Hydrogen Peroxide Produced by Angiopoietin-1 Mediates Angiogenesis

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Abstract

Angiopoietin-1 (Ang1) mediates angiogenesis by enhancing endothelial cell survival and migration. It is also known that Ang1 activates Tie2, an endothelial-specific tyrosine kinase receptor, but the molecular mechanism of this process is not clear. In this study, we investigated whether reactive oxygen species (ROS) production plays a role in Ang1-mediated angiogenesis. We found that human umbilical vein endothelial cells treated with Ang1 produce ROS transiently, which was suppressed by NADPH oxidase inhibitor, diphenyleneiodonium chloride, and rotenone. The Ang1-induced ROS was identified as hydrogen peroxide (H2O2) using adenosine-catalase infection. Removal of H2O2 by adenosine-catalase significantly suppressed Ang1-induced in vitro endothelial cell migration, in vivo tubule formation and angiogenesis, and activation of p44/42 mitogen-activated protein kinase (MAPK), involved in cell migration, and delayed the deactivation of Akt phosphorylation involved in cell survival. Supporting in vitro data, Ang1-induced vascular remodeling in castalase (−/−) mice was more prominent than in castalase (+/+) mice: Ang1-induced increases of the diameter of terminal arterioles and the postcapillary venules in castalase (−/−) mice were significant compared with castalase (+/+) mice. These results show that Ang1-induced H2O2 plays an important role in Ang1-mediated angiogenesis by modulating p44/42 MAPK activity. (Cancer Res 2006; 66(12): 6167-74)

Introduction

Angiogenesis is essential for tumor growth, metastasis, embryonic development, wound healing, and arteriosclerosis (1–4). Angiogenesis is regulated by several factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and angiopoietin, whose activities, in turn, are associated with other factors (5–7). Of these, angiopoietin-1 (Ang1) was identified as a ligand of Tie2, a tyrosine kinase receptor with homology with immunoglobulin and EGF receptor. Tie2 is a member of the receptor tyrosine kinase family and is mainly expressed in vascular endothelial cells (8, 9). Ang1 and Tie2 play important roles in blood vessel formation by enhancing the survival, migration, sprouting, and network formation of vascular endothelial cells (10). Studies in Ang1- and Tie2-deficient mice (8) and experiments involving overexpression of Ang1 (11) confirm these findings. A series of in vitro experiments showed that Ang1-promoted endothelial cell survival is mediated mainly via Tie2-phosphotyrosylinositol 3-kinase (PI3K)/Akt signaling pathway (4, 12). Ang1 stimulates recruitment of downstream of kinase-R (Dok-R) in a Tie2-dependent manner. Phosphorylation of Dok-R also results in the recruitment of Ras GTPase-activating protein and adaptor molecule Nck, which regulate cell motility and rearrange cytoskeletal proteins (4).

Numerous studies suggest that a variety of growth factors, such as EGF, FGF, PDGF, and VEGF, induce the generation of reactive oxygen species (ROS) through tyrosine kinase receptor (13, 14). ROS generated by ligand binding to these receptors can phosphorylate tyrosine residues on several growth factor receptors and initiate the activation of downstream signaling cascades (14). In injured cells, ROS especially stimulates angiogenic response, and in cancer cells, it induces tubule formation, migration, and proliferation associated with neovascularogenesis (5, 15, 16). In endothelial cells, VEGF induces the generation of ROS by causing the activation of Rac1 and NADPH oxidase and thereby regulates cell proliferation and migration (16, 17). Another recent study shows that endothelial-derived nitric oxide (NO) is required for Ang1-induced angiogenesis (18). However, the role of ROS in Ang1-induced angiogenesis has not been examined.

In this study, we examined the possible role of Ang1-induced ROS generation in Ang1-induced angiogenesis-related response as well as the signaling pathways leading up to it in endothelial cells. We found that Ang1 significantly stimulates ROS generation, which we identified to be mainly hydrogen peroxide (H2O2). Employing adenosinocatalase and catalase (−/−) mice, we further showed that Ang1-induced H2O2 plays essential roles in angiogenesis and related responses by modulating the signaling pathways of Akt and mitogen-activated protein kinase (MAPK) phosphorylations.

Materials and Methods

Cell culture. Human umbilical vein endothelial cells (HUVEC; AngioLab, Daejon, Korea) and primary endothelial cells were prepared 80% confluent in 100-mm dishes at passages 4 to 6 in Medium 199 (M-199; Life Technologies, Stafford, TX) containing 10% fetal bovine serum (FBS), heparin (100 μg/mL; Sigma, St. Louis, MO), endothelial cell growth supplement (50 μg/mL; Sigma), 100 μg/mL streptomycin, and 100 units/mL penicillin G at 37°C in an atmosphere of 5% CO2 and 95% air.

Treatment of HUVECs with cartilage matrix protein Ang1. The HUVECs were incubated in M-199 containing 2% FBS overnight (16 hours), treated with 200 ng/mL cartilage matrix protein Ang1 (COMP-Ang1) for 30 minutes at 37°C (12, 19), and recovered for various times. To avoid problems due to insolubility and aggregation of native Ang1 recombinant protein, we used Ang1 variant COMP-Ang1, which is soluble and stable Ang1 (20, 21). Treated cells were harvested after washing twice with ice-cold PBS.
Measurement of intracellular ROS. HUVECs treated with COMP-Ang1 were incubated with 5 μmol/L 2',7'-dichlorofluorescein (DCF) diacetate (DCFH-DA) for 5 minutes, which is converted to DCF by intracellular esterase. The latter was then oxidized by ROS to the highly fluorescent DCF. The fluorescence of each dish was immediately analyzed at excitation wavelength of 488 nm and emission wavelength of 515 or 540 nm by Zeiss confocal laser scanning fluorescence microscope (LSM 510, Zeiss, Oberkochen, Germany). All measurements were at least triplicated.

Measurement of intracellular mitochondrial ROS. Cells were pre-treated with 1 μmol/L rotenone for 1 hour, stimulated with 200 ng/mL COMP-Ang1 for 30 minutes, and then recovered for various times. The recovered cells were stained with 15 μmol/L dye dihydrorhodamine 123 (DHR123, Molecular Probes, Carlsbad, CA) for 10 minutes with washed with HBSS (13). The fluorescence of each dish was immediately analyzed as described above. All measurements were at least triplicated.

Adenoviral catalase infection to hydrolyze H2O2. Confluent HUVECs were infected in 35-mm dish with adenovirus encoding human catalase (Lab Frontier, Seoul, Korea) or empty adenoviral vector in M-199 containing 2% FBS for overnight at 37°C. After removal of the adenovirus, the cells were treated with various reagents for further studies. Catalase overexpressed by adenovirus infection was detected with anti-human catalase (Lab Frontier) in Western blot analysis.

Western blot analysis. Lysates from cells with treated COMP-Ang1 were analyzed with Western analysis using various antibodies, including anti-phosphorylated extracellular signal-regulated kinase, anti-phosphorylated p38, anti-phosphorylated Akt (New England Biolabs, Inc., Ipswich, MA), anti-phosphorylated e-Jun NH2-terminal kinase (JNK; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-catalase (Lab Frontier), at 1:1,000 dilution in PBS with 0.3% Triton X-100 (PBST) at 4°C for overnight. The protein-antibody complex was visualized with horseradish peroxidase (HRP)-conjugated secondary antibody at a 1:2,000 dilution. The immunoblots were then incubated for 30 seconds in the Enhanced Chemiluminescence Plus kit (Amersham Biosciences, Piscataway, NJ) and detected by LAS 3000 (Fuji Photo Film Co., Tokyo, Japan).

In vitro tubule formation. In vitro tubule formation in HUVECs was assayed on Matrigel (Becton Dickinson, Franklin Lakes, NJ). Matrigel (200 μL) was coated on 24-well plate and incubated to form a solid gel for 30 minutes at 37°C. The serum-starved cells were incubated with or without 20 μmol/L diphenylene-iodonium chloride (DPI) for 30 minutes and then treated with COMP-Ang1 (200 ng/mL) for 30 minutes. The stimulated cells were seeded onto Matrigel-coated wells in M-199 containing 10% FBS and incubated for 7 to 24 hours. The images of tubule formation were observed with an Olympus digital camera and analyzed for tubule length and area with Image Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD).

In vitro cell migration assay. The migration assays were done using a 24-well chemotaxis chamber (Transwell, Costar, Corning, NY). The upper chamber of Transwell was coated with 50 μg with Matrigel (Matrigel basement membrane matrix, BD Biosciences, Franklin Lakes, NJ) for 1 hour at 37°C and then placed into 24-well chambers. The lower chamber was filled with M-199 medium containing 10% FBS. Cells stimulated with COMP-Ang1 (107/150 μL) were seeded in each well in the upper chamber and then incubated for 24 hours at 37°C. The nonmigrating cells after incubation were removed from the upper side of the filters with a cotton ball. The filters were stained with DiffQuik staining solution (Fisher Photo Film Co., Tokyo, Japan).

Adv-COMP-Ang1. Recombinant adeno virus expressing COMP-Ang1 or LacZ was constructed using the pAdEasy vector system (Qbiogene, Morgan Irvine, CA) as described previously (23).

Studies with catalase (−/− and +/+ mice). Specific pathogen-free catalase (−/− and +/+ mice), which have been back-crossed to C57BL/6 mice for 10 generations and wild-type (WT) generated as described previously (24), and their WT C57BL/6, catalase (+/+ mice, were fed and bred under specific pathogen-free conditions. Animal care and experimental procedures used were approved by the Animal Care Committees of Ewha Womans University (Seoul, Korea) and Korea Advanced Institute of Science and Technology (Daejon, Korea). Male mice, 8 to 10 weeks old, were used in this study.

Adenoviral treatment and immunohistochemistry. Adv-COMP-Ang1 or Adv-LacZ [1 × 109 plaque-forming units (pfu)] diluted in 50 μL sterile 0.9% NaCl was injected i.v. through the tail vein. Two weeks later, the treated mice were anesthetized and perfused with 1% paraformaldehyde in PBS. The tracheas were removed and immuno stained as whole mounts. After incubation for 1 hour at room temperature with a blocking solution containing 5% normal goat serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) in PBST, the tracheas were incubated overnight at 4°C with anti-platelet/endothelial cell adhesion molecule 1 (PECAM-1) antibody, hamster clone 2H8 (1:1,000; Chemicon International, Inc., Temecula, CA). After several washes with PBST, the tracheas were incubated for 4 hours at room temperature with FITC-conjugated anti-hamster IgG secondary antibody (1:500. Jackson Immunoresearch Laboratories). After several washes with PBST, the tracheas were flat mounted on a glass slide with Vectashield (Vector Laboratories, Inc., Burlingame, CA). For controls, the primary antibody was omitted or substituted with preimmune serum. Signals were visualized, and digital images were obtained with a Zeiss Apotome microscope and a Zeiss LSM 510 confocal microscope.

Morphometry and statistical analysis. Morphometric measurements of postcapillary venules and terminal arterioles I of the PECAM-1-stained trachea were made as described previously (25) by using a Zeiss Apotome microscope coupled to a monochrome charge-coupled device camera and image analysis software (Axiovision, Zeiss). Measurements of the vessel diameters were made on 10 fields at the rostral end of each trachea. Using an overlay grid with 15-mm spacing, the diameters of postcapillary venules and terminal arterioles in the mucosa between the rings were measured for the quantification of vessel diameters. Four to five mice were used per group as described previously (25). Values presented are mean ± SD. Significance of differences between mean was tested by ANOVA followed by the Student-'s-Newman-Keuls’ test. Statistical significance was set at P < 0.05.

Results

Ang1 induces ROS generation. To characterize the events underlying Ang1-induced angiogenesis, we first examined the generation of ROS after Ang1 treatment in HUVECs. HUVECs were exposed to Ang1 (200 ng/mL) for 30 minutes, and DCF fluorescence produced was measured for various recovery times after stimulation. We found that ROS was generated immediately after Ang1 treatment (Fig. 1A) in a time-dependent manner (Supplementary Fig. S1). Ang1 also induced tubule formation in endothelial cells (Fig. 1B). DCFH-DA is converted to DCF by intracellular esterase and oxidized to highly fluorescent DCF by various ROS, including H2O2 and NO.

To determine the nature of the ROS generated by Ang1, we pretreated HUVECs with various inhibitors of ROS generation, such as DPI (20 μmol/L), rotenone (1 μmol/L), or l-Nω-nitroarginine methyl ester (l-NAME; 3 mmol/L) for 1 hour before treating with Ang1. DCF fluorescence of cells immediately after Ang1 treatment is shown in Fig. 2A. DPI, a NAPDH oxidase inhibitor, significantly suppressed both endogenous and Ang1-induced ROS generation because NADPH oxidase is a major source of ROS generation. Rotenone, a specific inhibitor of site I NADH oxidase, significantly suppressed both endogenous and Ang1-induced ROS generation because NADPH oxidase is a major source of ROS generation. Therefore, the specific inhibitor of site I of the mitochondrial electron transport chain, partially abolished...
Ang1-induced ROS generation. However, L-NAME, a nonspecific endothelial NO synthase (eNOS) inhibitor, did not affect the Ang1-induced ROS generation. These results summarized in Fig. 2 suggest that Ang1 possibly stimulates the generation of cytosolic and mitochondrial H₂O₂ but not NO. Previous reports suggested that NO is required for Ang1-induced angiogenesis (18).

We then examined whether Ang1-induced ROS generation is blocked by L-NAME in concentration-dependent and recovery time-dependent manners. Ang1-induced DCF fluorescence with or without pretreatment of 3 mmol/L L-NAME was monitored during the recovery for various times (0, 0.5, and 1 hour) after Ang1 treatment (Fig. 2C). Ang1-induced ROS generation was initially increased and then slowly decreased during recovery but was not affected by pretreatment of L-NAME. This was confirmed by varying the concentrations of L-NAME. HUVECs pretreated with various concentrations of L-NAME (3, 6, and 12 mmol/L) for 16 hours were stimulated with Ang1, and DCF fluorescence was measured immediately after the treatment. Pretreatment at lower concentrations of L-NAME did not affect Ang1-induced ROS generation, whereas higher concentrations of L-NAME themselves increased ROS generation (Fig. 2D). This suggests that

Figure 1. Effect of Ang1 on ROS generation and in vitro tubule formation. A, serum-starved HUVECs were treated with 200 ng/mL Ang1 for 30 minutes and recovered at indicated times (0, 0.5, 1, and 2 hours) at 37°C. Ang1-treated cells were incubated for 5 minutes with 5 μmol/L DCFH-DA. The fluorescence of each dish was analyzed by Zeiss confocal laser scanning fluorescence microscope (LSM 510). B, Ang1-stimulated (200 ng/mL) cells were seeded onto Matrigel-coated wells in M-199 containing 10% FBS and incubated for 7 to 24 hours for tubule formation. All measurements were at least triplicated.

Figure 2. Ang1 induces cytosolic or mitochondrial ROS but not NO. A, cells were incubated with medium with or without 20 μmol/L DPI, 1 μmol/L rotenone, and 3 mmol/L L-NAME for 1 hour and then with Ang1 (200 ng/mL) for 30 minutes at 37°C. The treated cells were further incubated for 5 minutes with 5 μmol/L DCFH-DA. The fluorescence of each dish was analyzed as described. B, DCF fluorescence of each sample compared with control cells. C, cells incubated with (NAME +) or without (NAME −) 3 mmol/L L-NAME for 1 hour were treated with Ang1 (200 ng/mL) for 30 minutes and recovered at indicated times (0, 0.5, and 1 hour) at 37°C. Treated cells were incubated for 5 minutes with 5 μmol/L DCFH-DA. The fluorescence of each dish was analyzed as described. D, cells were incubated with or without L-NAME containing indicated concentrations (0, 3, 6, and 12 mmol/L) for overnight (>16 hours). ROS was measured as described. All measurements were at least triplicated. *, P < 0.05; #, P < 0.05.
Ang1-induced ROS generation was not affected by eNOS inhibition. To explain the observed partial inhibition by rotenone of Ang1-induced ROS generation, we examined whether Ang1 induces mitochondrial ROS generation. To specifically detect mitochondrial ROS, we used dye DHR123, which reacts with mitochondrial ROS and emits fluorescence. We found that mitochondrial ROS was generated by Ang1. Rotenone significantly inhibited ROS generation by Ang1 in early stages, but this inhibitory effect disappeared after 1 hour (Fig. 3). Because the amount of mitochondrial ROS generated by Ang1 is quite low compared with cytosolic ROS, we conclude that Ang1-induced ROS detected using DCFH-DA (Fig. 1) is mainly cytosolic ROS.

Identification of Ang1-induced ROS as mainly H2O2. HUVECs were infected with various amounts of adenovirus-catalase (Fig. 4A) and then treated with Ang1. As shown in Fig. 4B and C, ROS generation by Ang1 in adenovirus-catalase-infected HUVECs decreased to the control level. This suggests that Ang1-induced ROS is H2O2 and that its generation can be blocked by catalase overexpression.

H2O2 is an important regulator of the Ang1-induced angiogenesis. To determine whether H2O2 generated by Ang1 can affect Ang1-induced angiogenesis, we examined tubule formation and migration in endothelial cells in the presence or absence of adenovirus-catalase infection. HUVECs infected with control adenovirus or adenovirus-catalase were treated with Ang1, and cell migrations and tubule formations were examined. Results of in vitro cell migration assay using Transwell to examine angiogenesis-related response are shown in Fig. 5A. Ang1-induced cell migrations were completely abolished in adenovirus-catalase-infected cells. We further found that tubule formations in terms of area and length were suppressed to control levels in adenovirus-catalase-infected cells (Fig. 5B). Only data on areas, but not on lengths, are shown in Fig. 5B.

Next, we confirmed, using CAM assay, the effect of H2O2 induced by Ang1 on in vivo angiogenesis. As shown in Fig. 5C, CAM treated with adenovirus control vector and Ang1 caused outgrowth capillary neovascularization near core region. On the other hand, CAM treated with adenovirus-catalase and Ang1 exhibited little or no neovascularization. This suggests that H2O2 generated by Ang1 specifically regulates Ang1-induced angiogenesis and related response.

Ang1 activates p44/42 MAPK and p38 MAPK in a ROS (H2O2)-dependent manner. To determine how Ang1-induced H2O2 regulates angiogenesis, we examined the kinetics of activation of various kinases, including Akt, p44/42 MAPK, p38 MAPK, and JNK by Ang1 (data not shown). The activation kinetics agreed well with those reported previously by others (4, 19, 26). To explore the relationship between the activations of the various kinases and ROS generation, we examined kinase activations resulting from treatment with Ang1 for 30 minutes of HUVECs infected with control adenovirus and adenovirus-catalase. The observed activation kinetics of various kinases, including Akt, p44/42 MAPK, p38 MAPK, and JNK, is shown in Fig. 6. The activation of p44/42 MAPK by Ang1 was significantly inhibited in adenovirus-catalase-infected cells (Fig. 6B, c), and p38 MAPK activation was delayed (Fig. 6B, b). However, there was no difference of JNK activation by Ang1 between control and adenovirus-catalase-infected cells (Fig. 6B, d). Ang1 treatment...
caused a dramatic early activation of Akt in 1 hour followed by a slow deactivation in control as well as adenovirus-infected HUVECs. On the other hand, the activation of Akt was unexpectedly sustained much longer in adenovirus-catalase-infected cells than in the control (Fig. 6B, a). These results suggest that the activations of p44/42 MAPK and p38 MAPK were regulated by Ang1-induced ROS, whereas the activation of Akt by Ang1 occurred in a ROS-independent manner and that the deactivation was regulated in a ROS-dependent manner. To confirm that H2O2 generated by Ang1 plays a role in Ang1-induced angiogenesis, we also examined the effect of Adv-COMP-Ang1 on vascular enlargement in tracheal vessels of catalase (+/−) and catalase (−/−) mice because vascular enlargement is a characteristic feature of Ang1-induced changes in adult blood vessels. We found previously that COMP-Ang1 given by adenoviral vector induced long-lasting vascular enlargement, which resulted from endothelial cell proliferation (23).

Catalase (+/−) and catalase (−/−) mice were treated with Adv-COMP-Ang1, and vascular enlargement in tracheal vessels was examined at 2 weeks after the treatment. COMP-Ang1-induced enlargements of postcapillary venules, collecting venules, venous end of capillaries, venules, and terminal arterioles in catalase (−/−) mice were larger than in catalase (+/+) mice (Fig. 7A). The diameter of terminal arterioles increased 1.18-fold in catalase (+/+) mice compared with 1.38-fold in catalase (−/−) mice.

Discussion

The present study shows that exposure of endothelial cells to Ang1 increases intracellular ROS especially H2O2 and that H2O2 affects the signaling pathway of MAPK activation and regulates cell migration, tubule formation, and angiogenesis both in vitro and in vivo. This is the first report on the role of H2O2 in Ang1-induced angiogenesis. A report that appeared after this article was submitted (24), and several others (25–27) confirm the involvement of ROS as well as Ang1 in signaling (27).

A variety of growth factors, such as EGF, FGF, PDGF, and VEGF, have been shown to induce the generation of ROS through binding to tyrosine kinase receptor (13, 14). ROS generated via phosphorylation of tyrosine residues initiates the activation of downstream signaling cascades in several growth factor receptors (14). ROS has been shown to play an important role as mediator of angiogenesis (28). ROS seems to stimulate angiogenic response in injured cells during repair process and induce tubule formation, cell migration, and proliferation associated with angiogenesis and vasculogenesis involved in cancer progression (5, 16). VEGF has been shown to regulate the proliferation and migration of endothelial cells and induce the generation of ROS by causing the activation of Rac1 and

![Figure 5](link)

**Figure 5.** Cell migration and tubule formation by Ang1 is mediated by Ang1-induced H2O2. Cells infected by 10 or 50 MOI Adv-vector (Adv) or adenovirus-catalase (Adv-Cat) were incubated with 200 ng/mL Ang1 for 30 minutes at 37°C. The stimulated cells were seeded onto Matrigel-coated Transwell (A) or plates (B) in M-199 containing 10% FBS. Cells were incubated for 7 to 24 hours. C, BSA (300 ng/mL) and Ang1 (300 ng/mL) with 50 MOI control adenovirus or with 50 MOI adenovirus-catalase was loaded on Thermanox coverslips and air dried. The reagent-coated coverslips were applied to the CAM surface of 9-day-old chick embryo. After 3 days, the neovasculature was observed under a microscope. Each sample was measured with more than six chick embryos, and the best image of CAM was presented. Arrows, neovasculature. *, P < 0.05; #, P < 0.05.
NADPH oxidase (16, 17). However, the exact role of ROS generation in angiogenesis is not clear (17, 29).

To determine whether Ang1 mediates ROS signaling pathway in angiogenesis, we explored the effects of DPI (a NADPH oxidase inhibitor), L-NAME (an eNOS inhibitor), and rotenone (a mitochondrial complex I inhibitor) on Ang1-induced ROS production. We found that Ang1-induced ROS generation was inhibited significantly by DPI, only slightly by rotenone, and not at all by L-NAME. This suggests that Ang1-induced ROS in endothelial cells is mainly H2O2, which was produced in a NADPH oxidase-dependent manner. Recently, it has been reported that endothelial-derived NO plays a role in Ang1-induced angiogenesis through PI3K/Akt-dependent signaling pathway (18). To confirm the effect of NO in Ang1-induced angiogenesis, we treated endothelial cells with the eNOS inhibitor, L-NAME. However, in our study conditions, L-NAME did not affect Ang1-induced ROS generation in concentration-dependent manner nor did it affect the recovery in a time-dependent manner (Fig. 2). We could not detect Ang1-induced phosphorylation of eNOS (Supplementary Fig. S2) and found that Ang1-induced angiogenesis was not affected in eNOS or inducible NO synthase knockout mice (data not shown). Although these results are at variance with some previous studies, they suggest that H2O2 plays a key role in Ang1-induced ROS. Recent finding (21) that ROS production in response to Ang1 exposure originates mainly from a Rac1-dependent NADPH oxidase confirms that Ang1 induces the activation of Rac1 and stimulates endothelial cells to migrate and change the shape through PI3K (10). Ang1/Tie2 signaling is essential for both angiogenesis and cell survival in endothelial-specific manner (30). Stable interaction of Tie2 receptor with α5β1 integrin regulates Ang1-induced angiogenesis by recruiting PI3K and FAK (31). On the other hand, recent reports show that Ang1 can induce cell survival, adhesion, and migration through integrin in Tie2-lacking cardiac and skeletal myocyte (32) and fibroblast (33). This indicates that Tie2 in Ang1 response is largely endothelial specific. These results suggest that ROS generated by Ang1 through Tie2 receptor or integrin activation seems to trigger angiogenic response by initiating the activation of downstream signaling cascades, such as PI3K.

To confirm the role of H2O2 in Ang1-induced angiogenesis, we examined catalase overexpression using adenoviral catalase. We found that the ROS generation can be completely blocked by adenovirus-catalase infection (Fig. 4). We also studied the effects of Ang1-induced H2O2 on angiogenesis and signaling pathways and in adenovirus (control) or adenovirus-catalase-infected endothelial cells. When Ang1-induced H2O2 generation was significantly suppressed in catalase-overexpressed endothelial cells, cell migration, tubule formation, and in vivo angiogenesis were abrogated (Fig. 5). These results suggest that Ang1-induced H2O2 acts as a critical mediator in angiogenesis and related responses.

It has been reported that angiogenic factor regulates remodeling of cytoskeletal proteins and migration through Akt activation (34, 35) and that Ang1 and Tie2 system protects cells against the damage, promotes their survival, and maintains the integrity of nonproliferating endothelial cells in normal adult blood vessels (19, 36). The cell survival promoted by Ang1 and Tie2 seems to be mediated mainly by PI3K/Akt (4, 12) and p44/42 MAPK (20). However, because signaling pathways involved in the angiogenic effect of Ang1 are not well understood, we examined Ang1-induced activation of Akt and MAPK in catalase-overexpressed cells. Activation of p44/42 MAPK by Ang1 was significantly inhibited in adenovirus-catalase-infected cells, and p38 MAPK activation was delayed. Similar effects were found when PDGF was treated with extracellular catalase (37). However, JNK activation by Ang1 was similar in control as well as adenovirus-catalase-infected cells. Our studies thus show that Ang1-induced activations of p44/42 MAPK and p38 MAPK parallel the generation of H2O2 by Ang1. Akt phosphorylation activated by various growth factors and cytokines occurring downstream of PI3K regulates cell survival (38). Our finding that Ang1-induced Akt activation was not affected by removal of H2O2 in adenovirus-catalase-infected cells and that Akt dephosphorylation was significantly delayed in these cells suggests...
that Akt activation is a ROS-independent phenomenon, whereas Akt deactivation is a ROS-dependent process. These results are not in accord with a previous report that VEGF-induced ROS phosphorylates the receptor and that the phosphorylated receptor activates PI3K/Akt signaling pathway (16). It is possible that the growth factor activates PI3K and affects ROS generation and Akt pathways among others (39). Although Akt possibly appears downstream of ROS, our results suggest that factors other than ROS may regulate the activation of Akt through PI3K. According to recent reports, Akt activated by Ang1 does not show any significant effect on Ang1-induced phosphorylation of p44/42 MAPK and p38 MAPK (40), and PI3K inhibitor blocks the activation of both Akt and MAPK by inhibiting ROS generation (41). These reports support our postulation of a different mechanism for the regulation of Akt and MAPKs facilitated by a cross-talk between ROS and Akt deactivation. When we examined the role of Ang1-induced ROS in the expression of VEGF and Ang1, we found that the expression of VEGF was specifically inhibited in adenovirus-catalase-infected cells, whereas Ang1 expression was not affected (data not shown). These results suggest that Ang1-induced ROS caused the expression of VEGF protein.

These effects by Ang1 observed in vitro were confirmed in vivo using catalase (+/+ ) and catalase (−/−) mice. Because COMP-Ang1 given by adenoviral vector induced long-lasting vascular enlargement by endothelial cell proliferation (23), the effects of Adv-COMP-Ang1 on vascular enlargement in tracheal vessels of catalase (+/+) and catalase (−/−) mice were examined in Fig. 7. Ang1-induced enlargement of postcapillary venules, collecting venules, venous end of capillaries, venules, and terminal arterioles in catalase (−/−) mice was larger than in catalase (+/+) mice. Ang1-induced vascular enlargement in catalase (−/−) mice is more sensitive than in catalase (+/+) mice.

In summary, these studies show for the first time that Ang1 generates H2O2 and that Ang1-induced H2O2 modulates the activation of p44/42 MAPK and p38 MAPK, thereby playing critical roles in tubule formation, cell migration, and angiogenesis. Further studies on the details of Ang1-induced signaling pathways, cross-talk between the pathways, and cellular roles of each pathway should help identify the functional differences of various angiogenic factors.

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Figure 7. $H_2O_2$ increase in catalase (−/−) mice increased the COMP-Ang1-induced generation on postcapillary venules and terminal arterioles. Catalase (+/+) mice and catalase (−/−) mice were treated with $1 \times 10^3$ pfu Adv-LacZ or Adv-COMP-Ang1. A, green, after 2 weeks, the tracheal vessels were visualized with PECAM-1 immunostaining. The diameters of terminal arterioles (B, yellow arrowheads) and postcapillary venules (C, white arrowheads) were measured. Diameters of 35-40 PV/5 and 10-12 TA/10 fields were measured at the edge of cartilage rings in each mouse. Columns, mean from two to three mice; bars, SD, *, $P < 0.05$ versus Adv-LacZ; #, $P < 0.05$ versus catalase (+/+) mice treated with Adv-COMP-Ang1. Bar, 100 μm.
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