interaction between endothelial cells and pericytes in the enlarged blood vessels of the trachea by double-immunostaining for endothelial cells and pericytes at 4 weeks after Ade-LacZ or Ade-COMP-Ang1 treatment. The interaction of endothelial cells and pericytes in most of tracheal blood vessels (except postcapillary venules) in mice that received Ade-COMP-Ang1 was similar to that in mice that received Ade-LacZ (Figure 8). Although less interaction of endothelial cells with pericytes was found on the enlarged postcapillary venules than elsewhere, the number of pericytes of the enlarged postcapillary venules was similar to the control postcapillary venules (Figure 8). Thus, COMP-Ang1 did not promote pericyte recruitment to the COMP-Ang1–induced enlarged venules in the trachea.

Discussion

The most important and novel finding in this study is that enlargement of tracheal blood vessels and enhancement of tracheal tissue blood flow induced by long-term and sustained exposure to COMP-Ang1 had not regressed for up to 16 weeks, despite the fact that exposure to COMP-Ang1 had already been discontinued at 6 to 7 weeks in adult mice. In comparison, enlargement of tracheal blood vessels induced by short-term intermittent exposure to COMP-Ang1 regressed on discontinuation of recombinant COMP-Ang1 treatment. Therefore, long-lasting vascular enlargement and enhancement of blood flow can be achieved by long-term and sustained exposure to COMP-Ang1.

Like other therapeutic proteins, circulating COMP-Ang1 rapidly disappeared in the plasma, probably because of its trapping by the Tie2 receptor of lung endothelial cells.15
However, we were able to achieve long-term (>4 weeks) and sustained (>1000 ng/mL) circulating COMP-Ang1 in mice by a single intravenous injection of $1 \times 10^9$ pfu Ade-COMP-Ang1. Throughout these experiments, we learned that long-term (=6 weeks) and sustained exposure to COMP-Ang1 produced long-lasting enlargement of postcapillary venules and terminal arterioles in the tracheal mucosa, whereas short-term (=2 weeks) and intermittent exposure to COMP-Ang1 produced reversible enlargements of these vessels. Similar to our results, another study found that long-term (4 weeks) sustained exposure to vascular endothelial growth factor (VEGF) produced long-lasting acquired vascular remodeling in liver, whereas short-term (2 weeks) sustained exposure to VEGF produced reversible vascular remodeling. What are the major mechanisms and factors that produce long-lasting and reversible vascular remodeling? Is there a threshold stimulation of Tie2 by COMP-Ang1 that can produce permanent enlargement? Our results suggest that auto-amplification of Tie2 expression by treatment with COMP-Ang1 above a certain dose and exposure period could be one of the mechanisms. Once Tie2 expression is activated by a long-term and excess exposure to COMP-Ang1, after discontinuation of COMP-Ang1, the subsequent activation of Tie2 might be achieved by endogenous circulating Ang1 or increased shear stress caused by increased blood flow. However, auto-amplification of Tie2 expression cannot be achieved below a certain dose and exposure period of COMP-Ang1, as evidenced by the experiments with intravenous administration of COMP-Ang1 recombinant protein. Therefore, the dose and the exposure period of COMP-Ang1 or VEGF should be considered in any therapeutic approaches where permanent vascular enlargements are needed to alleviate dysfunctions of ischemic tissues.

Tie1, an endothelial-specific receptor tyrosine kinase, shares a high degree of homology with Tie2. Although Tie1 was isolated more than a decade ago, no ligand had been found to activate it. Recently, Saharinen et al demonstrated that COMP-Ang1 stimulated Tie1 phosphorylation in cultured endothelial cells. Moreover, they showed that COMP-Ang1–induced Tie1 activation was amplified via Tie2 and was more efficient than native Ang1- and Ang4–induced Tie1 activation. Thus, COMP-Ang1 and Ang1 are now known to be activating ligands for both Tie1 and Tie2. However, our data indicate that COMP-Ang1–induced vascular remodeling in adult tracheal vessels is mainly mediated through activation of Tie2, not by Tie1. (See expanded Discussion section in the online data supplement.)

Although Ang1 induces vascular enlargement and has therapeutic benefits to ischemic tissues in several experimental animal models, little is known about whether the vascular enlargement is accompanied by enhanced blood flow. Our results showed that COMP-Ang1–induced vascular enlargement was accompanied by enhanced tissue blood flow in the trachea. Therefore, enhanced blood flow through arteriolar and venular enlargements induced by COMP-Ang1 could provide a great therapeutic benefit to ischemic peripheral tissues. In fact, Ang1-induced vessel enlargement is a unique characteristic among many growth factors. Our immunohistological examination of phosphohistone H3 revealed that COMP-Ang1–induced vascular enlargements were evidently the result of endothelial proliferation, which is consistent with a recent report. Thus, arteriolar and venular enlargements are achieved mainly by circumferential endothelial proliferation, which is a unique phenomenon and is different from multidirectional endothelial cell proliferation during vasculogenesis and angiogenesis. Moreover, our results revealed that different organs showed different sensitivities to long-term and sustained COMP-Ang1. In fact, blood vessels in the skin, heart, adrenal cortex, and liver, among other organs, are relatively sensitive to the COMP-Ang1–induced vascular enlargement. Therefore, COMP-Ang1 could provide a great therapeutic benefit to patients with delayed skin wound healing and ischemic heart diseases through its ability to promote vascular remodeling. Nevertheless, the mice treated with long-lasting and sustained COMP-Ang1 did not show any significant changes in body weight, systemic blood pressure, or heart rate. More detailed analysis will be necessary to clarify how it is possible that the mice with enlarged blood vessels caused by long-term and sustained COMP-Ang1 have normal blood pressure and heart rate.

Ang1 is known to be a strong growth factor for pericyte recruitment to nascent endothelial cells during development. This Ang1–induced pericyte recruitment is related to the Ang1–induced antileakage effect on VEGF and proinflammatory stimuli. However, our results show a lower number and poorer covering of pericytes in COMP-Ang1–
induced enlarged postcapillary venules. In fact, in a mouse model that completely blocks pericyte recruitment to developing vessels by injection of antagonistic monoclonal antibody against platelet-derived growth factor receptor-β, Ang1 is able to restore a hierarchical architecture of growing blood vessels and rescues retinal edema and hemorrhage even in the absence of pericyte recruitment. Thus, COMP-Ang1 may be able to assemble endothelial cells in a frame of hierarchical architecture without pericyte recruitment.\(^{20}\) Thus, COMP-Ang1 may be able to assemble endothelial cells in a frame of hierarchical architecture without pericyte recruitment in the COMP-Ang1–induced enlarged blood vessels.

In conclusion, long-lasting vascular enlargement and enhancement of blood flow can be achieved by long-term and sustained exposure to COMP-Ang1.

Acknowledgments

Supported, in part, by the Bio-Challenge Program and the National Research Laboratory Program (2004-02376 to G.Y.K.) of the Korean Ministry of Science and Technology, the Korea Health R&D Project (0405-DB01-0104-0006 to G.Y.K.), the Ministry of Health & Welfare, and the Korea Science and Engineering Foundation (R01-2004-000-10045-0 to B.H.J.). Also supported by National Institutes of Health grants HL-24136 and HL-59157 from the National Heart, Lung and Blood Institute (to D.M.D.).

References

Long-Term and Sustained COMP-Ang1 Induces Long-Lasting Vascular Enlargement and Enhanced Blood Flow
Chung-Hyun Cho, Kyung Eun Kim, Jonghoe Byun, Hyung-Suk Jang, Duk-Kyung Kim, Peter Baluk, Fabienne Baffert, Gyun Min Lee, Naoki Mochizuki, Jin Kim, Byeong Hwa Jeon, Donald M. McDonald and Gou Young Koh

Circ Res. 2005;97:86-94; originally published online June 16, 2005;
doi: 10.1161/01.RES.0000174093.64855.a6
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/97/1/86

An erratum has been published regarding this article. Please see the attached page for:
/content/98/9/e69.full.pdf

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2005/06/16/01.RES.0000174093.64855.a6.DC1
In an article by Cho et al (Circ Res. 2005;97:86–94) the authors mistakenly duplicated panel B (16w) in Figure 3, and erroneously presented panel D (LacZ) in Figure 3.

The authors mistakenly duplicated the image of panel B (16w) in Figure 3 from panel B in Figure 1. In addition, the authors mistakenly selected the representative image for panel D (LacZ) in Figure 3 from images obtained from the 1+T2 experiment in panel D of Figure 3. These errors occurred inadvertently during the process of selecting representative images from mislabeled multiple images. The corrected Figure 3 appears here, and the authors apologize for these errors.
Online Supplements

Long-Term and Sustained COMP-Ang1 Induces Long-lasting Vascular Enlargement and Enhanced Blood Flow

(COMP-Ang1 in Vascular Remodeling)

Chung-Hyun Cho, Kyung Eun Kim, Jonghoe Byun, Hyung-Suk Jang, Duk-Kyung Kim, Peter Baluk, Fabienne Baffert, Gyun Min Lee, Naoki Mochizuki, Jin Kim, Byeong Hwa Jeon, Donald M McDonald, Gou Young Koh

Biomedical Research Center and Department of Biological Sciences, Korea Advanced Institute of Science and Technology. Daejeon, 305-701, Korea
(C-H.C., K.E.K., G.M.L., G.Y.K)
Department of Medicine, Samsung Medical Center and Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea
(J.B., H-S.J., D-K.)
Cardiovascular Research Institute, Comprehensive Cancer Center, and Department of Anatomy, University of California, San Francisco, CA 94143, U.S.A.
(P.B., F.B., D.M.M)
Department of Structural Analysis, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan (N.M)
Department of Anatomy, College of Medicine, The Catholic University of Korea Seoul, 137-701, Korea (J.K.)
Department of Physiology, College of Medicine, Chungnam National University Daejeon, 301-131, Korea (B.H.J)

Subject codes: 129, 147, 95

Address correspondence to:
Gou Young Koh
Biomedical Research Center
Korea Advanced Institute of Science and Technology
373-1, Guseong-dong, Daejeon, 305-701
Republic of Korea
Phone: 82-42-869-2638; Fax: 82-42-869-2610
E-mail: gykoh@kaist.ac.kr
Supplemental Materials and Methods

Generation of COMP-Ang1 recombinant protein and Ade-COMP-Ang1

Recombinant Chinese hamster ovary (rCHO) cells expressing COMP-Ang1 (CA1-2) were established following a previously described method (1). Briefly, CA1-2 cells were established by transfection of a vector containing the dihydrofolate reductase (\textit{dhfr}) and FLAG-COMP/CC-Ang1/FD (hereafter COMP-Ang1) genes into \textit{dhfr}-deficient CHO cells (CRL-9096, American Type Culture Collection, Manassas, Virginia, USA). This was followed by \textit{dhfr}/methotrexate (MTX)-mediated gene amplification (1). The stable rCHO cells were selected with 1 \text{\mu M} MTX (Sigma-Aldrich, St. Louis, Missouri, USA). CA1-2 cells were grown and maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% dialyzed fetal bovine serum (Invitrogen, Carlsbad, California, USA) and 1 \text{\mu M} MTX (Sigma-Aldrich). For recombinant COMP-Ang1 protein production, CA1-2 cells were inoculated at 2 x 10^5 cells/mL in 125-ml Erlenmeyer flasks containing 50 mL of medium on an orbital shaker (Vision, Bucheon, Korea) at 110 rpm in a humidified 5% CO\textsubscript{2} incubator at 37\degree\text{C}. After 3 days, the culture medium containing FLAG-tagged COMP-Ang1 recombinant protein was purified by column chromatography on anti-FLAG M1 antibody agarose affinity gel (Sigma-Aldrich) with excess FLAG peptide (Sigma-Aldrich). After purification, the protein was quantitated using the Bradford assay and confirmed with Coomassie blue staining of an SDS-PAGE gel. These analyses showed that approximately 30 mg/L of COMP-Ang1 was harvested.

Recombinant adenovirus expressing COMP-Ang1 or LacZ was constructed using the pAdEasy\textsuperscript{TM} vector system (Qbiogene, Carlsbad, California, USA). Briefly, the COMP-
Ang1 gene or bacterial β-galactosidase (hereafter LacZ) gene was excised from the pcDNA3.1(+)‐FLAG‐COMP/CC‐Ang1/FD plasmid or pcDNA3.1(+)‐FLAG LacZ (2) via the Hind III (Klenow)/Sal I sites. The resulting fragment was then inserted into the Bgl II (Klenow)/Sal I sites of the pShuttle‐CMV (Qbiogene) transfer vector to give pShuttle‐CMV‐COMP‐Ang1 or pShuttle‐CMV‐LacZ. Homologous recombination between the linearized pShuttle‐CMV‐COMP‐Ang1 or pShuttle‐CMV‐LacZ and the adenoviral plasmid pAdEasy‐1 was mediated by co‐transformation in *E. coli* (BJ5183) (Qbiogene). The selected recombinant plasmid was linearized by Pac I digestion and then transfected into human embryonic kidney (HEK)‐293 cells (American Type Culture Collection). Several days later, viral plaques were assayed for protein expression and further purified by two more rounds of plaque purification. Large‐scale production of recombinant adenovirus was accomplished in HEK‐293 cells grown in 150‐mm culture dishes. Viral particles were precipitated with polyethylene glycol solution followed by further purification by cesium chloride density centrifugation. The purified virus solutions were dialyzed against phosphate buffered saline (PBS) and finally against 10 mM Tris‐HCl buffer (pH 8.0) containing 2 mM MgCl₂ and 4% sucrose. The viral stock was aliquoted and stored at −80°C. Viral titers were determined by the Tissue Culture Infectious Dose 50 (TCID₅₀) method, as described in the manual for the pAdEasy™ vector system (Qbiogene).

**Animals and treatment**

Specific pathogen‐free FVB/N mice and Tie2‐GFP transgenic mice (FVB/N) (3) were purchased from Jackson Laboratory and bred in our pathogen‐free animal facility. Male mice 8‐10 weeks old were used for this study. Animal care and experimental procedures
were performed under approval from the Animal Care Committees of KAIST. For protein treatment, 200 µg of COMP-Ang1 recombinant protein or BSA dissolved in 50 µL of sterile 0.9% NaCl was injected daily through the tail vein for 2 weeks. For adenoviral treatment, the indicated amount of Ade-COMP-Ang1, Ade-LacZ, or Ade-sTie2-Fc or Ade-sTie1-Fc (generous gift from Dr. Gavin Thurston and Dr. Ella Ioffe at Regeneron Pharmaceuticals) diluted in 50 µL of sterile 0.9% NaCl was injected intravenously through the tail vein.

**Measurement of systemic blood pressure and heart rate**

Mice were anesthetized by intraperitoneal injection of a combination of anesthetics (80 mg/kg ketamine and 12 mg/kg xylazine) and placed supine on a warming pad. Left carotid artery was cannulated with a PE-10 catheter serially connected to a pressure transducer, preamplifier, and analog-to-digital converter (Axon Instrument). After calibration of the system, blood pressure and heart rate were continuously recorded for 10 minutes. Data were analyzed using Axoscope software (Axon Instrument).

**Enzyme-linked immunosorbent assay (ELISA)**

Approximately 50 µL of blood was obtained from the tail vein into a heparinized capillary tube at the indicated times. ELISA was adopted for precise detection of COMP-Ang1 in plasma. Each well of a 96-well plate (NUNC-Immuno™ Plate, MaxiSorp™ surface, NUNC, Roskilde, Denmark) was coated with 100 µl of soluble Tie2-Fc (2 µg/ml in PBS) at 4°C overnight, washed with PBS, incubated with blocking buffer (2% BSA in PBS) at 37°C for 2 hrs, and washed again with PBS. Diluted plasma (1:50-1:1000) was added into the well and incubated at 37°C for 2 hrs, washed with
PBS, and then anti-FLAG polyclonal antibody (Sigma-Aldrich) was added because COMP-Ang1 being tagged with FLAG peptide. The amount of bound anti-FLAG polyclonal antibody was colorized with serial incubations of biotin-conjugated anti-rabbit IgG, avidin-alkaline phosphatase conjugate, and p-nitrophenyl phosphate. The concentration was determined by a SpectraMax microplate reader (Molecular Devices Corp., Sunnyvale, California, USA) at an optical density of 415 nm.

**Immunohistochemical staining**

Mice were anesthetized by intraperitoneal injection of a combination of anesthetics (80 mg/kg ketamine and 12 mg/kg xylazine). After fixation by vascular perfusion of 1% paraformaldehyde (PFA) in PBS, tracheas were removed and incised along the ventral midline and pinned on Sylgard plates (Dow Corning Corp.). Tracheas were stained as whole mounts. After incubation for 1 hr at room temperature with a blocking solution containing 5% normal goat serum (Jackson ImmunoResearch Laboratories Inc.) in PBS with 0.3% Triton X-100 (PBST), tracheas were incubated overnight at 4°C with one or more primary antibodies: (a) blood vessels: anti-PECAM-1 antibody, hamster clone 2H8, 1:1000 (Chemicon International Inc.); (b) dividing cells: anti-phosphohistone H3 antibody, rabbit polyclonal antibody, 1:500 (Upstate Biotechnology Inc.); (c) pericytes: anti-desmin antibody, rabbit polyclonal antibody, 1:500 (DAKO) and anti-NG2 antibody, rabbit polyclonal antibody, 1:500 (Chemicon International Inc.). After several washes with PBST, tracheas were incubated for 4 hours at room temperature with one or more secondary antibodies: (a) Cy3-conjugated anti-hamster IgG antibody, 1:500 (Jackson ImmunoResearch Laboratories Inc.); (b) FITC-conjugated anti-rabbit antibody, 1:500 (Jackson ImmunoResearch Laboratories Inc.). After several washes with PBST, tracheas
were flat-mounted on a slide glass with Vectashield (Vector Laboratories Inc.). For control experiments, the primary antibody was omitted or substituting preimmune serum. Signals were visualized and digital images were obtained with a Zeiss Apotome microscope and a Zeiss LSM 510 confocal microscope equipped with argon and helium-neon lasers (Zeiss).

**Measurement of tracheal tissue blood flow**

After the mice were anesthetized by intraperitoneal injection of the mixture of ketamine (80 mg/kg) and xylazine (12 mg/kg), the mice were placed on a heated table for maintenance of body temperature at 37°C. After midline incisions in neck, a type N flowprobe (Transonic Systems, Inc. Ithaca, New York, USA) was placed on tracheal wall along second, third and forth cartilage rings without applying pressure, as this would occlude the vessels and reduce perfusion in the area of interest. The flowprobe was kept in place on the position of the highest sensitivity by a micromanipulator and connected to a laser-Doppler flowmeter (model BLF21; Transonic Systems, Inc), which can measure microcirculation in 1 mm³ of tissue for real-time assessment of perfusion. These analog signals were digitized at 100 Hz (Digidata 1200, Axon Instrument, USA) and continuously displayed by a data-acquisition program, and then mean tissue perfusion rate (ml/min/100g of tissue) was analyzed using Axoscope 9.0 software. Using this method, the tissue blood flow was measured for three consecutive seconds of each 20-second interval (total 1 min).

**Morphometric measurements and statistics**

Morphometric measurements of postcapillary venules and terminal arterioles in mouse
trachea were made on whole mounts with PECAM-1 immunostaining using a Zeiss Apotome microscope coupled to a monochrome charge-coupled device (CCD) camera and image analysis software (Axiovision, Zeiss). Measurements of the vessel diameters were made on 10 fields at the rostral end of each trachea, and 4 to 5 mice were used per group as previously described (4). Using an overlay grid with 15-mm spacing, the diameters of capillaries, postcapillary venules and collecting venules overlying cartilage ring and terminal arterioles in the mucosa between the rings were measured for the quantification of vessel diameters. Area densities (percentage of tissue area) of Tie2-GFP fluorescence viewed in real-time fluorescence images of tracheal whole mounts were measured by stereological point counting of 5 regions at a screen magnification of x200, each 0.21 mm² in area, per trachea. For each trachea, Numbers of PH3 immunopositive endothelial cells were counted at a screen magnification of x200 in 5 regions, each 0.21 mm² in area, per trachea. PECAM-1-immunopositive blood vessels and desmin/NG2-immunopositive pericytes were measured at a magnification of x200 in 5 regions, each 0.21 mm² area, per trachea. Values were expressed per mm². Values presented are mean ± standard deviation (SD). Significance of differences between mean was tested by unpaired t-test or analysis of variance followed by the Student-Newman-Keuls test. Statistical significance was set at P<0.05.

**Supplemental Discussion**

In agreement with a recent report (5), our additional results indicate that COMP-Ang1 also activates Tie1 and Tie2 in lung endothelial cells in adult mice (Supplementary Figure 3). Therefore, COMP-Ang1-induced vascular remodeling could be mediated through Tie1 and Tie2 activation. To determine the involvement of each
specific receptor on ligand action, a dimeric and soluble extracellular binding domain of receptor is generally used. Dimeric and soluble extracellular binding domains of Tie1 (sTie1-Fc) and Tie2 (sTie2-Fc) are available. However, sTie1-Fc does not bind to COMP-Ang1 \textit{in vitro} (2), thereby it could not block the action of COMP-Ang1 \textit{in vivo}. In comparison, sTie2-Fc binds to COMP-Ang1 and could block the action of COMP-Ang1 \textit{in vivo}. Consistent with our prediction, we found that pretreatment with excess Ade-sTie1-Fc ($5 \times 10^8$ pfu) did not change the Ade-COMP-Ang1 ($1 \times 10^8$ pfu)-induced vascular remodeling, while excess Ade-sTie2-Fc ($5 \times 10^8$ pfu) almost completely suppressed the Ade-COMP-Ang1 ($1 \times 10^8$ pfu)-induced vascular remodeling. Although we could not completely exclude the role of Tie1 activation in COMP-Ang1-induced vascular remodeling, we concluded that COMP-Ang1-induced vascular remodeling is mainly mediated through Tie2 activation in adult tracheal vessels, because the excess Ade-sTie2-Fc almost completely blocked the COMP-Ang1-induced vascular remodeling.

\textbf{References}


**Supplemental Figure Legends**

**Supplemental Figure 1.** Effect of adenoviral COMP-Ang1 on vascular remodeling in renal cortex, renal medulla, and small intestine at 16 weeks after treatment. FVB/n mice were treated with 1x10^9 pfu Ade-LacZ or Ade-COMP-Ang1. Sixteen weeks later, blood vessels in renal cortex (A, B, C, and D), renal medulla (E, F, G, and H), and villi of jejunum (I, J, K, and L) were visualized with PECAM-1 (CD31) immunostaining (red), and the sections were stained with H&E. There are no overt differences in blood vessels between the mice treated with Ade-COMP-Ang1 and the mice treated with Ade-LacZ. The results from 4 experiments were similar. Scale bar = 50 µm.

**Supplemental Figure 2.** Induction of Tie2 expression in COMP-Ang1-induced vascular remodeling in skin and liver. Tie2-GFP transgenic mice (10 weeks old) were treated with a single injection of 1x10^9 pfu Ade-LacZ (A and C) or Ade-COMP-Ang1
(B and D). At 16 weeks after treatment, Tie2 expression in blood vessels of abdominal skin and liver was visualized with GFP expression (green) and PECAM-1 immunostaining (red), and the views were merged. The results from 4 experiments were similar. Arrowheads, arteries; arrows, veins (A and B) or capillaries (D). Tie2 expression was notably higher in the enlarged veins of abdominal skin and the sinusoidal capillaries in liver of the mice treated with Ade-COMP-Ang1 than the mice treated with Ade-LacZ. Scale bar = 25 µm.

**Supplemental Figure 3.** *In vivo* phosphorylations of Tie1 and Tie2 stimulated by intravenous injection of COMP-Ang1 (CA1, 30 µg) into FVB/n mice. Tie1 or Tie2 was immunoprecipitated from lung protein lysate and immunoblotted with anti-phosphotyrosine to detect phosphorylated Tie1 or Tie2 (pTie1 or pTie2, upper panels). The membrane was stripped and reprobed with anti-Tie1 antibody or anti-Tie2 antibody (lower panels) to verify equal loading of protein in each lane. The ratio indicates the densitometric analyses presented as the relative ratio of pTie2 to Tie2 with BSA (B, 30 µg) arbitrarily given as 1. Folds represent the mean ± S.D. from 4 experiments. *, $P<0.05$ versus BSA.
Supplemental Table 1. Comparison of Hemodynamic Parameters between control- and COMP-Ang1-treated mice.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>COMP-Ang1</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>30.5 ± 1.9</td>
<td>29.0 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>87.8 ± 5.8</td>
<td>90.7 ± 3.6</td>
<td>NS</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>51.7 ± 4.9</td>
<td>59.2 ± 4.2</td>
<td>NS</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>70.1 ± 6.8</td>
<td>74.1 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>190 ± 16</td>
<td>212 ± 1.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

FVB/N mice were treated with 1x10⁹ pfu Ade-LacZ (n=5) or Ade-COMP-Ang1 (n=5). Sixteen weeks later, mice were anesthetized, and their systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate were measured. Values are mean ± SD from 5 mice. NS, not significant.
Supplementary Figure 3

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>CA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio</td>
<td>1.0</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>±0.0</td>
<td>±1.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>CA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio</td>
<td>1.0</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>±0.0</td>
<td>±2.8</td>
</tr>
</tbody>
</table>