HSP90 and Akt modulate Ang-1-induced angiogenesis via NO in coronary artery endothelium

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Chen, Jian-xiong, Mayme Lee Lawrence, Gary Cunningham, Brian W. Christman, and Barbara Meyrick. HSP90 and Akt modulate Ang-1-induced angiogenesis via NO in coronary artery endothelium. J Appl Physiol 96: 612–620, 2004. —This study examines the notion that heat shock protein (HSP) 90 binding to nitric oxide (NO), endothelial NO synthase (eNOS), and PI3K-Akt regulate angiopoietin (Ang)-1–induced angiogenesis in porcine coronary artery endothelial cells (PCAEC). Exposure to Ang-1 (250 ng/ml) for periods up to 2 h resulted in a time-dependent increase in eNOS phosphorylation at Ser 1177 that occurred by 5 min and peaked at 60 min. This was accompanied by a gradual increase in NO release. Ang-1 also led to stimulation of HSP90 binding to eNOS and a significant increase in Akt phosphorylation. Thirty minutes of pretreatment of cells with either 1 µg/ml geldanamycin (a specific inhibitor of HSP90) or 500 nM wortmannin [a specific phosphatidylinositol 3 (PI3)-kinase (PI3K) inhibitor] significantly attenuated Ang-1-stimulated eNOS phosphorylation and NO production. Exposure to Ang-1 caused an increase in endothelial cell migration, tube formation, and sprouting from PCAEC spheroids, and pharmacological blockade of HSP90 function or inhibition of PI3K-Akt pathway completely abolished these effects. Inhibition of nitric oxide synthase by Nω-nitro-L-arginine methyl ester (2.5 mM) also resulted in a significant decrease in Ang-1-induced angiogenesis. We conclude that stimulated HSP90 binding to eNOS and activation of the PI3K-Akt pathway contribute to Ang-1-induced eNOS phosphorylation, NO production, and angiogenesis in PCAEC.

phosphorylated endothelial nitric oxide synthase; Tie-2 receptor; neovascularization; signal transduction

ANGIOGENESIS IS THE FINAL common pathway in ischemia-induced neovascularization as well as formation of collateral vessels in cardiovascular diseases. Ligand-initiated signaling in several classes of receptor tyrosine kinases has been identified as crucial mediators of angiogenesis in the heart (28). One of them, Tie-2, is specifically expressed in vascular endothelium and plays a crucial role in angiogenesis and stabilization of vascular integrity. Angiopoietin (Ang)-1 binding to the Tie-2 receptor (10) controls numerous signaling pathways that are involved in diverse cellular processes, including endothelial cell migration, capillary tube assembly, and recruitment of perivascular support cells to form mature and functional vessels (13, 20, 43). Ang-1 has been shown to have specific effects on endothelial cells. For example, it has been shown that Ang-1 prevents endothelial cell apoptosis induced by serum deprivation, irradiation, and mannitol via activation of the PI3K-protein kinase B (PI3K-Akt)–signaling pathway (24, 29).

The PI3K-Akt pathway is also known to increase nitric oxide (NO) production by direct phosphorylation of endothelial NO synthase (eNOS) (11, 15, 31). NO is highly regulated by various stimuli and growth factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (1, 3), and has been considered a downstream mediator of VEGF and other angiogenic factors. Previous work has established the involvement of PI3K-Akt-NO signaling in VEGF, bFGF, transforming growth factor (TGF)–β, ephrin-B4, and sphingosine-1-phosphate-induced angiogenesis (1, 33, 35, 37, 42). In coronary arteries, Ang-1 has been shown to protect endothelial cells against oxidized low-density lipoprotein-induced injury via a PI3K-Akt-dependent mechanism (25). More recently, Babaei and colleagues (2) demonstrated, using human umbilical vein endothelial cells, that Ang-1-induced angiogenesis is NO dependent. To date, little is known about the functional significance and molecular mechanisms of NO in Ang-1-induced angiogenesis in porcine coronary artery endothelial cells (PCAEC).

In vitro and in vivo evidence suggests that heat shock protein (HSP) 90 plays an important role in the regulation of eNOS and NO production. In bovine aortic endothelial cells, exposure to VEGF increases HSP90 binding to eNOS, eNOS activity, and NO production (16). Our previous data in PCAEC revealed that hypoxic stimulation of eNOS activity and NO production involved an increase in eNOS-associated HSP90 (8). Moreover, an HSP90–eNOS interaction has been considered a key target to modulate NO-dependent angiogenesis (5). However, the role of HSP90–eNOS interaction in Ang-1-induced angiogenesis has not been examined.

The present study uses PCAEC to explore the possible molecular mechanisms of Ang-1–stimulated NO production and the role of NO on Ang-1–induced angiogenesis. Our results demonstrate that Ang-1 stimulates eNOS phosphorylation and increases NO production via stimulation of HSP90 binding to eNOS and activation of the PI3K-Akt pathway. We also demonstrate that PI3K-Akt and HSP90–eNOS interactions play a pivotal role in Ang-1–induced PCAEC migration, tube formation, and sprouting.

MATERIALS AND METHODS

Culture of Coronary Artery Endothelial Cells

Coronary arteries were isolated from normal pig hearts as previously described (23). Briefly, the coronary arteries were cleaned of connective tissues, and endothelial cells were carefully removed from the luminal surface of the coronary arteries with a sterile cotton bud. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Cells attached to the cotton were agitated in growth medium and cultured at 37°C in a humidified 95% air-5% CO₂ incubator. Single colonies of cells were subcultured in 10% fetal bovine serum (FBS) in endothelial basal growth medium (EGM, Clonetics). Each of the endothelial lines had a typical cobblestone morphology, showed uptake of acetylated low-density lipoprotein, and exhibited factor VIII-related antigen. Primary cultures of PCAEC between passages 5 and 10 were used in all experiments.

**Experimental Protocols**

PCAEC were pretreated for 30 min with one of the following pharmacological inhibitors: 1 μg/ml geldanamycin (GA; HS990 inhibitor; Sigma Chemical, St. Louis, MO), 500 nM wortmannin (Wort; PI3K inhibitor; Sigma Chemical), and 2.5 mM N'–nitro-l-arginine methyl ester [l-NAME; NO synthase (NOS) inhibitor; Sigma Chemical]. All time course and pharmacological interventions were carried out in 0.4% FBS in MEM. All pharmacological interventions were dissolved and diluted in 0.4% FBS in MEM. After 30 min of pretreatment with each of the interventions, 250 ng/ml Ang-1 (recombinant human Ang-1, R&D System) was added, and the cells were incubated for 4 h at 37°C. Each assay was carried out in duplicate.

**Western Blot Analysis**

PCAECs were lysed in 300 μl of lysis buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.25% Na deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM orthovanadate). Twenty-five or 50 μg of proteins were subjected to SDS-PAGE on 12% polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was performed with anti-phospho-eNOS (1:1,000; Cell Signaling Technology), mouse anti-eNOS (1:2,500; BD Transduction Laboratories), mouse anti-HSP90 (1:1,000; BD Transduction Laboratories), or rabbit anti-phospho-Akt antibody (1:1,000; Santa Cruz Biotechnology) for 1 h at room temperature. The membrane was then washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:4,000 for eNOS, 1:2,000 for HSP90; BD Transduction Laboratories) or horseradish peroxidase-conjugated anti-rabbit IgG (1:2,000 dilution for p-Akt and p-eNOS; Promega). Total level of Akt was detected by using anti-Akt (1:1,000; Cell Signaling Technology). The membranes were developed using a Western blot chemiluminescence detection reagent (Perkin-Elmer, Life Science Products). The density of the band was measured by densitometric analysis was carried out by using image acquisition and analysis software (LabWorks, UVP, Upland, CA).

**Immunoprecipitation of eNOS and Blotting with HSP90**

Endothelial cells were washed and incubated in lysis buffer containing 20 mM Tris·HCl (pH, 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM orthovanadate for 30 min on ice and briefly sonicated. Immunoprecipitation was carried out by incubating the cell lysates (200 μg) with eNOS monoclonal antibody (2 μg/ml of total cell protein) for 16 h at 4°C, followed by a 2-h incubation with a 1:1 protein A-to-protein G ratio sepharose slurry. After centrifugation, the immunoprecipitates were washed in lysis buffer, resuspended in loading buffer, boiled for 5 min, subjected to SDS-PAGE on 12% polyacrylamide gel, and transferred to a nitrocellulose membrane. The primary antibody used for immunoblotting was anti-HSP90 (1:1,000, BD Transduction Laboratories). The membrane was then washed and incubated with secondary antibody. The membrane was then developed by using the Western blot chemiluminescence detection reagent (Perkin-Elmer, Life Science Products). The density of the band was measured by using image acquisition and analysis software (LabWorks, UVP).

**Measurement of NO Release**

NO release was measured by using a specific chemiluminescence method and a NO analyzer (NOA 280i, Sievers, Boulder, CO) as previously described (7). Briefly, the sample was injected into a reflow chamber that contained saturated vanadium chloride in 1 N HCl. The mixture was refuxed at 55°C while being continuously flushed with nitrogen gas, which in turn was transferred to the NO analyzer, where NO reacted with ozone to produce light. Light was measured by a photomultiplier, converted into voltage, and recorded on a standard flatbed recorder. A standard curve of NO was determined by using known concentrations of sodium nitrite, and the sample NO concentrations (NOₓ) were calculated by using the standard curve. Each assay was carried out in duplicate.

**Cell Migration Assay**

Migration assays were performed as previously described (45). Briefly, polycarbonate filter wells (Transwell, Costar, 8-μm pores, 6.5-mm diameter) were coated with 10 μg/ml rat tail type I collagen (BD Biosciences). PCAEC were plated (1 × 10⁶ cells in a volume of 200 μl) in the upper chamber of the filter well with or without each pharmacological intervention. The bottom chamber was filled with 600 μl of 0.4% MEM containing 250 ng/ml Ang-1 with or without each intervention. The cells were then allowed to migrate for 4 h at 37°C. Cells 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). Cells adhering to the top of the transwell filter were removed with a cotton swab. Only those cells that migrated completely to the lower surface of the filter insert were counted. Ten randomly selected fields were counted on each filter by using a ×25 objective. Experiments were performed in duplicate for each intervention.

**Capillary-like Tubule Formation**

PCAEC were trypsinized, counted, and placed into flat-bottom 24-well tissue culture plates (2.5 × 10⁴ cells/well) in serum-free EGM. Cells were incubated with 250 ng/ml Ang-1 for 3 h. In the pharmacological intervention experiments, cells were incubated with each pharmacological intervention for 30 min before adding Ang-1. After cells were seeded, 300 μl of extracellular matrix gel (Sigma Chemical) was placed onto the surface of the cells, and the plate was incubated at 37°C for 1 h to allow the gel to set. After solidification, the gel was covered with EGM medium or EGM medium containing 250 ng/ml Ang-1 with or without each intervention for a further 24 h. The gels were photographed by using a phase-contrast microscope (44). Capillary tubule formation was quantified by counting the number and cumulative length of tubular structures in eight fields by using image acquisition and analysis software (LabWorks, UVP). Each assay was conducted in duplicate.

**Endothelial Cell Spheroid Model of Angiogenesis**

Generation of PCAEC spheroids. We utilized the recently described cell spheroid model of angiogenesis (19, 26). This model has been reported to involve the aggregation of endothelial cells into three-dimensional spheroids when embedded in collagen gels with subsequent formation of sprouts or capillary-like projections and complex networks (19). A confluent monolayer of PCAEC was trypsinized, and the cells were centrifuged at 800 rpm for 5 min. The cell pellet was suspended in the cell in 0.2% methycellulose-10% FBS EGM medium, and 1 × 10⁶ of the suspended cells were seeded into nonadhesive 96-well plates and cultured at 37°C. After 24 h, spheroid formation and morphology were assessed by using phase-contrast microscopy. The spheroids were harvested by centrifugation at 800 rpm for 3 min and carefully vacuum aspirated to leave the spheroids in 10% of the methocel medium.
Spheroid-based in vitro angiogenesis assay. One-half milliliter of the spheroid suspension was mixed with 250 ng/ml of Ang-1 with or without each of the pharmacological interventions in 0.2% methocell-20% FBS in EGM medium. A 0.5-ml aliquot of collagen gel solution was then added to the suspension, mixed thoroughly, and added to each well of a 24-well cell culture plate. The gel was solidified at 37°C for 30 min, and 0.5 ml of 20% FBS in EGM containing 250 ng/ml Ang-1 with or without each pharmacological intervention was added to the top of the gel. After 24 h, the length of capillary sprouts from each spheroid was measured with image acquisition and analysis software (LabWorks, UVP). At least 10 spheroids per experimental group were analyzed. Experiments were performed in duplicate for each intervention.

Morphological analysis. Collagen gel-embedded PCAEC spheroids were fixed for 24 h with 4% paraformaldehyde followed by dehydration through graded ethanol at 4°C for 24 h. The gel was then vacuum infiltrated in 1:1 paraffin-to-xylene solution at 60°C for 10 h. The infiltration was completed by using fresh low melting paraplast X-tra at 60°C for 24 h before embedding. The blocks were cooled at 4°C, and serial 10-μm sections were cut and stained with hematoxylin.

Statistical Analysis

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

RESULTS

Ang-1 Stimulates eNOS Phosphorylation and NO Release

Exposure to Ang-1 (250 ng/ml) for various periods of time (5, 15, 30, 60, and 120 min) resulted in a time-dependent increase in eNOS phosphorylation at Ser1177. Phosphorylated eNOS was apparent at 5 min, was significantly increased by 15 min, peaked at 60 min, and then started to decline (Fig. 1, A and B). Exposure to Ang-1 had little effect on total eNOS protein expression over the 120 min of exposure (Fig. 1A). The time frame of Ang-1-stimulated NOX-release NO production showed a similar pattern as eNOS phosphorylation (Fig. 1C).

Ang-1 Increases HSP90 Binding to eNOS and Upregulates Phosphorylated eNOS

To examine the time course of HSP90 binding to eNOS after treatment with Ang-1, we immunoprecipitated with an eNOS antibody followed by immunoblotting with a HSP90 antibody. Ang-1 led to a gradual increase in HSP90 binding to eNOS that was apparent within 5 min, compared with untreated controls, was significantly elevated by 15 min, peaked at 30 min, and then gradually decreased (Fig. 2). Ang-1 had little effect on total intracellular HSP90 levels (Fig. 2). We next examined whether pharmacological blockade of HSP90 suppressed Ang-1-induced eNOS phosphorylation. Cells were pretreated with GA (1 μg/ml) for 30 min and were exposed to Ang-1 for 60 min. GA completely abolished Ang-1-induced eNOS phosphorylation to a level similar to controls (Fig. 3, A and B). Pretreatment with GA had no effect on eNOS protein expression. Pretreatment with GA also significantly suppressed Ang-1-stimulated NOX release (Fig. 3C).

Ang-1 Activates PI3K-Akt Pathway and Stimulates eNOS Phosphorylation

Exposure to Ang-1 for periods up to 120 min led to a dramatic increase in phosphorylation of Akt at Ser473, which was observed at 5 min, peaked at 30 min, and then declined toward baseline (Fig. 4). Pretreatment with the PI3K inhibitor Wort (500 nM) completely suppressed Ang-1-stimulated eNOS phosphorylation (Fig. 3, A and B), and this was accompanied by significant inhibition of Ang-1-stimulated NO production (Fig. 3C).

HSP90, PI3K/Akt, NOX, and Ang-1-Stimulated Endothelial Cell Migration and Capillary Tubule Formation

To investigate the effects of HSP90, PI3K-Akt, and NOX on Ang-1-induced angiogenesis in vitro, we first examined...
endothelial cell migration by using a modification of the Boyden chamber assay. Stimulation of PCAEC with Ang-1 for 4 h caused a 2.7-fold increase in endothelial cell migration compared with untreated controls (Fig. 5). Cells treated with Ang-1 + Wort (500 nM), Ang-1 + GA (1 μg/ml), or Ang-1 + L-NAME (2.5 mM) resulted in significant suppression of Ang-1-stimulated endothelial cell migration (Fig. 5).

Next, we determined the effect of HSP90, PI3K-Akt, and NO on Ang-1-induced capillary tubule structure formation in three-dimensional collagen gels. Exposure to Ang-1 for 24 h resulted in a 2.7- to 3-fold increase in both the number and length of capillary tubule formation in the collagen gel. Pretreatment with either Wort (500 nM) or GA (1 μg/ml) completely abolished Ang-1-induced tubule formation in three-dimensional collagen gel. Pretreatment with L-NAME (2.5 mM) also led to a significant decrease in Ang-1-stimulated capillary tubule formation (Fig. 6).

Ang-1 Stimulates Sprouting of Capillary-like Structures

To further test the angiogenic activity of Ang-1, we examined spheroid formation and sprouting by using the multicellular three-dimensional spheroid technique (Fig. 7, top). Untreated collagen gel-embedded PCAEC spheroids had a low level of spontaneous sprouting (Fig. 7A). Twenty-four-hour exposure to Ang-1 led to an approximately threefold increase in length of sprouts from the spheroids compared with controls (Fig. 7B). Exposure to Ang-1 for 48–72 h resulted in an organized network of sprouts and branches from the collagen-embedded spheroids (Fig. 7, C and D). Morphological analysis of cross sections through Ang-1-stimulated spheroid-embedded gels revealed a single layer of endothelial cells lining the lumens of capillary-like structures (Fig. 7, bottom).

Fig. 2. A: representative time course for Ang-1-stimulated heat shock protein (HSP) 90 binding to eNOS. PCAECs were exposed to Ang-1 for the indicated times; cell lysates were immunoprecipitated (IP) with eNOS antibody and immunoblotted (IB) with HSP90 antibody. Western blot analyses revealed that Ang-1 stimulates HSP90 binding to eNOS, reaching a peak at 30 min, and then declining toward baseline. B: densitometric data from Western blot analyses demonstrating that exposure of PCAECs to Ang-1 for periods up to 120 min caused a gradual increase in HSP90 binding to eNOS (●). Total intracellular HSP90 levels showed little change over the 120 min of the study (▲) (n = 3; data are means ± SD; *P < 0.05 compared with controls (time 0)).

Fig. 3. A: effect of phosphatidylinositol 3 (PI3) kinase and HSP90 inhibitors on Ang-1-stimulated eNOS phosphorylation. Pretreatment with wortmannin (Wort; 500 nM) or geldanamycin (GA; 1 μg/ml) for 30 min was followed by exposure to Ang-1 (250 ng/ml) for 60 min. Cell lysates were immunoblotted with phospho-eNOS (p-eNOS) antibody. Western blot analysis demonstrated that Wort and GA significantly suppressed Ang-1-stimulated eNOS phosphorylation. B: densitometric data from Western blots revealed that pretreatment with Wort and GA significantly suppressed Ang-1-induced eNOS phosphorylation (data are means ± SD; n = 3; *P < 0.05 compared with Ang-1 treatment). C: effect of PI3 kinase and HSP90 inhibitors on Ang-1-stimulated NO production. Pretreatment of cells with Wort (500 nM) or GA (1 μg/ml) for 30 min was followed by Ang-1 for 60 min. Pretreatment with Wort and GA significantly suppressed Ang-1-induced NO, release (data are means ± SD; n = 4; *P < 0.05 compared with Ang-1 treatment).
stimulation with angiogenic factors such as VEGF, shear stress, and, more recently, Ang-1 (2, 12, 15, 31). Phosphorylation of eNOS correlates directly with electron flow through the eNOS enzyme, increases in eNOS activity, and NO production by endothelial cells (30). Our study demonstrates that Ang-1 stimulates both phosphorylation of eNOS at Ser1177 and NO release from PCAEC and that these changes can be suppressed by pretreatment with a specific PI3K inhibitor. These data demonstrate and confirm the notion that eNOS phosphorylation at Ser1177 mediates Ang-1-induced NO release.

Ang-1 is a unique growth factor that induces Tie-2 receptor phosphorylation and interacts with other signal transduction molecules. Recent studies show that Ang-1 induces diverse angiogenic activities and that activation of PI3K-Akt pathway is necessary for Ang-1-induced endothelial cell migration, cell survival, and tube formation (2, 14, 24, 29, 34). Consistent with these findings, our data reveal that Ang-1 regulates endothelial cell migration and capillary formation via a PI3K-Akt-dependent mechanism in PCAEC. Furthermore, this is the first demonstration that Ang-1 stimulates sprouting of capillary-like structures with patent endothelial-lined lumens from PCAEC spheroids with formation of complex three-dimensional networks and branches. These data indicate that Ang-1 plays an essential role in the angiogenesis and formation of mature, intact coronary capillaries.

The molecular mechanisms by which the PI3K-Akt pathway initiates angiogenesis remain largely unknown. Because the PI3K-Akt pathway is only transiently increased by Ang-1, it is likely that the downstream mediators of this pathway play a critical role in angiogenesis. So far, relatively little is known about the downstream target molecules of Akt in angiogenesis, although several candidate molecules have been identified, including Bad, procaspase-9, survivin, and eNOS (2, 6, 9, 12, 15, 24, 31, 34). It is possible that combinations of these and other unidentified Akt target molecules cooperatively and synergistically modulate vascular angiogenesis. Further studies are needed to dissect the PI3K-Akt pathway and elucidate its downstream mediators.

**DISCUSSION**

The present study in PCAEC demonstrates that Ang-1 stimulates eNOS phosphorylation at Ser1177 and is significantly elevated as early as 15 min of exposure, peaks at 60 min, and remains elevated for periods up to 120 min. This is accompanied by a time-dependent increase in NO release. In addition, our study demonstrates that Ang-1 stimulates HSP90 binding to eNOS without affecting total cellular HSP90. Furthermore, inhibition of HSP90 significantly suppresses Ang-1-stimulated eNOS phosphorylation and NO release. Ang-1 also results in a time-dependent increase in Akt phosphorylation and inhibition of PI3K significantly suppresses Ang-1-stimulated eNOS phosphorylation and NO release. Furthermore, our data demonstrate that either inhibition of PI3K-Akt pathway or disruption HSP90 binding to eNOS inhibits Ang-1-induced endothelial migration, capillary sprouting, and tube structure formation. Use of the NOS inhibitor L-NAME also attenuates Ang-1-induced angiogenesis.

Phosphorylation of eNOS at Ser1177 is widely considered to be an important mechanism for increased NO production after stimulation with angiogenic factors such as VEGF, shear stress, and, more recently, Ang-1 (2, 12, 15, 31). Phosphorylation of eNOS correlates directly with electron flow through the eNOS enzyme, increases in eNOS activity, and NO production by endothelial cells (30). Our study demonstrates that Ang-1 stimulates both phosphorylation of eNOS at Ser1177 and NO release from PCAEC and that these changes can be suppressed by pretreatment with a specific PI3K inhibitor. These data demonstrate and confirm the notion that eNOS phosphorylation at Ser1177 mediates Ang-1-induced NO release.

Fig. 4. A: representative time course of Ang-1-stimulated Akt phosphorylation. Cells were exposed to Ang-1 for periods up to 120 min; the cell lysates were immunoblotted with an antibody directed against phosphorylated Akt (p-Akt) at Ser473. Ang-1 led to an increase in phosphorylation of Akt by 5 min and peaked at 30 min. Total Akt level was unaltered during the 120-min study. B: densitometric data from Western blot analyses demonstrated that Ang-1 significantly suppresses Ang-1-stimulated eNOS phosphorylation at Ser1177 and is significantly elevated as early as 15 min of exposure, peaks at 60 min, and remains elevated for periods up to 120 min. This is accompanied by a time-dependent increase in NO release. In addition, our study demonstrates that Ang-1 stimulates HSP90 binding to eNOS without affecting total cellular HSP90. Furthermore, inhibition of HSP90 significantly suppresses Ang-1-stimulated eNOS phosphorylation and NO release. Ang-1 also results in a time-dependent increase in Akt phosphorylation and inhibition of PI3K significantly suppresses Ang-1-stimulated eNOS phosphorylation and NO release. Furthermore, our data demonstrate that either inhibition of PI3K-Akt pathway or disruption HSP90 binding to eNOS inhibits Ang-1-induced endothelial migration, capillary sprouting, and tube structure formation. Use of the NOS inhibitor L-NAME also attenuates Ang-1-induced angiogenesis.

Phosphorylation of eNOS at Ser1177 is widely considered to be an important mechanism for increased NO production after

Fig. 5. Effect of PI3 kinase, HSP90, and NOS inhibitors on Ang-1-stimulated endothelial cell migration. Exposure of cells to Ang-1 (250 ng/ml) resulted in a 2.7-fold increase in endothelial cell migration as assessed by the Boyden chamber assay. Treatment with Wort (500 nM), GA (1 μg/ml), or the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 2.5 mM) showed significantly suppressed Ang-1-induced endothelial migration (data are means ± SD; n = 3; *P < 0.05 compared with Ang-1 treatment).
NO is known to mediate many physiological and pathological functions and is considered to be an important mediator of angiogenesis. For instance, stimulation of human umbilical vein endothelial cells with bFGF or VEGF increased NO production and induced formation of capillary-like structures in three-dimensional fibrin gels; these effects were inhibited by pretreatment with the NOS inhibitor l-NAME (1, 35, 48). Furthermore, l-NAME has been demonstrated to inhibit VEGF-induced angiogenesis in the rabbit cornea model of angiogenesis (47), and the recent study of Rikitake et al. (37) demonstrated that sphingosine-1-phosphate induced angiogenesis via a NO-dependent mechanism. These data highlight the importance of NO in modulating angiogenesis. Our data are in line with those findings and further demonstrate that Ang-1-induced NO production is a key event in the angiogenesis in PCAEC.

In the last few years, the PI3K-Akt signal transduction pathway has emerged as one of the main signaling routes that coordinate complex events leading to changes in cell metabolism, cell growth, cell movement, and cell survival. Akt also has been reported to regulate initiation of angiogenesis through its stimulatory effects on endothelial cell migration and actin reorganization, proliferation, and survival (12, 17, 22, 27, 32, 38, 40, 41, 46). Recent studies (11, 15, 31) have shown that stimulation with VEGF leads to transient activation of PI3K and phosphorylation of Akt, thereby regulating eNOS gene expression and NO production. The present studies are in agreement with those showing that the PI3K-Akt pathway regulates Ang-1-stimulated eNOS phosphorylation and NO production in PCAEC.

Although many studies have defined the angiogenic properties of Ang-1, the role that PI3-Akt-NO signaling axis plays on
Fig. 7. Top: representative images of the effect of Ang-1 on the sprouting of capillary-like structures from collagen-embedded PCAEC spheroids over time. A: 24-h control; B: Ang-1 (250 ng/ml) 24 h; C: Ang-1 (250 ng/ml) 48 h; D: Ang-1 (250 ng/ml) 72 h. Untreated PCAEC spheroids had a low level of spontaneous sprouting. Twenty-four-hour exposure to Ang-1 led to growth of sprouts from the spheroids. By 48–72 h, exposure to Ang-1 resulted in a complex network of tubules sprouting from the spheroids. Bottom: light micrograph showing Ang-1-stimulated capillary-like structures with endothelial cells lining the lumen that originated from PCAEC spheroids. Line = 200 μm.

Fig. 8. A: representative images of the effect of PI3K, HSP90, and NOS inhibitors on Ang-1-stimulated sprouting of capillary-like structures from PCAEC spheroids at 24 h. Exposure to Ang-1 resulted in a striking increase in spheroids sprouting; treatment with Wort, GA, and L-NAME suppressed Ang-1-stimulated sprouting of PCAEC spheroids. B: quantitative analysis of the sprout from PCAEC spheroids. Treatment with Wort (500 nM) or GA (1 μg/ml) completely suppressed Ang-1-stimulated spheroid sprouting. Treatment with L-NAME (2.5 mM) caused a significant decrease in Ang-1-stimulated capillary sprouting (data are means ± SD; n = 4; *P < 0.05 compared with Ang-1 treatment).
Ang-1-induced angiogenesis in PCAEC has been little studied. However, recently Babaei and colleagues (2) demonstrated that Ang-1-induced NO production and capillary formation could be inhibited in human umbilical vein endothelial cells by inhibitors of NOS and PI3K and that Ang-1-induced neovascularization was reduced in eNOS knockout mice. Our study in PCAEC reveals that inhibition of the PI3K-Akt-eNOS-NO signaling pathway by Wort significantly suppresses Ang-1-induced endothelial cell migration and tubule structure formation and completely blocks Ang-1-induced sprouting of complex capillary-like structures from PEAEC spheroids. These findings confirm that the PI3K-Akt-eNOS-NO pathway plays an important role in capillary formation and branching.

HSP90 has long been known to affect the activity and function of other proteins by acting as a molecular chaperone. Recent in vitro evidence shows that overexpression of HSP90 enhanced eNOS phosphorylation and NO production; an increase in HSP90 binding to eNOS was central to these effects (4, 16, 18). We have recently reported a similar mechanism in response to hypoxia, which showed an increase in eNOS activity, NO release, and eNOS-associated HSP90 (8). To investigate the HSP90 and eNOS interaction, a well-recognized and specific inhibitor of HSP90, the ansamycin antibiotic GA, was used in our present study. GA binds to the ATP binding site of the chaperone HSP90 to block the assembly of HSP90 heterocomplexes and inhibit its folding, as well as ATPase activity, leading to cell-cycle disruption (36, 39). Recently, GA also has been reported to prevent HSP90 and Akt binding to eNOS, and block phosphorylation of eNOS at Ser1179 and inhibition of NO production (4, 16, 18). In the present study, we further demonstrate that exposure to Ang-1 leads to a time-dependent increase in HSP90 binding to eNOS, and treatment with GA significantly suppresses Ang-1-induced eNOS phosphorylation and NO production. To our knowledge, this is the first demonstration that an interaction of HSP90 with eNOS modulates Ang-1-induced upregulation of eNOS and increases NO production.

Recently, Brouet and colleagues (5) demonstrated that HSP90 plays a key role in NO-dependent angiogenesis. They found that inhibition of HSP90 function or overexpression of HSP90 regulated NO production and NO-induced angiogenesis. Inhibition of HSP90 function has also been reported to reduce VEGF gene expression and suppress hypoxia-induced angiogenesis (21). Consistent with those studies, our data demonstrate that pharmacological inhibition of HSP90 significantly suppressed Ang-1-induced endothelial cell migration and tubule structure formation and completely blocked Ang-1-induced sprouting of capillary-like structures from spheroids. Thus it would seem that an HSP90-eNOS interaction plays a key role in Ang-1-induced angiogenesis in PEAEC.

In summary, this study demonstrates that Ang-1 stimulates HSP90 binding to eNOS, activates the PI3K-Akt pathway, leads to eNOS phosphorylation at Ser1177, and increases NO production by PEAEC. Furthermore, we demonstrated that disruption of the HSP90-eNOS interaction and of the PI3K-Akt pathway blocks Ang-1-stimulated endothelial cell migration and formation of complex capillary networks. We conclude that both a HSP90-eNOS interaction and activation of the PI3K-Akt pathway play a critical role in the regulation of Ang-1-induced eNOS phosphorylation, NO production, and angiogenesis in PEAEC. Unraveling the complex molecular mechanisms that contribute to angiogenesis should provide the foundation for future therapies for the prevention and reversal of coronary artery disease.

GRANTS

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REFERENCES

19. Haspel HC, Scieli GM, McMahon G, and Scieli AG. Inhibition of vascular endothelial growth factor-associated tyrosine kinase activity with


