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Induction of Angiogenesis by Heat Shock Protein 90 Mediated by Protein Kinase Akt and Endothelial Nitric Oxide Synthase

Jianxin Sun and **James K. Liao**
Vascular Medicine Research Unit (J.S., J.K.L.), Brigham & Women's Hospital and Harvard Medical School, Boston, Mass; and the Department of Pharmacology (J.S.), Second Military Medical University, Shanghai, China

Abstract

Objective—A specific inhibitor of heat shock protein 90 (Hsp90), 17-AAG, has been shown to inhibit tumor growth through cell cycle arrest, differentiation, or apoptosis. Because angiogenesis is important for tumor growth, we hypothesize that inhibition of angiogenesis by 17-AAG may mediate some of its antitumor effects.

Methods and Results—Because protein kinase Akt and endothelial nitric oxide synthase (eNOS) are critical for angiogenesis, we studied the effects of 17-AAG on the phosphorylation and expression of Akt and eNOS in human umbilical vein endothelial cells. In a concentration- and time-dependent manner, inhibition of Hsp90 by 17-AAG decreased Akt and eNOS expression by 74% and 81%, respectively. Inhibition of eNOS expression by 17-AAG occurred at the transcriptional level as determined by eNOS promoter activity and nuclear run-on assay. Furthermore, treatment with 17-AAG decreased basal and vascular endothelial growth factor-stimulated Akt and eNOS phosphorylation. This corresponded with decreased NO production and inhibition of endothelial cell migration and angiogenesis. The anti-angiogenic effect of 17-AAG was partially reversed by the NO donor, SNAP.

Conclusions—These findings indicate that Hsp90 is important not only for Akt and eNOS phosphorylation but also for eNOS gene transcription and suggests that Hsp90 may be a novel target for anti-angiogenic therapy.

Keywords

angiogenesis; endothelium; nitric oxide; heat shock protein; protein kinase Akt

Endothelial cells play critical roles in angiogenesis, a physiological or pathological neovascularization process in response to tissue ischemia and tumor growth or metastasis.[1,2] Nitric oxide (NO) mediates diverse biological functions, which include regulation of endothelial cell growth,[3,4] apoptosis,[5] and migration.[6-8] Recent studies suggest that NO is essential for ischemia-induced angiogenesis.[9,10] NO is a physiological metabolite of L-arginine to citrulline conversion by the 3 NO synthases (NOS): neuronal NOS, inducible NOS, and endothelial NOS (eNOS).[11] eNOS produces NO constitutively at low levels but can be transiently stimulated to produce high levels of NO by hormones or environmental stimuli.[12,13] Several angiogenic factors, including vascular endothelial growth factor (VEGF), stimulate the release of endothelium-derived NO.[14-16] The release of NO by these factors is believed to mediate some of their angiogenic actions.

Correspondence to James K. Liao, Brigham & Women's Hospital, 65 Landsdowne Street, Room 275, Cambridge, MA 02139. E-mail jliao@rics.bwh.harvard.edu.

Hsp90 has been shown to associate with eNOS and Akt in vascular endothelial cells.[17-19] Direct interaction of Hsp90 with eNOS augments eNOS enzymatic activity, probably through an allosteric modulation of eNOS, resulting in enhanced affinity of calcium/calmodulin for the enzyme.[17,20] Furthermore, Hsp90 may mediate eNOS phosphorylation through regulating calmodulin binding to eNOS and Akt.[18,19,21]

Hsp90 is a ubiquitously expressed and abundant molecular chaperone that participates in the folding, assembly, maturation, and stabilization of diverse cellular proteins, particularly those involved in signal transduction, cell-cycle regulation, and survival decisions.[22] A number of signaling molecules and oncogenic proteins, including v-Src, Bcr-Abl, Raf-1, ErbB2, cyclin-dependent kinases, some growth factor receptors, steroid receptors, and numerous others, have been found to associate with Hsp90.[23-28] Additionally, pharmacological intervention of Hsp90 function by its specific inhibitors has been shown to target these client proteins for ubiquitination and protesomal degradation.[28,29] Because many Hsp90 client proteins are classified as oncogenic proteins, which promote cancer cell growth or survival or both, Hsp90 has emerged as one of the most exciting targets for cancer drug development.[30,31]

The ansamycin antibiotics, including geldanamycin and its closely related analogue, allyamino-17-demethoxygeldanamycin (17-AAG), bind to the ATP site of Hsp90 and disrupt its association with client proteins.[32,33] However, it is not known whether Hsp90 inhibitors can exert antitumor effects via inhibition of angiogenesis. The aim of the present study, therefore, is to investigate the effects of Hsp90 inhibitor 17-AAG on angiogenesis and to define the mechanisms involved.

Materials and Methods

All tissue culture reagents were purchased form Life Technologies Gibco BRL. 5,6-Dichlorobenzimidazole riboside and Triton X-100 were purchased form Sigma Chemical Corp. Calcein AM was purchased from Molecular Probes, Inc. [α^{32}P]-CTP (3000 Ci/mmol) was purchased from New England Nuclear Life Science Products. NO donors S-nitroso-N-acetylpenicillamine (SNAP) and VEGF were obtained from Calbiochem. 17-AAG and geldanamycin were generously provided by the Cancer Treatment and Evaluation Program, National Cancer Institute (Bethesda, Md).

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics and cultured in medium 199 (M-199; BioWhittaker) containing endothelial cell growth supplement, 10% fetal bovine serum, and antibiotics (100 U/mL penicillin, 10 μg/mL streptomycin, and 20 μg/mL neomycin). Bovine aortic endothelial cells were freshly prepared as previously described and used at passage 2. Cellular viability was determined by cell count, morphology, and trypan blue exclusion.

Western Blotting and Immunoprecipitation

Proteins were prepared and separated on SDS-PAGE as described.[34,35] Immunoblotting was performed using monoclonal antibodies to eNOS (1:1000 dilution; Transduction Laboratories, Lexington, Ky), to p85 antiserum (1:500 dilution; Upstate Biotech), to phospho-eNOS (Ser1177), phospho-Akt (Ser473), and Akt (1:1000 dilution; Cell Signaling Tech), to hsp90 (1:200 dilution; Santa Cruz Biotech), and to α -tubulin (DM1A) (1:5000 dilution; Sigma). Immunodetection was accomplished using a sheep anti-mouse secondary antibody (1:2000 dilution) or donkey anti-rabbit secondary antibody (1:2000 dilution) and the enhanced chemiluminescence (ECL) kit (Amersham Corp). Immunoprecipitation was performed essentially as previously described.[34]

Northern Blotting

Equal amounts of total RNA (15 μg) were separated by 1% formaldehyde-agarose gel electrophoresis, and hybridization and washing were performed as described.[36]

Nuclear Run-on Assay

Confluent endothelial cells (5×10^7 cells) grown in lipoprotein-deficient serum were treated with 17-AAG (200 nmol/L) or vehicle for 24 hours. Nuclei were isolated and in vitro transcription was performed as previously described.[37] Equal amounts (1 μg) of full-length human eNOS, β-actin, and pGEM-3z cDNA were vacuum-transferred onto nitrocellulose membranes with a slot blot apparatus (Schleicher & Schuell). The relative intensity of eNOS band was determined as the ratio of eNOS to β-actin intensity.

Assessment of Cell Apoptosis

Annexin V staining was used for assessment of apoptotic cells. Briefly, endothelial cells were washed once with 1× binding buffer and stained with EGFP-annexin V solution (1:40; Clontech) and propidium iodide (50 mg/mL; Sigma) for 15 minutes at room temperature in the dark. Cells were then washed with 1× binding buffer and annexin V-EGFP-positive cells were evaluated by fluorescence microscopy.

Determination of NO Production by Endothelial Cells

The NO production by endothelial cells was assessed by quantification of the nitrite content in the supernatant with a commercially available fluorometric kit (Cayman Chemicals).[38]

Transfection Assay

Bovine endothelial cells (70% confluent) were transfected with the use of SuperFect Transfection Reagent (QIAGEN Inc) with 6 μg of the indicated cDNA constructs: the empty vector (pcDNA3) or a [-1.8 kb] eNOS promoter linked to the luciferase reporter gene.[39] As a control for transfection efficiency, 2 μg of pCMV.β-gal plasmid was cotransfected. For luciferase activity, cells were harvested 48 hours after transfection and treated with the indicated conditions. The β-galactosidase and luciferase activities were determined by a chemiluminescence assay (Dual-light; Tropix) with the use of Berthold L9501 luminometer. The eNOS promoter activity was expressed as the ratio of luciferase to β-galactosidase activity.

Cell Migration Assay

Endothelial cell migration was estimated in a Transwell with 24 wells (Costar). HUVEC at passage 4 were grown to confluence in M-199. Inhibitors were added 2 hours before assays. Then, 2×10^4 cells in suspension were added to the upper chamber with or without the inhibitors indicated in the Figure legends. The bottom chamber was filled with 500 μL medium containing SNAP, VEGF, and inhibitors when indicated. The assembly was then incubated at 37°C to allow cell migration. After 4 hours, the cells were fixed and stained by Diff-Quik solutions (VWR Scientific). Cells that did not migrate through the membrane were gently removed from the upper surface. Cell migration was scored in 4 representative fields (magnification, 400×), and each group was performed in triplicate.

Tube Formation Assay

The 96-well culture plates were coated with 105 μL of growth factor-reduced Matrigel per well and then allowed to polymerize for 30 minutes at 37°C. HUVECs cultured for 24 hours in M-199 with 1% fetal bovine serum were seeded on coated plates at a density of 2×10^4 cells per well in M-199 supplemented with 1% fetal bovine serum and the agents as indicated in the Figure legend and then incubated for 18 hours at 37°C. Cells were washed with Hank balanced

salt solution and stained with Calcein AM (8 μg/mL in Hank balanced salt solution). Pictures were taken at ×40 magnification with a digital output camera (Olympus DP11) attached to an inverted phase-contrast microscope (Olympus IX70); total tube length was measured by using the NIH Image program (National Institutes of Health, Bethesda, Md).

Statistical Analyses

All data were analyzed by means of 1- or 2-way ANOVA and Fisher exact test for post hoc analyses. A value of $P<0.05$ was considered statistically significant.

Results

Effects of Hsp90 Inhibition on eNOS Protein Expression

HUVEC were treated with various concentrations of the selective hsp90 inhibitor 17-AAG for 24 hours. In a concentration-dependent manner, 17-AAG inhibited eNOS protein expression with an IC_{50} of 90 nmol/L (Figure 1A). At 400 nmol/L, 17-AAG inhibited eNOS protein expression by 81%. Significant effects of 17-AAG on eNOS expression were also observed at concentrations as low as 50 nmol/L. However, 17-AAG, at concentrations of 20 nmol/L and 10 nmol/L did not affect eNOS protein expression. A similar effect was observed with another structurally related but less selective Hsp90 inhibitor, geldanamycin (GM) (data not shown). In addition, Hsp90 inhibitor 17-AAG inhibited eNOS protein expression in a time-dependent manner. At concentration of 400 nmol/L, 17-AAG inhibited eNOS protein expression by 51% after 6 hours of treatment, and this inhibitory effect was sustained for at least 24 hours (Figure 1B). In contrast to its effects on eNOS, the cytoskeleton protein α-tubulin was unchanged and the cyclin-dependent kinase inhibitor, $p27^{Kip1}$, was increased by 17-AAG treatment, suggesting cell-cycle arrest (data not shown). To determine whether 17-AAG affects Hsp90 binding to eNOS, HUVECs were treated with 400 nmol/L of 17-AAG for 30 minutes and coimmunoprecipitation was performed with eNOS specific antibody and immunoblotted with hsp90 antibody. Treatment with 17-AAG did not affect hsp90 binding to eNOS (Figure 1C), suggesting that the inhibitory effect on eNOS expression is mostly likely caused by direct inhibition of hsp90 activity by 17-AAG.

Effects of 17-AAG on eNOS mRNA Expression and Stability

To determine whether changes in eNOS protein levels correlated with changes in eNOS steady-state mRNA levels, we performed Northern blotting on endothelial cells exposed to vehicle and 17-AAG for 24 hours. Treatment with 17-AAG concentration-dependently attenuated eNOS mRNA levels, with maximal effects observed at 400 nmol/L (inhibition by 78±7%, n=4, $P<0.01$) (Figure 2A). To further dissect the mechanism underlying inhibitory effects of 17-AAG on eNOS mRNA, we first studied the effects of 17-AAG on eNOS gene promoter activity. bovine aortic endothelial cells were transfected with a functional eNOS promoter linked to the luciferase gene.[39] Treatment with 17-AAG for 24 hours significantly inhibited eNOS promoter activity in a concentration-dependent manner (Figure 2B). At concentrations of 100 nmol/L, 200 nmol/L, and 400 nmol/L, the eNOS promoter activities were inhibited by 52%, 61%, and 72%, respectively ($P<0.01$ for all values, n=6).

To further confirm the effects of 17-AAG on eNOS gene transcription by a different method, we performed nuclear run-on assays using endothelial cells treated with 17-AAG (400 nmol/L) for 24 hours. Preliminary studies using different amounts of radiolabeled RNA transcripts demonstrate that under our experimental conditions, hybridization was linear and nonsaturable. The density of each eNOS band was standardized to the density of its corresponding α-actin. The specificity of each band was determined by the lack or weak of hybridization to the nonspecific pGEM cDNA vector. Treatment with 17-AAG (400 nmol/L) significantly attenuated eNOS gene transcription compared with that of untreated cells ($P<0.01$, n=3)

(Figure 2C). To determine whether the decrease in eNOS expression was also caused by posttranscriptional mechanisms, eNOS mRNA stability was examined in endothelial cells treated with 17-AAG for 24 hours in the presence of the mRNA synthase inhibitor 5,6-dichlorobenzimidazole riboside. The eNOS mRNA half-life was unchanged by 17-AAG (400 nmol/L) (data not shown). Taken together, theses results suggest that 17-AAG decreases eNOS mRNA expression through suppressing eNOS gene transcription.

Effects of Hsp90 Inhibition on Akt Protein Expression and Phosphorylation

Akt was shown recently to associate with the Hsp90 and inhibition of Hsp90 function caused Akt degradation and rapid inactivation of Akt.[18] Whether Hsp90 inhibitor 17-AAG affects Akt signaling particularly in HUVECs is undefined. To this end, Western blot analysis was performed with cell lysates obtained from HUVECs treated with various concentrations of 17-AAG for 24 hours. In a concentration-dependent manner, 17-AAG significantly attenuated Akt protein expression (Figure 3A). At 400 nmol/L, 17-AAG inhibited total Akt protein expression by 74±7%. In contrast, PI3-kinase subunit p85 was barely altered and inhibition of Akt protein expression by 17-AAG was observed at 6 hours of treatment and sustained for at least 24 hours. Likewise, the phosphorylation of Akt was also inhibited by the treatment with 17-AAG in a concentration-dependent manner. Interestingly, treatment with 17-AAG (400 nmol/L) for 3 hours significantly attenuated phosphorylation of Akt, whereas the total Akt protein expression was barely changed (Figure 3B), suggesting that inhibition of Hsp90 function could produce rapid dephosphorylation of Akt in HUVECs.

To further verify this conclusion, the effect of 17-AAG on VEGF-stimulated Akt phosphorylation was also investigated. HUVECs were pretreated with 17-AAG for 2 hours and VEGF-induced Akt phosphorylation at 10 minutes was probed by Western blot analysis. As expected, VEGF (50 ng/mL) treatment caused a significant increase in Akt phosphorylation, which was also markedly inhibited in the presence of 17-AAG (400 nmol/L). In contrast, Akt protein levels were unchanged by treatment of the cells with either VEGF or 17-AAG or both.

Effects of 17-AAG on Phosphorylation of eNOS and NO Release

To investigate whether 17-AAG inhibition of eNOS protein expression could cause a decrease in NO release, NO release was assessed by measuring its final stable equimolar degradation products, nitrite and nitrate. Expectedly, basal NO production at 24 hours was markedly attenuated by 17-AAG (Figure 4A). In addition to inhibiting basal NO production, 17-AAG (400 nmol/L) also inhibited VEGF-stimulated NO production ($P<0.01$) (Figure 4A). Previous studies have revealed that VEGF-stimulated activation of PI3-kinase/Akt/eNOS pathway plays an essential role in the regulation of eNOS activity. Therefore, measuring effects of 17-AAG on Akt/eNOS pathway will help us dissect the mechanism underlying 17-AAG-mediated inhibition of VEGF-stimulated NO production. Phosphorylation of Akt, determined by Western blot analysis, was significantly increased by VEGF treatment for 10 minutes, which, however, was markedly attenuated by 17-AAG pretreatment of HUVECs for 2 hours ($P<0.01$, compared with VEGF) (Figure 4B). Likewise, VEGF-stimulated phosphorylation of eNOS was also substantially attenuated by 17-AAG ($P<0.01$) (Figure 4C). Together, these results suggest that in addition to inhibiting eNOS protein expression, 17-AAG may also attenuate NO production through dephosphorylation of Akt and eNOS.

Effects of 17-AAG on Cell Viability and Apoptosis

Cell viablity, assessed by trypan blue exclusion, was similar in 17-AAG-treated cells versus vehicle-treated cells for 24 hours (data not shown). 17-AAG treatment (200 nmol/L) for 24 hours, however, result in greater proportion of apoptotic cells, determined by EGFP-Annexin V/PI doubling staining (Figure 5). This effect was partially reversed by NO donor SNAP (10 μmol/L), suggesting that NO, at least in part, involves in 17-AAG-induced apoptosis.

Effects of 17-AAG on In Vitro Angiogenesis

Because NO plays a central role in the process of angiogenesis and because 17-AAG decreased NO production, it prompted us to investigate whether 17-AAG could inhibit NO-mediated downstream events, such as angiogenesis under both basal and VEGF-stimulated conditions. Angiogenesis was evaluated by EC migration and capillary-like tube formation assays. Treatment with 17-AAG (400 nmol/L) inhibited EC migration under both basal and VEGF-stimulated condition ($P<0.01$) (Figure 6A). This effect appeared to be NO-dependent because it was reversed by cotreatment with the NO donor, SNAP. Likewise, 17-AAG markedly attenuated both basal and VEGF-stimulated tube formation ($P<0.01$) (Figure 6B). This inhibitory effect of 17-AAG was partially reversed by NO donor SNAP. Taken together, these data suggest that 17-AAG inhibited in vitro angiogenesis in an NO-dependent manner.

Discussion

This study demonstrates that pharmacological inhibition of Hsp90 function attenuates the expression and activity of eNOS leading to inhibition of endothelial cells migration and in vitro capillary-like tube formation. The reduction in eNOS expression was achieved by 2 structurally related Hsp90 inhibitors, including geldanamycin and its analogue, Allyamino-17-demethoxygeldanamycin (17-AAG). The antitumor effects of Hsp90 inhibitor 17-AAG are thought to closely associated with its direct effects on tumor cells, including inhibiting proliferation and inducing apoptosis. However, our present study suggests an additional mechanism underlying 17-AAG antitumor effects, ie, inhibition of angiogenesis.

Endothelial cells play critical roles in angiogenesis, a physiological or pathological neovascularization process in response to tissue ischemia and tumor growth or metastasis.[2] NO generated by the eNOS has been shown to regulate endothelial cell growth, apoptosis, migration, and is essential for angiogenesis.[40] In vascular endothelial cells, Hsp90 has been reported to associate with eNOS and participate in the regulation of eNOS activity.[17] For example, acute treatment of endothelial cells with relatively high concentrations of geldamycin (17.8 μmol/L, 15 minutes) caused inhibition of eNOS activity, probably through an allosteric mechanism.[17] Furthermore, geldanamycin (17.8 μmol/L) treatment has been shown to increase eNOS-dependent superoxide anionproduction by specifically inhibiting Hsp90 association with eNOS, thereby shifting NO and superoxide anion balance at a fundamental level in bovine coronary endothelial cells.[41] In contrast, treatment of HUVEC with lower-concentration 17-AAG (400 nmol/L, 30 minutes) did not alter the association of eNOS with Hsp90, suggesting that the effect is caused by inhibition of Hsp90 activity rather than its ability to associate with eNOS. The expression of eNOS, however, was markedly inhibited by treatment with 17-AAG. This effect was evident after 6 hours treatment, with concentrations of 17-AAG as low as 50 nmol/L.

To determine the mechanism of eNOS downregulation, we examined the effects of 17-AAG on eNOS gene transcription and eNOS mRNA stability. We show that 17-AAG caused a marked downregulation of eNOS mRNA via an effect of 17-AAG to attenuate eNOS gene transcription, as determined by both eNOS promoter activity analysis and nuclear run-on assays. The eNOS promoter used in this study contains putative *cis*-acting elements for activator protein-1 and activator protein-2, sterol regulatory element-1, retinoblastoma control element, shear stress response element, nuclear factor-1, and cAMP response element.[39] Recently, Hsp90 inhibitor geldanamycin has been demonstrated to inhibit activator protein-1-mediated signaling through attenuating basal and hypoxia induced c-jun expression in HT29 human colon adenocarcinoma cells.[42] Of particular importance, a recent study reported that Hsp90 inhibition regulates HIF-1-dependent transcriptional activity,[43] which could also have implications for the regulation of eNOS expression. However, the precise mechanism by which

Hsp90 inhibitor 17-AAG inhibits eNOS gene transcription in vascular endothelial cells is still undergoing investigation.

In addition to the specific regulation of eNOS expression, our data show that 17-AAG markedly inhibits Akt protein expression in HUVEC, which is in line with previous notion observed in some cancer cells,[44-46] whereas the PI3-kinase subunit p85 expression is unaltered. 17-AAG also markedly inhibits basal and VEGF induced phosphorylation of Akt in HUVEC. PI3 kinase/Akt pathway plays an essential role in the regulation of eNOS activity. Stimuli such as VEGF, insulin, estrogen, or shear stress induce phosphorylation at serine 1177 (for human)/1179 (for bovine) through PI3-kinase-dependent activation of Akt, with an ensuing increase in enzyme activity that can be mimicked with recombinant Akt in vitro or overexpressed constitutively active Akt in intact cells.[13,47,48] It has been established that VEGF-induced angiogenesis is NO-dependent, because VEGF-induced angiogenesis is defective in $eNOS^{-/-}$ mice.[49] In this study, our data revealed that 17-AAG substantially inhibited VEGF-induced eNOS phosphorylation and NO production. Accordingly, VEGF-induced NO-dependent angiogenesis, including endothelial cells migration and tube formation, are also attenuated.

In summary, Hsp90 inhibitor 17-AAG may inhibit NO production and NO-dependent angiogenesis via 2 distinct pathways, ie, inhibition of eNOS gene transcription and Akt-mediated phosphorylation of eNOS. Therefore, Hsp90 may represent another important target for anti-angiogenic therapy.

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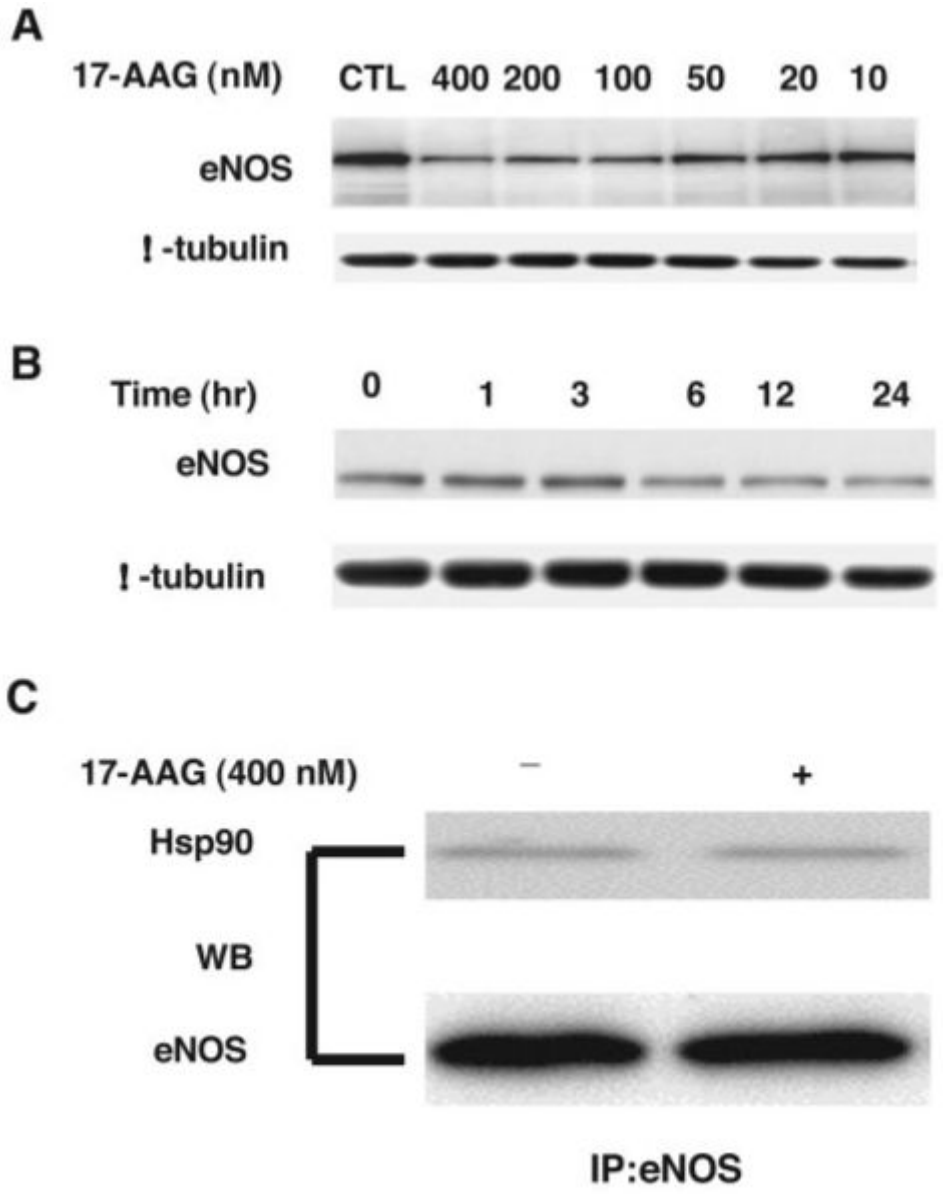

Figure 1.

A, Western blot (15 μg of protein per lane) showing concentration-dependent effects of Allyamino-17-demethoxygeldanamycin (17-AAG, 20 to 400 nmol/L) on eNOS protein expression in human umbilical vein endothelial cells (HUVECs) at 24 hours. *P<0.01 versus control condition. B, Western blot analysis showing the time-dependent effect of 17-AAG (400 nmol/L) on eNOS protein expression at 24 hours. C, Coimmunoprecipitation showing the effect of 17-AAG (400 nmol/L) on Hsp90 association with eNOS.

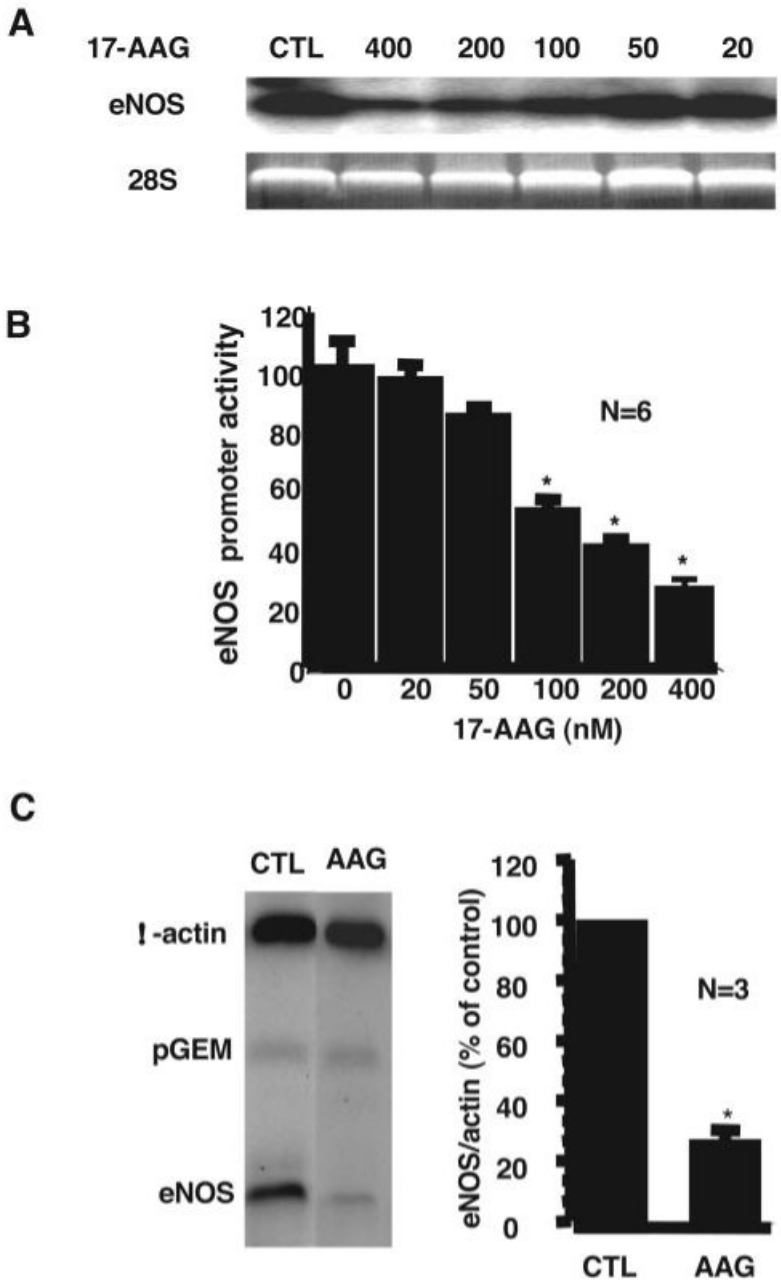

Figure 2.

A, Northern blot (15 μg of total RNA/lane) analysis showing concentration-dependent effect of 17-AAG on eNOS mRNA levels in human umbilical vein endothelial cells at 24 hours. B, Effect of 17-AAG (10 to 400 nmol/L) on eNOS gene transcription. Bovine aortic endothelial cells were transiently transfected with vector alone or a -1.8-kDa eNOS promoter construct. eNOS promoter activity was standardized to β-galactosidase activity and expressed as fold induction. *P<0.01 versus untreated condition. C, Nuclear run-on assay showing the effect of 17-AAG (400 nmol/L) on eNOS gene transcription at 24 hours. The β-actin gene transcription and lack of pGEM band served as internal controls for standardization and nonspecific binding. Blots shown are representative of 4 separate experiments. *P<0.01 versus untreated condition.

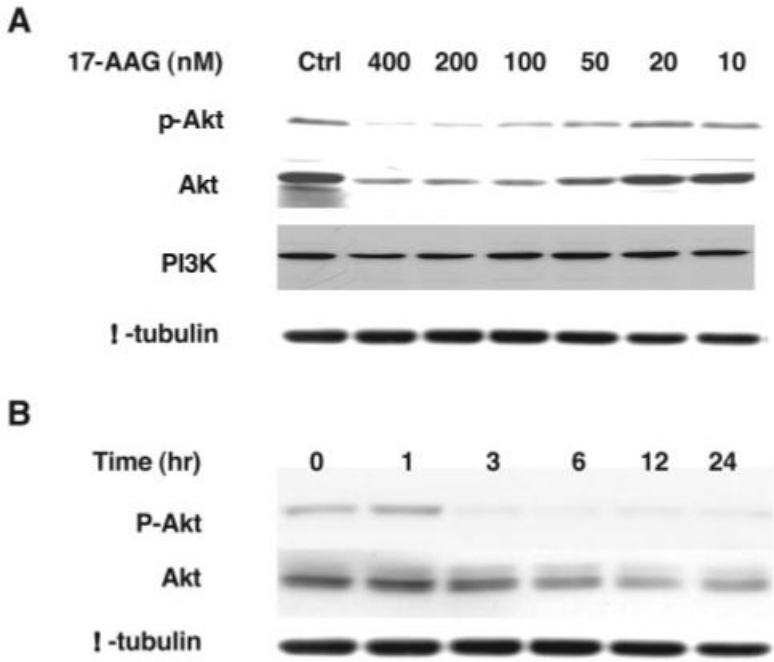

Figure 3.
Western blots (30 μg of protein/lane) analysis showing concentration-dependent (A) and time-dependent (B) effects of 17-AAG (400 nmol/L) on Akt, phospho-Akt, and PI3-kinase p85 subunit levels.

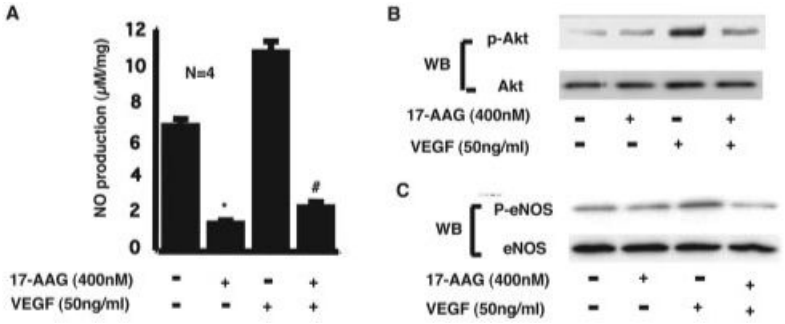

Figure 4.

A, Effects of 17-AAG on basal and VEGF (50 ng/mL)-stimulated NO production as assessed by measuring total nitrite and nitrate in human umbilical vein endothelial cells at 24 hours. B, Western blot (30 μg of total protein/lane) showing effects of 17-AAG (400 nmol/L) on total Akt and VEGF (50 ng/mL) induced phospho-Akt levels at 10 minutes in human umbilical vein endothelial cells. C, Western blot (30 μg of total protein/lane) showing effects of 17-AAG (400 nmol/L) on total eNOS and VEGF (50 ng/mL)-induced phospho-eNOS levels at 10 minutes in human umbilical vein endothelial cells.

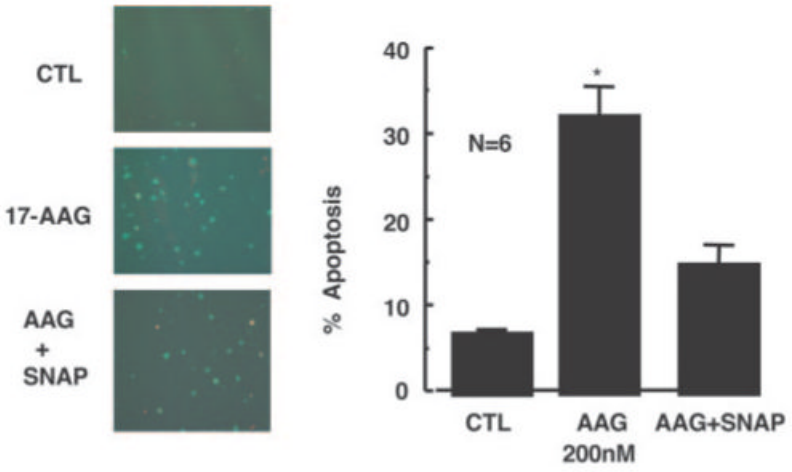

Figure 5.

Effect of 17-AAG on cell apoptosis as measured by annexin V conjugated with EGFP staining in human umbilical vein endothelial cells at 24 hours. *P<0.01 versus control and 17-AAG +SNAP.

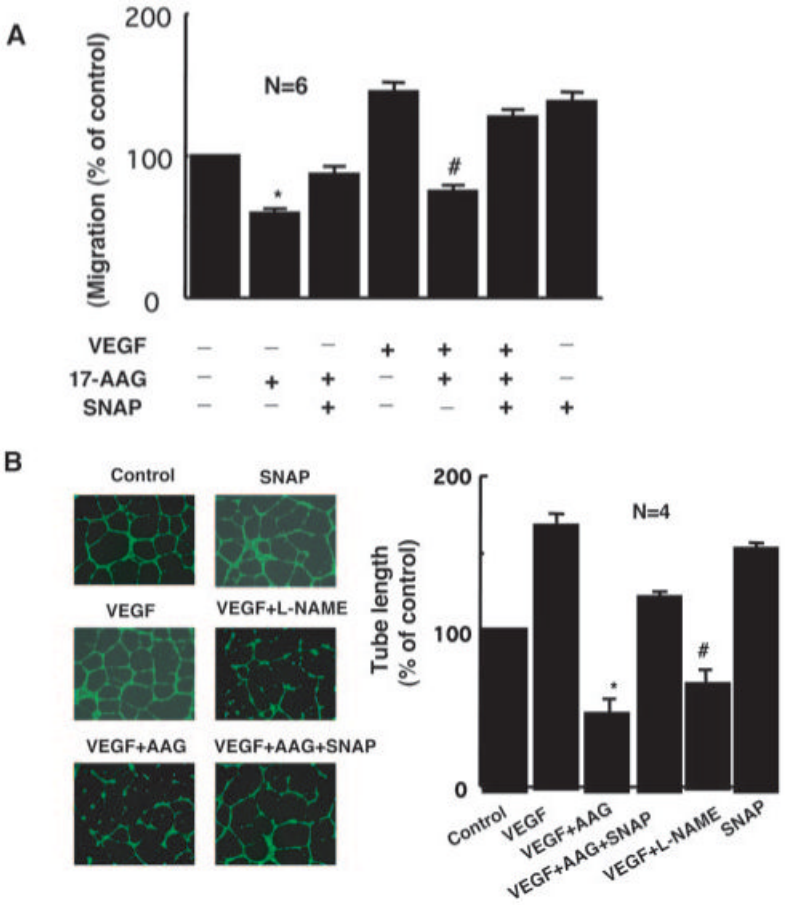

Figure 6.

A, Effect of 17-AAG on basal and VEGF (50 ng/mL)-stimulated endothelial cell migration. Cell migration was measured in unstimulated and VEGF-stimulated human umbilical vein endothelial cell in the presence or absence of 17-AAG (400 nmol/L), SNAP (10 μmol/L), or both. *P<0.01 versus control and 17-AAG + SNAP. #P<0.01 versus VEGF alone or VEGF +SNAP. B, Effect of 17-AAG on angiogenesis as assessed by tube formation in vitro. Human umbilical vein endothelial cells (n=8 per group) were treated with indicated reagents and capillary-like tube-formation was assessed (see Materials and Methods for details). Representative fields for Matrigel-based capillary-like tube formation at ×40 magnification are shown in the left panel. The addition of 17-AAG (400 nmol/L) inhibited VEGF (50 ng/mL) stimulated tube-length, which was partially reversed by addition of NO donor SNAP (10 μmol/L). An inhibitor of NOS, L-NAME (1 mmol/L) was used as a positive control. *P<0.01 versus VEGF alone and VEGF+AAG+SNAP. #P<0.01 versus VEGF alone.