Angiopoietin-1-induced angiogenesis is modulated by endothelial NADPH oxidase

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Chen, Jian-Xiong, Heng Zeng, Mayme L Lawrence, Timothy S. Blackwell, and Barbara Meyrick. Angiopoietin-1-induced angiogenesis is modulated by endothelial NADPH oxidase. Am J Physiol Heart Circ Physiol 291: H1563–H1572, 2006.—Reactive oxygen species (ROS) have been shown to play a central role in the pathogenesis of many cardiovascular diseases, such as atherosclerosis, diabetes, and hypertension. Recent studies (1, 4, 6, 8, 36) show that low levels of ROS serve as intracellular signaling molecules that modulate vascular smooth muscle cell (VSMC) proliferation (6). NADPH oxidase is the major source of intracellular ROS. The present study uses porcine coronary artery endothelial cells (PCAECs) to Ang-1 (250 ng/ml) for periods up to 30 min led to a transient and dose-dependent increase in intracellular ROS. Thirty minutes of pretreatment with the NADPH oxidase inhibitors diphenylene iodonium (DPI, 10 μM) and apocynin (200 μM) suppressed Ang-1-stimulated ROS. Pretreatment with either DPI or apocynin also significantly attenuated Ang-1-induced Akt and p44/42 MAPK phosphorylation. Inhibition of NADPH oxidase significantly suppressed Ang-1-induced endothelial cell migration and sprouting from endothelial spheroids. Using mouse heart microvascular endothelial cells from wild-type (WT) mice and mice deficient in the p47phox component of NADPH oxidase (p47phox−/−), we found that although Ang-1 stimulated intracellular ROS, Akt and p42/44 MAPK phosphorylation, and cell migration in WT cells, the responses were strikingly suppressed in cells from the p47phox−/− mice. Furthermore, exposure of aortic rings from p47phox−/− mice to Ang-1 demonstrated fewer vessel sprouts than WT mice. Inhibition of the Tie-2 receptor inhibited Ang-1-induced endothelial migration and vessel sprouting. Together, our data strongly suggest that endothelial NADPH oxidase-derived ROS play a critical role in Ang-1-induced angiogenesis.

angiogenic signaling; porcine coronary artery endothelium; Akt; extracellular signal-regulated kinase 1/2; Tie-2

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role of endothelial NADPH oxidase-derived ROS in Ang-1 signaling and angiogenesis. Our results demonstrate that Ang-1 stimulates transient intracellular generation of ROS and leads to activation of Akt and p42/44 MAPK (ERK-1/2). We also demonstrate that endothelial NADPH oxidase-derived ROS play a pivotal role in Ang-1/Tie-2-induced vascular endothelial cell migration and vessel sprouting via modulation of Akt and ERK-1/2 phosphorylation.

MATERIALS AND METHODS

The experimental protocol was reviewed and approved by the Institutional Animal Care and use Committee at Vanderbilt University (Nashville, TN).

Culture of coronary artery endothelial cells. Endothelial cells were carefully removed from the luminal surface of normal porcine coronary arteries and cultured as previously described (10, 11). Single colonies of cells were subcultured in 10% FBS in EGM (Clonetics). Only those cells having a typical cobblestone morphology, showing uptake of acetylated low-density lipoprotein, and exhibiting factor VIII-related antigen were used in these experiments (10, 11). Primary cultures of PCEs, between passages 5 and 10, were used in all experiments.

Mouse heart microvascular endothelial cells. The p47^phox^ knockout mice were provided by Dr. Steve Holland at the National Institute of Allergy and Infectious Diseases. C57/BL6 wild-type (WT) mice and p47^phox^ knockout mice were anesthetized by inhalation of carbon dioxide, and the heart was removed. The left ventricle was dissected, cut into small pieces, washed in PBS, and suspended in HBSS. Six mouse hearts were pooled and used for each mouse heart microvascular endothelial cell (MHMEC) preparation. The small pieces of heart were digested with collagenase A (2 mg/ml in HBSS) for 1 h at 37°C. Released cells were centrifuged and suspended in 5 ml of suspension buffer (Ca^2+^- and Mg^2+^-free PBS containing 0.5 g/100 ml BSA and 2 mM EDTA) and filtered through a 200-μm filter. The filtered cells were washed and suspended in 10 ml of 10% FBS in DMEM (5 mmol/l glucose). The cells were then transferred to a 100-mm culture dish and allowed to grow for several days. Clones of endothelial cells were identified by phase microscopy; the cells were trypsinized and transferred to culture dishes. Cells having typical cobblestone morphology, showing uptake of acetylated low-density lipoprotein, and exhibiting factor VIII-related antigen were used in these experiments. Primary cultures of MHMECs, between passages 4 and 10, were used in all experiments.

Experimental protocols. Before exposure to 250 ng/ml Ang-1 (recombinant human Ang-1, R&D System) for periods between 5 and 60 min, PCEs and MHMECs were pretreated for 30 min with the NADPH oxidase inhibitors diphenylene iodonium (DPI, 5–10 μM, CalBiochem, San Diego, CA) and apocynin (Apo, 200 and 600 μM, CalBiochem) or the SOD mimetic 4-hydroxy-TEMPO (Tempol, 5 mM, Sigma) and anti-rabbit Tie-2 antibody (15 μg/ml, Santa Cruz, CA). All time course and pharmacological interventions were carried out in 0.4% FBS in DMEM. Untreated cells served as controls.

Measurement of ROS. Intracellular ROS were determined by oxidative conversion of cell permeable chloromethyl-2,7^-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Molecular Probes, Eugene, OR) to fluorescent dichlorofluorescein (DCF) (22). Briefly, endothelial cells, cultured in two-well chamber slides with 10 μM CM-H2DCFDA in PBS for 30 min. DCF fluorescence was measured over the whole field of vision using a Zeiss fluorescence microscopy connected to an imaging system. In addition, intracellular levels of superoxide were measured by using a Luminax superoxide kit (Stratagene, La Jolla, CA) according to the manufacturer’s specifications.

Western blot analysis. PCEs and MHMECs were lysed in 300 μl of lysis buffer, and total protein concentrations were determined by using a BCA protein assay kit (Pierce). Protein (25 μg) was subjected to SDS-PAGE on 12% polyacrylamide gels and transferred to a nitrocellulose membrane. Western blot analysis was performed with each of the specific antibodies as follows: for phosphorylated Akt, the membrane was incubated with rabbit anti-phospho-Akt antibody (1:1,000 dilution, Santa Cruz Biotech) and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2,000 dilution, BD Biosciences), and densitometric analysis was carried out using an image acquisition and analysis software (LabWorks, UVP).

Immunoprecipitation of Tie-2 and Western blot analysis with p-Tie-2. After 15 min of treatment with Ang-1 (250 ng/ml) without 30 min of pretreatment with either DPI (10 μM) or Apo (200 μM), endothelial cells were washed and incubated in lysis buffer and briefly sonicated. The lysates were immunoprecipitated with anti-rabbit Tie-2 antibody (2 μg/ml of total cell protein, Santa Cruz Biotech) for 16 h at 4°C, followed by a 2-h incubation with 1:1 protein A:protein G-sepharose slurry. After centrifugation, the immunoprecipitates were washed, resuspended in loading buffer, boiled for 5 min, and subjected to SDS-PAGE on 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The primary antibody used for Western blot analysis was anti-p-Tie-2 (1:1,000 Cell Signaling Technology). The membranes were washed and incubated with secondary antibody coupled to HRP, and densitometric analysis was carried out using an image acquisition and analysis software (LabWorks, UVP).

Cell migration assay. Migration assays were performed as previously described (10). Polycarbonate filter wells (8-μm pores, 6.5 mm diameter, Transwell, Costar) were coated with type I collagen (BD Biosciences), and either PCEs or MHMECs were plated (1 × 10^5 cells) in the upper chamber with or without each pharmacological intervention. The bottom chamber was filled with 600 μM 0.4% MEM containing 250 ng/ml Ang-1 (R&D system) with or without each intervention. The cells were then allowed to migrate for 4 h at 37°C. Cells on the filter were fixed in 10% formalin ( Fisher) for 20 min, rinsed in PBS, stained with 0.2% Crystal violet dye (Fisher) for 30 min, and mounted in Cytoseal-60 (Richard-Allan Scientific, Kalamazoo, MI). Ten randomly selected fields were counted on each filter using a ×25 objective. Experiments were performed in duplicate for each intervention.

Endothelial spheroid angiogenesis assay. PCEs and MHMECs were plated (1 × 10^5 cells) in the upper chamber with or without each pharmacological intervention. The bottom chamber was filled with 600 μM 0.4% MEM containing 250 ng/ml Ang-1 (R&D system) with or without each intervention. The cells were then allowed to migrate for 4 h at 37°C. Cells on the filter were fixed in 10% formalin ( Fisher) for 20 min, rinsed in PBS, stained with 0.2% Crystal violet dye (Fisher) for 30 min, and mounted in Cytoseal-60 (Richard-Allan Scientific, Kalamazoo, MI). Ten randomly selected fields were counted on each filter using a ×25 objective. Experiments were performed in duplicate for each intervention.

Mouse aortic ring spraying assay. Thoracic aortas from C57/BL6 (WT) and p47^phox^ knockout mice were isolated, dissected from connective tissues, and washed in PBS extensively under aseptic conditions. The aortas were maintained in MEM plus 10% FBS and antibiotic-antimycotic (Gibco). Clotted blood inside the aorta was flushed with media, and the aortae were cut into rings ~1 mm in thickness. The rings were placed in the middle of the 24-well plate,
overlaid with 300 µl of type I collagen solution, and left to polymerize for 1–2 h at 37°C before the addition of 10% FBS MEM. Vessel outgrowth was examined using an Olympus 1/H11003/70 microscope. After three days of culture, the area of vessel outgrowth was quantified by using image acquisition and analysis software (LabWorks, UVP).

Statistical analysis. The results are expressed as means ± SD. Statistical analysis was performed using one-way ANOVA followed by the Duncan’s multiple-comparison test. P < 0.05 was taken as significant.

RESULTS

Ang-1 stimulates intracellular ROS formation. Exposure of PCAECs to different concentrations of Ang-1 (150–500 ng/ml) for 5 min resulted in a dose-dependent increase in intracellular ROS. A modest increase in ROS was apparent at 150 ng/ml and was further increased at doses of 250 and 500 ng/ml (data not shown). Based on this information, a concentration of 250 ng/ml was used in these experiments. Exposure of PCAECs to Ang-1 for various time periods (2, 5, 10, 15, and 30 min) resulted in a transient increase in intracellular ROS formation. Intracellular ROS were increased at 2 min, peaked between 5 and 10 min, and gradually declined to baseline.

Similarly, exposure of WT MHMECs to Ang-1 (250 ng/ml) for 5 min led to an increase in intracellular ROS. This increase was markedly attenuated in p47phox−/− MHMECs (n = 4 cell lines). Pretreatment of WT MHMECs with either the NADPH oxidase inhibitors DPI (10 µM) and Apo (200 µM) or a SOD mimetic Tempol (Temp, 5 mM), followed by exposure to Ang-1 (250 ng/ml), significantly suppressed Ang-1-induced superoxide formation. Temp alone also resulted in a significant reduction in basal superoxide production (n = 3 cell lines, data are means ± SD. *P < 0.05 when compared with Ang-1 alone; #P < 0.05 compared with baseline). Con, control; KO, knockout; RLU, relative light units.

Fig. 1. A: time course for angiopoietin-1 (Ang-1; 250 ng/ml)-stimulated intracellular reactive oxygen species (ROS) generation in porcine coronary artery endothelial cells (PCAECs). ROS formation was increased at 2 min, peaked between 5 and 10 min, and gradually declined to baseline. B: pretreatment of PCAECs with either diphenylene iodonium (DPI; 10 µM) or apocynin (Apo; 200 µM) caused striking suppression of Ang-1-induced ROS generation. C: in wild-type (WT) mouse heart microvascular endothelial cells (MHMECs), exposure to Ang-1 (250 ng/ml) for 5 min caused a striking increase in intracellular ROS. This increase was markedly attenuated in p47phox−/− MHMECs (n = 4 cell lines). D: pretreatment of WT MHMECs with either the NADPH oxidase inhibitors DPI (10 µM) and Apo (200 µM) or a SOD mimetic Tempol (Temp, 5 mM), followed by exposure to Ang-1 (250 ng/ml), significantly suppressed Ang-1-induced superoxide formation. Temp alone also resulted in a significant reduction in basal superoxide production (n = 3 cell lines, data are means ± SD. *P < 0.05 when compared with Ang-1 alone; #P < 0.05 compared with baseline). Con, control; KO, knockout; RLU, relative light units.
effect on basal ROS production, treatment with Tempol alone significantly reduced basal intracellular superoxide levels (Fig. 1D).

NADPH oxidase modulates Ang-1-stimulated ERK and Akt phosphorylation. Previous studies (10, 42) demonstrated that activation of Akt and p42/44 MAPK mediates Ang-1-induced angiogenesis. To further explore the role of NADPH oxidase in Ang-1-induced angiogenic signaling, we examined the effects of NADPH oxidase inhibitors on Ang-1-stimulated p42/44 phosphorylation. Exposure of PCAECs to Ang-1 for periods up to 60 min caused a significant increase in phosphorylated ERK-1/2. Increased phosphorylation was apparent at 15 min, peaked at 30 min, and by 60 min had declined, although the densitometric value remained significantly above control levels (Fig. 2, A and B). Pretreatment with the NADPH oxidase inhibitors DPI and Apo (200 or 600 μM) attenuated Ang-1-stimulated p42/44 ERK phosphorylation (Fig. 2, C and D). Treatment with DPI and Apo alone had little effect on basal p42/44 MAPK phosphorylation (Fig. 2, C and D). Studies in WT MHMECs showed a similar increased in ERK-1/2 phosphorylation, peaking at 30 min of Ang-1 treatment; this increase was inhibited in the p47phox-deficient cells (Fig. 2E).

Stimulation of PCAECs with Ang-1 for 30 min caused a dramatic increase in phosphorylated Akt at Ser473 but had little effect on Akt phosphorylation at Thr308. Pretreatment with DPI (10 μM) and Apo (200 and 600 μM) for 30 min completely suppressed Ang-1-induced Akt phosphorylation at Ser473 (Fig. 3, A and B) but failed to alter Akt phosphorylation at Thr308 (data not shown). Treatment with DPI and Apo alone had little effect on basal Akt phosphorylation (Fig. 3, A and B). Similarly, exposure of WT MHMECs to Ang-1 resulted in an increase in phosphorylated Akt at 30 and 60 min; the increase was not apparent in the p47phox-deficient cells (Fig. 3C).

Ang-1-stimulated Tie-2 phosphorylation is not dependent on NADPH oxidase. To ascertain whether NADPH oxidase modulates phosphorylation of Tie-2, MHMECs were exposed to Ang-1 with and without pretreatment with either DPI (10 μM)
MHMECs from p47phox-deficient mice failed to show this increase in spheroid sprouting that was suppressed by treatment with either DPI or Apo alone. Exposure to DPI, Apo, and Tempol alone failed to elicit any effect on WT MHMECs (Fig. 5, left, A and B, and right, top), pretreatment of PCAECs with DPI (10 μM) or Apo (200 μM) significantly inhibited Ang-1-stimulated PCAEC spheroid sprouting (Fig. 5, left, C and D, respectively). Treatment with either DPI or Apo alone had little effect on basal spheroid migration (Fig. 5, left, E and F, respectively, and right, top). Similarly, with the use of WT MHMECs, Ang-1 caused an increase in spheroid sprouting that was suppressed by treatment with DPI, Apo, and Tempol (Fig. 5, right, bottom). Spheroids of p47phox−/− mice showed only a modest increase in sprouting that was significantly attenuated by DPI, Apo, and Tempol (Fig. 5, right, top).}

Effect of NADPH oxidase on endothelial cell migration and sprouting are dependent on NADPH oxidase. To explore the role of NADPH oxidase on Ang-1-induced angiogenesis, we first examined endothelial cell migration in the presence of NADPH oxidase inhibitors. PCAECs treated with Ang-1 + DPI (10 μM) or Ang-1 + Apo (200 μM) for 4 h resulted in significant suppression of Ang-1-induced cell migration. DPI at a concentration of 5 μM failed to inhibit migration. Treatment with DPI and Apo alone had little effect on basal endothelial migration (Fig. 4A).

We further examined the effect of NADPH oxidase on Ang-1-stimulated endothelial cell migration using WT and p47phox−/− MHMECs. MHMECs from WT mice showed a significant increase in migration in response to Ang-1, whereas MHMECs from p47phox−/− mice failed to show this increase (Fig. 4B). Exposure of WT MHMECs to Ang-1 caused a similar approximate doubling in endothelial migration that was significantly attenuated by DPI, Apo, and Tempol. Treatment with anti-Tie-2 also significantly attenuated Ang-1-stimulated cell migration, and this effect was more striking than after pretreatment with either DPI or Apo. Exposure to DPI, Apo, and Tempol alone failed to elicit any effect on WT MHMECs basal migration (Fig. 4C).

Effect of NADPH oxidase on spheroid sprouting. Similarly, using a PCAEC spheroid sprouting assay, we found that Ang-1 caused an increase in vessel sprouting (Fig. 5, left, A and B, and right, top); pretreatment of PCAECs with DPI (10 μM) or Apo (200 μM) significantly inhibited Ang-1-stimulated PCAEC spheroid sprouting (Fig. 5, left, C and D, respectively). Treatment with either DPI or Apo alone had little effect on basal sprouting (Fig. 5, left, E and F, respectively, and right, top). Similarly, with the use of WT MHMECs, Ang-1 caused an increase in spheroid sprouting that was suppressed by treatment with DPI, Apo, and Tempol (Fig. 5, right, bottom). Spheroids of p47phox−/− deficient cells showed only a modest response to Ang-1 (Fig. 5, right, bottom).

or Apo (200 μM), and Tie-2 was immunoprecipitated with a Tie-2 antibody followed by Western blot analysis with an antibody to phosphorylated Tie-2. Ang-1 resulted in an ∼60% increase in Tie-2 phosphorylation (Fig. 3, D and E). The increase in Ang-1-stimulated Tie-2 phosphorylation was little changed by pretreatment with either DPI or Apo. Neither Apo nor DPI alone altered basal levels of Tie-2 phosphorylation.

Ang-1-stimulated endothelial cell migration and sprouting are dependent on NADPH oxidase. To explore the role of NADPH oxidase on Ang-1-induced angiogenesis, we first examined endothelial cell migration in the presence of NADPH oxidase inhibitors. PCAECs treated with Ang-1 + DPI (10 μM) or Ang-1 + Apo (200 μM) for 4 h resulted in significant suppression of Ang-1-induced cell migration. DPI at a concentration of 5 μM failed to inhibit migration. Treatment with DPI and Apo alone had little effect on basal endothelial migration (Fig. 4A).

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Effect of NADPH oxidase on vascular sprouting. We next examined the role of NADPH oxidase on Ang-1-mediated angiogenesis using a mouse aortic ring vessel outgrowth assay from WT and p47phox−/− mice. By 3 days, stimulation of WT rings with Ang-1 resulted in a dramatic increase in vessel outgrowth compared with that of controls (Fig. 6, left, A and B, and right), and pretreatment with DPI (10 μM) or Apo (200 μM) significantly reduced the area of Ang-1-induced vessel outgrowth (Figs. 6, left, C and D, and right). The decrease in area after pretreatment with Apo was the result of a decrease in number of sprouts; length of sprouts, especially after treatment with Apo, seemed little affected. Treatment with DPI and Apo alone tended to reduce basal vessel outgrowth, although the total area of outgrowth was not significantly different (Fig. 6, left, E and F, and right). In addition, treatment with anti-Tie-2 significantly attenuated Ang-1 induced vessel outgrowth (Fig. 6, left, G, and right). Aortic rings from p47phox−/− mice exhibited only rare and short vessel outgrowths both at baseline (Fig. 6, left, H) and after stimulation with Ang-1 (Fig. 6, left, I). Calculation of the area of vessel outgrowth confirmed these findings (Fig. 6, right).

DISCUSSION

This study demonstrates that NADPH oxidase-derived ROS regulate Ang-1-induced endothelial cell migration, spheroid sprouting, and angiogenesis using both in vitro and ex vivo models. With the use of PCAECs and MHMECs, our studies demonstrate that exposure to Ang-1 leads to a time- and dose-dependent increase in intracellular ROS that is abolished by pretreatment with the NADPH oxidase inhibitors DPI and Apo, a deficiency of the p47phox subunit of NADPH oxidase and the superoxide dismutase mimetic Tempol. Similarly, Ang-1 results in an increase in p44/42 MAPK and Akt at Ser473 phosphorylation that is suppressed by both NADPH oxidase inhibitors and p47phox deficiency. Our data also demonstrate that inhibition of NADPH oxidase activity, superoxide production, or p47phox deficiency suppresses Ang-1-induced endothelial cell migration as well as capillary sprouting from endothelial spheroids and mouse aortic rings. Furthermore, inhibition of Tie-2 inhibits Ang-1-induced endothelial migration and vessel growth.

Recent studies (17, 19) demonstrate that all the components of the phagocyte NADPH oxidase complex are present in endothelial cells. Furthermore, endothelial cells release a small amount of ROS as signaling messengers to modulate vascular homeostasis (2, 13, 17, 38). Stimulation of endothelial cells with growth factors, such as VEGF, increases NADPH oxidase activity and ROS production (38, 39). Consistent with those studies, our data demonstrate that Ang-1 leads to a transient increase in intracellular ROS, the majority of which are gene-

![Fig. 4. A: effect of NADPH oxidase inhibitors on Ang-1-stimulated PCAEC migration. Treatment with either DPI (10 μM, but not 5 μM) or Apo (200 μM) significantly suppressed Ang-1-induced endothelial migration. Apo and DPI alone had no effect on basal endothelial migration (n = 4 cell lines, data are means ± SD, *P < 0.05 compared with Ang-1 treatment; #P < 0.05 compared with baseline). B: effect of Ang-1 on WT and p47phox−/− MHMEC migration. In WT MHMECs (black bars), exposure to Ang-1 (250 ng/ml) for 4 h significantly increased migration. In p47phox−/− MHMECs (white bars), exposure to Ang-1 (250 ng/ml) failed to cause a significant increase in Ang-1-induced endothelial migration (n = 3 cell lines, data are means ± SD, *P < 0.05 compared with WT controls). C: effect of NADPH oxidase inhibitors on Ang-1-stimulated endothelial cell migration in WT MHMECs. Treatment with either NADPH oxidase inhibitors DPI (10 μM) and Apo (200 μM) or the SOD mimetic Temp (5 mM) and an antibody to Tie-2 (15 μg/ml) significantly suppressed Ang-1-induced endothelial migration. Apo, DPI, and Temp alone had no effect on baseline endothelial migration (n = 3 cell lines, data are means ± SD, *P < 0.05 compared with Ang-1 alone; #P < 0.05 compared with baseline).](http://ajpheart.physiology.org/)

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generated through an NADPH oxidase-dependent mechanism, confirming the recent studies (22) in human umbilical vein endothelial cells. However, our finding of an increase in intracellular superoxide after pretreatment with Tempol before the addition of Ang-1 compared with Tempol alone was surprising and suggests that ROS, other than superoxide, may also contribute to the Ang-1-induced changes.

NADPH oxidase is an important mediator of the angiogenic signaling cascades and is essential in the regulation of angiogenesis (2, 3, 38). With the use of a genetic approach, NADPH oxidase-derived ROS have been shown to trigger the angiogenic switch that modulates angiogenesis. Overexpression of the gp91phox homologue, Nox1, of NADPH oxidase leads to increased ROS formation and upregulation of VEGF mRNA and matrix metalloproteinase activity; the effect of overexpression of gp91phox on VEGF expression is eliminated by the coexpression of catalase (3). Further, the gp91phox subunit has been implicated in neovascularization of the hindlimb in response to ischemia (34). Phosphorylation of cytosolic p47phox has also been shown to be important in the activation of NADPH oxidase. NADPH oxidase-derived ROS can lead to activation of VEGF receptor, transactivation of epidermal growth factor receptors, and induction of HIF-1α and contribute to vascular angiogenesis (8, 18, 30, 37). Further, Rac1 has been reported to contribute to VEGF-induced ROS production and VEGF-induced angiogenesis (2, 38, 39). Our data demonstrate that both chemical inhibition of NADPH oxidase activity and a deficiency in p47phox significantly suppress Ang-1-induced endothelial cell migration and vascular sprouting. Whereas the effect of Apo pretreatment on vascular sprouting from aortic explants seems less effective than that for endothelial migration and sprouting from endothelial spheroids, the significant reduction in area after this treatment reflects the decreased number of sprouts. Pretreatment with a SOD mimic also effectively blocked basal and Ang-1-induced superoxide generation as well as Ang-1-induced endothelial migration and spheroid sprouting. Together, these data demonstrate that NADPH oxidase-derived superoxide is required for the
Ang-1-induced angiogenesis. In addition, our studies after inhibition of Tie-2 confirm that the Ang-1/Tie-2 complex is necessary for the angiogenesis.

Animal models and human specimens suggest that NADPH oxidase plays a key role in the development of atherosclerosis. Angiogenesis of the vessel wall is a consistent feature of atherosclerotic plaque development. The corneal micropocket angiogenesis assay reveals that homogenates of atherosclerotic aortae contain angiogenic activity that promotes corneal angiogenesis (29). Recent studies (7) in p47phox−/− mice on an ApoE−/− background reveal a significant reduction in atherosclerotic lesion area. Our finding that inhibition of NADPH oxidase activity attenuates Ang-1-induced p42/44 MAPK phosphorylation suggests that Ang-1 activation of p42/44 MAPK is also redox sensitive.

Akt is activated by a number of growth factors and cytokines and modulates many aspects of cellular function such as cell motility, migration, endothelial nitric oxide (NO) synthase (eNOS) phosphorylation, and NO production (5, 10, 14, 16, 28, 33). Compelling evidence also suggests that PI3K/Akt is an

In a previous study, it was shown that both p42/44 MAPK and PI3/Akt play major roles in Ang-1-stimulated angiogenesis (41). MAPK are key regulator proteins that control cell growth, apoptosis, and stress signaling. For example, ROS induce activation of MAPK and proliferation of VSMC (4, 32). PDGF induces p42/44 MAPK activation that is inhibited by pretreatment with exogenous catalase (40), and exposure of endothelial cells to shear stress leads to p42/44 MAPK phosphorylation that is suppressed by pretreatment with antioxidants (15). Our finding that inhibition of NADPH oxidase activity attenuates Ang-1-induced p42/44 MAPK phosphorylation suggests that Ang-1 activation of p42/44 MAPK is also redox sensitive.
important regulator of endothelial cell proliferation and survival and is the major signaling mediator in Ang-1-induced angiogenesis (5, 10, 25, 27, 41). Our study demonstrates that Ang-1-induced Akt phosphorylation at Ser473, like p42/44 MAPK, was significantly blunted by pretreatment with NADPH oxidase inhibitors and by deletion of p47phox. Thus endothelial NADPH oxidase is involved in the Ang-1-induced activation of Akt and p42/44 MAPK and modulation of endothelial cell migration and angiogenesis.

Our findings for ERK-1/2 phosphorylation are in contrast to those in human umbilical vein endothelial cells where DPI failed to suppress Akt phosphorylation. The reason for this is obscure but may reflect the different cell types and concentration of DPI (at least twice as concentrated as the dose used in the present study) utilized in these two studies (22).

In summary, the present study demonstrates that Ang-1 causes a transient increase in NADPH oxidase-derived intracellular ROS and that inhibition of NADPH oxidase blocks Ang-1-stimulated Akt and p42/44 MAPK phosphorylation. Furthermore, with the use of both in vitro and ex vivo models, inhibition of NADPH oxidase activity, superoxide production, or a deficiency of NADPH oxidase subunit p47phox suppresses Ang-1-induced angiogenesis. Additional studies after the inhibition of Tie-2 confirm the essential role of Tie-2 in this angiogenic response. We conclude that endothelial NADPH oxidase-derived ROS play a critical role in the regulation of Ang-1-induced angiogenic signaling cascades and angiogenesis.

GRANTS

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REFERENCES


