

# Inhibition of Vascular Endothelial Growth Factor-Induced Angiogenesis by Resveratrol through Interruption of Src-Dependent Vascular Endothelial Cadherin Tyrosine Phosphorylation

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## ABSTRACT

Resveratrol, a polyphenolic compound found in grapes and other fruits, has been reported to inhibit angiogenesis with an as yet elusive mechanism. Here, we investigate the detailed mechanism by which resveratrol inhibits vascular endothelial growth factor (VEGF)-induced angiogenic effects in human umbilical endothelial cells (HUVECs). Exposure of HUVECs to 1 to 2.5  $\mu$ M resveratrol significantly blocked VEGF-mediated migration and tube formation but not cell proliferation. Under the same concentrations, resveratrol failed to affect VEGF-stimulated activation of VEGF receptor, extracellular signal-regulated protein kinase 1/2, p38 mitogen-activated protein kinase, and Akt. Of interest, resveratrol, at the dose of 1 or 2.5  $\mu$ M, effectively abrogated VEGF-mediated tyrosine phosphorylation of vascular endothelial (VE)-cadherin and its complex partner,  $\beta$ -catenin. This inhibitory effect of resveratrol reflected on the retention of VE-cadherin at cell-cell contacts as demonstrated by immunofluorescence. Src kinase assay showed that VEGF-induced endogenous Src kinase activation was strongly inhibited

by 1 and 2.5  $\mu$ M resveratrol. Supportively, inhibition of Src activity by overexpression of Csk resulted in attenuation of the tyrosine phosphorylation of VE-cadherin and endothelial cell (EC) tube formation. Again, transfection with v-Src, an active form of Src, could reverse resveratrol inhibition of VE-cadherin tyrosine phosphorylation and EC tube formation. Reactive oxygen species (ROS) has been shown to be involved in VE-cadherin phosphorylation and its related functions. Flow cytometric analysis showed that VEGF stimulated an evident increase of peroxide, which was strongly attenuated by resveratrol. In addition, antioxidant *N*-acetyl-cysteine was demonstrated to strongly inhibit VEGF-mediated Src activation, VE-cadherin tyrosine phosphorylation, and HUVEC tube formation. Together, our data suggest that resveratrol inhibition of VEGF-induced angiogenesis was mediated by disruption of ROS-dependent Src kinase activation and the subsequent VE-cadherin tyrosine phosphorylation.

Angiogenesis is a process by which new blood vessels are formed from pre-existing vessels. New blood vessel formation by angiogenesis involves the degradation of extracellular matrix combined with sprouting and migration of endothelial cells from pre-existing capillaries (Risau, 1997). One of the first events that may occur during this process is the loss of stable cell-cell contacts between endothelial cells in the parent vessel and the transition of a quiescent to a dynamic migration of endothelial cells (Ausprunk and Folkman, 1977). Vascular endothelial (VE)-cadherin is an endothelial

cell-specific adhesion molecule that is localized within specialized structures of cell-cell contacts (Gotsch et al., 1997; Corada et al., 1999). It can transfer intracellular information by interacting with the cytoskeleton via several anchoring molecules (e.g.,  $\beta$ -catenin) (Aberle et al., 1996). Deletion or cytosolic truncation of VE-cadherin impairs remodeling and maturation of the vascular network, and on the cellular level it abolishes transmission of intracellular signals via VEGFR-2 (Carmeliet et al., 1999). Instead of deleting the VE-cadherin gene, some investigators recently have successfully inhibited tumor angiogenesis by targeting this molecule with monoclonal antibodies (Corada et al., 2002; Liao et al., 2002). Tyrosine phosphorylation of the cadherin-catenin complex has been proposed as another mechanism that regulates

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**ABBREVIATIONS:** VE, vascular endothelial; VEGFR, vascular endothelial growth factor receptor; VEGF, vascular endothelial growth factor; FAK-GST, focal adhesion kinase-glutathione S-transferase; HUVEC, human umbilical vein endothelial cell; DCF-DA, dichlorodihydrofluorescein diacetate; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine.

the stability of cell-cell junctions (Esser et al., 1998). For example, angiogenic factor VEGF stimulated increase of tyrosine phosphorylation of VEGFR-2 and VE-cadherin has been correlated with endothelial cell migration as well as tubular formation (Nawroth et al., 2002). Therefore, modulation of the tyrosine phosphorylation status of VE-cadherin would be critical for regulating angiogenesis.

Resveratrol (3,5,4'-trihydroxystilbene), a natural phytoalexin present in plants and many other human foods, has been found to play a role in decreasing coronary atherosclerosis and cancer (Pace-Asciak et al., 1995). Many epidemiological studies correlate a low incidence of coronary heart disease and atherosclerosis with a preferential consumption of red wine in the French population, a phenomenon known as "French paradox" (Renaud and de Lorgeril, 1992; Soleas et al., 1997). Resveratrol, an ingredient of red wine, is thought to be the major contributor of such an effect. Resveratrol has also been reported to inhibit the development of preneoplastic lesions in carcinogen-induced mammary carcinogenesis and the promotion stage of mouse skin carcinogenesis (Jang et al., 1997). Some biochemical characteristics, such as anti-oxidation, anti-lipogenesis, and anti-inflammation, have been proposed as possible explanations for the above-mentioned preventive effects of resveratrol (Frankel et al., 1993; Fauconneau et al., 1997). Recently, resveratrol has been found to be effective in inhibiting angiogenesis in certain assays as well as reducing the accompanied fibrosarcoma growth in C57B16/J mice (Brakenhielm et al., 2002). This finding highlighted the relevance of resveratrol to be used as a therapeutic modality for treating angiogenesis-related diseases. Mitogen-activated protein kinase has been proposed to be a possible target for resveratrol in inhibiting angiogenesis. However, this target seemed not enough to account for the detailed mechanism underlying resveratrol-inhibited angiogenesis.

In the present study, we demonstrate that abrogation of the tyrosine phosphorylation of VE-cadherin by resveratrol is a critical event for its angiogenesis inhibition. This reduction of the tyrosine phosphorylation of VE-cadherin by resveratrol led to the stabilization of VE-cadherin on cell-cell contacts. In vitro kinase assay showed that Src functioned up-stream of VE-cadherin and its activity was blocked by resveratrol. Overexpression of active v-Src significantly reversed the inhibitory effect of resveratrol on the tyrosine phosphorylation of VE-cadherin as well as endothelial cell tube formation. Our results suggest that resveratrol inhibited angiogenesis primarily through blocking Src-dependent tyrosine phosphorylation of VE-cadherin.

## Materials and Methods

**Reagents.** Resveratrol (>99%) was purchased from Sigma-Aldrich (St. Louis, MO). A stock solution of resveratrol was made in dimethyl sulfoxide at a concentration of 10 mM.

VEGF-A was obtained from R&D Systems (Minneapolis, MN), and Matrigel was from Collaborative Biotech (Bedford, MA). Anti-phospho-p44/p42 mitogen-activated protein kinase (extracellular signal-regulated protein kinase 1/2), anti-total extracellular signal-regulated protein kinase 1/2, anti-total Akt, anti-phospho-(Ser473)-Akt, and anti-phospho-(Thr308)-Akt rabbit polyclonal antibodies were obtained from Cell Signaling Technology (Bedford, MA). A rabbit polyclonal antibody raised against amino acids 3 to 18 of human Src (N-16; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for

immunoprecipitation for in vitro kinase assays, and monoclonal antibody against avian pp60c-src (Upstate Biotechnology, Lake Placid, NY) was used for Western blotting. The Src constructs were obtained from Dr. R. H. Chen (Institute of Biomedicine, College of Medicine, National Taiwan University, Taipei, Taiwan). FAK-GST fusion protein was from Dr. D. Schlaepfer (The Scripps Research Institute, San Diego, CA). All other reagents and media were from Sigma-Aldrich unless otherwise stated.

**[<sup>3</sup>H]Thymidine Incorporation Assay.** HUVECs were plated at a density of  $2 \times 10^4$  cells/well in a 24-well plate. Cells were incubated in growth media and allowed to attach for 24 h. Cells were washed twice with M199 and incubated for 6 h with M199 containing 1% fetal bovine serum. Cells were stimulated by the addition of indicated concentrations of resveratrol and/or 10 ng/ml VEGF for 24 h and followed by the addition of 1  $\mu$ Ci/ml [<sup>3</sup>H]thymidine for 6 h. High molecular mass <sup>3</sup>H-labeled radioactivity was precipitated using 5% trichloroacetic acid at 4°C for 30 min. After washing twice with ice-cold H<sub>2</sub>O, <sup>3</sup>H-labeled radioactivity was solubilized in 0.2 N NaOH containing 0.1% SDS and determined by liquid scintillation counter. Tube Formation Assay. Tube formation assays and quantification were performed essentially as described previously (Lee et al., 1999), using a minimal volume of Matrigel (0.24 mg/cm<sup>2</sup>) that both allowed HUVEC tubule formation and permitted vascular network visualization and quantification by image analysis. Briefly, HUVECs (passages 2–6) were cultured in endothelial growth medium containing 2% fetal bovine serum and bovine brain extract and plated ( $1.2 \times 10^5$  cells/ml) onto a layer of Matrigel. Resveratrol was added as indicated. Cells were washed, fixed in methanol, and stained in DifQuik solution 2 before tube area measurement. Three replicate fields of triplicate wells were digitally photographed. Tube area was quantified using MetaMorph software (Universal Imaging Corporation, West Chester, PA).

**Migration Assay (in Vitro Wounding).** In vitro scratched wounds were created by scraping the cell monolayer with a sterile disposable cell scraper. After injury of the monolayer, the cells were gently washed and stimulated by VEGF and resveratrol in combination. Endothelial cells migration from the edge of the injured monolayer was quantified by measurement of the distance between the wound edges before and after injury with a computer-assisted microscope (Carl Zeiss Inc., Thornwood, NY) at five distinct positions (every 5 mm).

**HUVEC Transfection.** Plasmids containing Csk and v-Src were transiently transfected into HUVEC P2 cells. Using an initial seeding density of  $1 \times 10^6$  cells/cm<sup>2</sup>, HUVECs were ready for transfection at 18 to 24 h after seeding. For each 75-cm<sup>2</sup> flask of cells to be transfected, 30  $\mu$ l of Lipofectin and 5  $\mu$ g of each DNA were diluted separately in 500  $\mu$ l of Opti-MEM I reduced serum medium. Solutions were combined, gently mixed, and incubated for 45 min at room temperature to allow formation of DNA-lipid complexes. The normal medium was replaced with 8 ml of Opti-MEM I reduced medium, and the DNA-lipid complexes were added subsequently to each flask and mixed gently by rocking the plate back and forth. Cells were incubated for 3 to 4 h at 37°C in humidified air in 5% CO<sub>2</sub>. The transfection mixture was then replaced with basal media containing supplements. Eighteen to 24 h after transfection, transfected cells were treated with VEGF and/or resveratrol for 1 h and then subjected to Src kinase activity assay.

**Immunoprecipitation.** Confluent HUVECs were incubated for 6 h in M199 containing 1% fetal bovine serum before addition of resveratrol and VEGF for a further 30 min or 1 h, depending upon the experimental design. Then cells were lysed in 1 ml of lysis buffer containing 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 137 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 1% Triton X-100. Lysates were clarified by centrifugation at 15,000g for 10 min, and the resulting supernatants were immunoprecipitated with 1  $\mu$ g/ml anti-VE-cadherin and anti- $\beta$ -catenin antibodies for 3 h at 4°C, followed by the addition of protein A-agarose beads for 1 h at 4°C. Immunoprecipitates were washed three times with lysis buffer,

solubilized in SDS-PAGE sample buffer, and further analyzed by Western blotting using anti-phosphotyrosine antibody.

**Western Blotting.** Cell lysates or immunoprecipitates from HUVECs were electrophoresed on SDS-PAGE gel and transferred to polyvinylidene difluoride membrane. The blocked membranes were then incubated with the indicated antibodies, and the immunoreactive bands were visualized using chemiluminescent reagent as recommended by Amersham Biosciences (Piscataway, NJ).

**Immunofluorescence Microscopy.** The procedure has been described in detail previously (Lampugnani and Dejana, 1997). Briefly, cells on fibronectin-coated glass coverslips were fixed, permeabilized, and then labeled with VE-cadherin antibodies. This was followed by incubation with fluorescein isothiocyanate-conjugated secondary antibodies and mounting using Mowiol 4-88 (Calbiochem, La Jolla, CA).

**Intracellular Peroxide Determination.** Intracellular peroxide production was quantified by flow cytometry using fluorescent dye DCF-DA (Shiah et al., 1999). Briefly, cells ( $2 \times 10^6$ ) were coincubated with 50 ng/ml VEGF and 50  $\mu$ M DCF-DA in the presence or absence of resveratrol for 30 min. After treatment, cells were subjected to flow cytometric analysis (BD Biosciences, San Jose, CA).

**Src Kinase Assays.** The kinase activity of endogenous Src kinase was assayed by the ability of immunopurified Src to phosphorylate an FAK-GST fusion protein in an in vitro assay (Eliceiri et al., 1998). Briefly, confluent HUVECs were exposed to different treatments for various times. Endogenous Src was immunoprecipitated from equivalent amounts of total protein and subjected to the Src kinase assay, and the samples were analyzed by 15% SDS-PAGE.

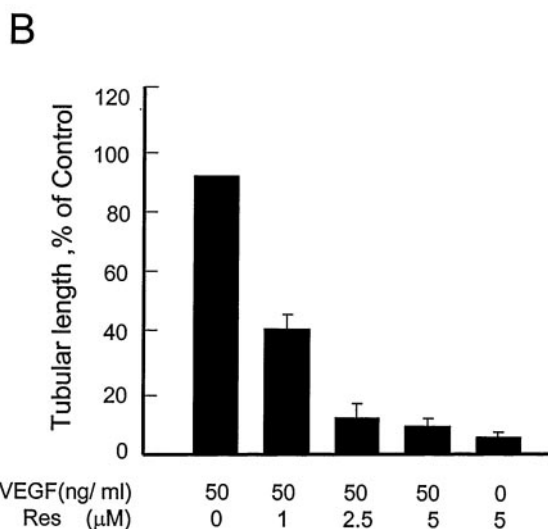
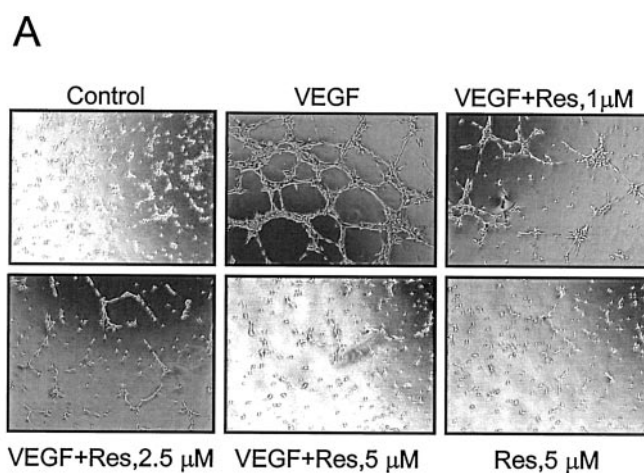
The in vitro Src kinase assays were performed in 96-well plates as a filter binding assay. Briefly, immunoprecipitated Src was incubated under optimized buffer conditions [20 mM Tris-HCl buffer, pH 7.5, 1–3 mM  $MnCl_2$ , 3–10 mM  $MgCl_2$ , 3–8  $\mu$ g/ml poly-(Glu:Tyr 4:1), 0.25 mg/ml polyethylene glycol 20,000, 8  $\mu$ M ATP, 10  $\mu$ M sodium vanadate, 1 mM dithiothreitol, and 0.2  $\mu$ Ci of [ $\gamma$ - $^{32}P$ ]ATP] in a total volume of 30  $\mu$ l in the presence or absence of resveratrol (1–5  $\mu$ M) for 20 min in ambient temperature. The reaction was stopped by adding 10  $\mu$ l of 250 mM EDTA. Using a 96-well filter system, half the volume (20  $\mu$ l) was transferred onto an Immobilon-polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA). The membrane was then washed extensively in 0.5%  $H_3PO_4$  and then soaked in ethanol. After drying, Microscint cocktail (PerkinElmer Life Sciences, Boston, MA) was added, and scintillation counting was performed (Wood et al., 2000).

## Results

**Effect of Resveratrol on VEGF-Induced HUVEC Tube Formation, Migration, and Proliferation.** To understand the possible mechanism underlying resveratrol inhibition of angiogenesis, we first used in vitro angiogenesis models to determine effects of resveratrol on VEGF-induced endothelial cell migration and tube formation, two critical steps in the process of angiogenesis. Capillary-like tube formation was assessed with HUVEC model on Matrigel. Treatment of HUVECs with various doses of resveratrol resulted in strong prevention of VEGF-induced tube formation (Fig. 1A). Quantitative results (Fig. 1B) showed that resveratrol had a 50 to 60% inhibitory effect on VEGF-induced tube formation at 1  $\mu$ M and almost completely inhibited tube formation at 2.5  $\mu$ M. In a wound-induced cell migration model, we found that resveratrol exhibited an equivalent potency of inhibiting endothelial cell migration compared with tube formation (Fig. 2). To clarify whether resveratrol inhibition of VEGF-induced tube formation and migration was caused by the impairment of endothelial cell proliferation, we examined the effect of resveratrol on DNA synthesis, one of the parameters of cell proliferation, by using 5'-bromo-

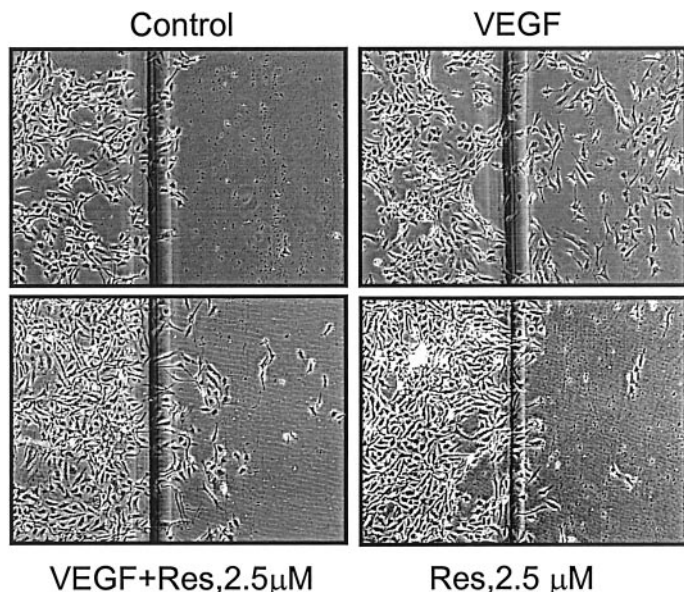
2'-deoxy-uridine incorporation assay. Resveratrol had no effect on VEGF-mediated DNA synthesis at 1 to 5  $\mu$ M, but it caused a 35 to 40% inhibition of DNA synthesis at 10  $\mu$ M (Fig. 3). This discrepancy in the doses required to produce either effect suggests that resveratrol inhibition of VEGF-induced cell migration and tube formation is not mediated by blocking of endothelial cell proliferation.

**Resveratrol Abolishes VEGF-Induced VE-Cadherin Tyrosine Phosphorylation and Redistribution.** It is well documented that VEGF-stimulated tyrosine phosphorylation of VE-cadherin is critical for the loosening of cell-cell contacts in established vessels and to allow sprouting and the formation of capillary networks during angiogenesis (Esser et al., 1998; Wright et al., 2002). Here, we tested the hypothesis that resveratrol could affect VEGF-mediated VE-cadherin tyrosine phosphorylation. Confluent cultures of HUVECs were exposed to 50 ng/ml VEGF alone and/or in combination

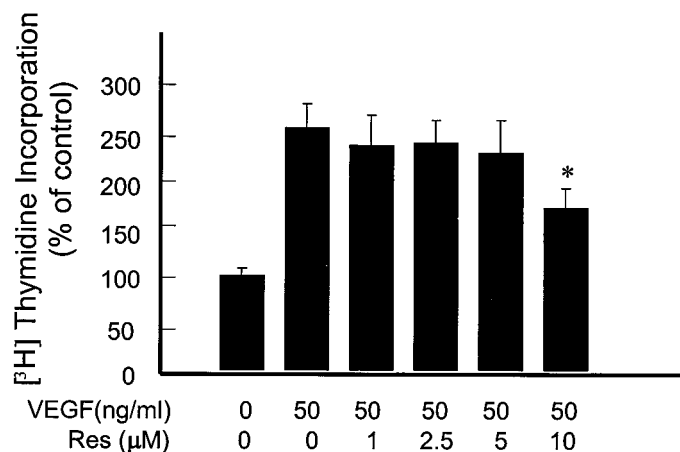


**Fig. 1.** Inhibitory effect of resveratrol (Res) on VEGF-induced tube formation. A, HUVECs were seeded onto the Matrigel layer in 24-well plate. The assay was performed in the presence of none, 50 ng/ml VEGF, or 50 ng/ml VEGF plus various concentrations of Res (1–5  $\mu$ M) for 12 h as described under *Materials and Methods*. The experiment was repeated four times with similar results (values are means  $\pm$  S.E.M.,  $n = 4$ ). B, quantitative results from A. Briefly, cells were washed, fixed in methanol, and stained in DifQuik solution 2 before tube area measurement. Three replicate fields of triplicate wells were digitally photographed. Tube area was quantified using MetaMorph software (Universal Imaging Corporation).

with various doses of resveratrol for 1 h. To determine the levels of tyrosine phosphorylation of VE-cadherin, we subjected HUVEC lysates from different treatments to immunoprecipitation with anti-VE-cadherin antibodies and then immunoblotted with anti-phosphotyrosine antibodies. VEGF treatment of HUVEC stimulated significant increase of tyrosine phosphorylation of VE-cadherin, which was effectively diminished by 1 and 2.5  $\mu\text{M}$  resveratrol (Fig. 4A). The tyrosine phosphorylation of  $\beta$ -catenin, a complex partner of VE-cadherin, was also greatly attenuated by 2.5  $\mu\text{M}$  resvera-



**Fig. 2.** Resveratrol inhibition of VEGF-induced HUVEC migration. Monolayers of confluent HUVECs were scraped with a sterile disposable cell scraper to create scratch wounds. After injury of the monolayer, the cells were gently washed and stimulated by VEGF (50 ng/ml) or/and resveratrol (Res) (2.5  $\mu\text{M}$ ) in combination for 24 h. Endothelial cells migration from the edge of the injured monolayer was quantified by measurement of the distance between the wound edges before and after injury with a computer-assisted microscope (Carl Zeiss) at five distinct positions (every 5 mm).

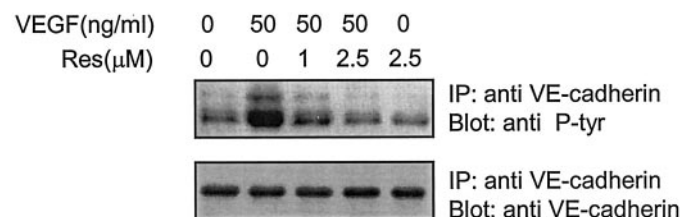


**Fig. 3.** Effect of resveratrol on VEGF-stimulated DNA synthesis in HUVECs. DNA synthesis was measured in quiescent HUVECs in 24-well plates after treatment with the indicated concentrations of Res or/and VEGF for 22 h, at which time [ $^3\text{H}$ ]thymidine (1  $\mu\text{Ci}/\text{well}$ ) was added. Radioactivity incorporated into cellular DNA was measured after 2-h incubation, as described under *Materials and Methods*. DNA synthesis was measured as [ $^3\text{H}$ ]thymidine incorporation. Each value represents mean  $\pm$  S.E.M. ( $n = 3$ ).

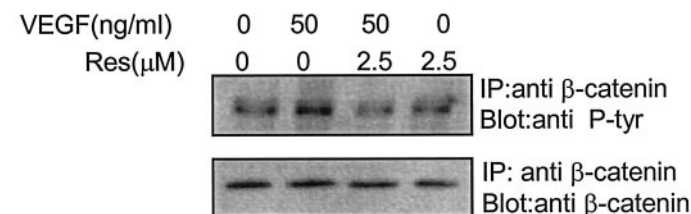
rol as analyzed by immunoblotting  $\beta$ -catenin immunoprecipitates with anti-phosphotyrosine antibodies (Fig. 4B). However, the complex status of VE-cadherin- $\beta$ -catenin was not changed by VEGF or resveratrol (data not shown). Because the VEGF receptor is located upstream of VE-cadherin, we further checked whether resveratrol inhibits VEGF-mediated VEGFR-2 tyrosine phosphorylation. Interestingly, resveratrol did not affect the VEGF-stimulated VEGFR-2 tyrosine phosphorylation (Fig. 5A), suggesting that resveratrol did not target the VEGFR-2 tyrosine kinase. Furthermore, we found that resveratrol also failed to affect VEGF-induced the phosphorylation of Erk1/2, p38, and Akt at the dose of 1 to 2.5  $\mu\text{M}$  (Fig. 5B).

Numerous studies have shown that tyrosine phosphorylated VE-cadherin would be lost from most cell-cell contact areas during VEGF-stimulated angiogenesis (Esser et al., 1998). Here, we examined whether resveratrol could interfere with the loss of VE-cadherin from cell-cell junctions by VEGF using immunofluorescent assay. In confluent endothelial cell monolayers, VE-cadherin was localized as bright immunostaining at cell-cell contacts (Fig. 6, I). However, within 1 h after stimulation with VEGF, the VE-cadherin fluorescence was greatly reduced and disappeared from cell-cell junctions (Fig. 6II, arrow). Interestingly, 2.5  $\mu\text{M}$  resveratrol treatment effectively retained the VE-cadherin fluorescence at cell-cell contacts (Fig. 6III). Resveratrol-treated cells did not show any difference in the fluorescent intensity at cell-cell junctions compared with control cells (Fig. 6IV). The

A



B



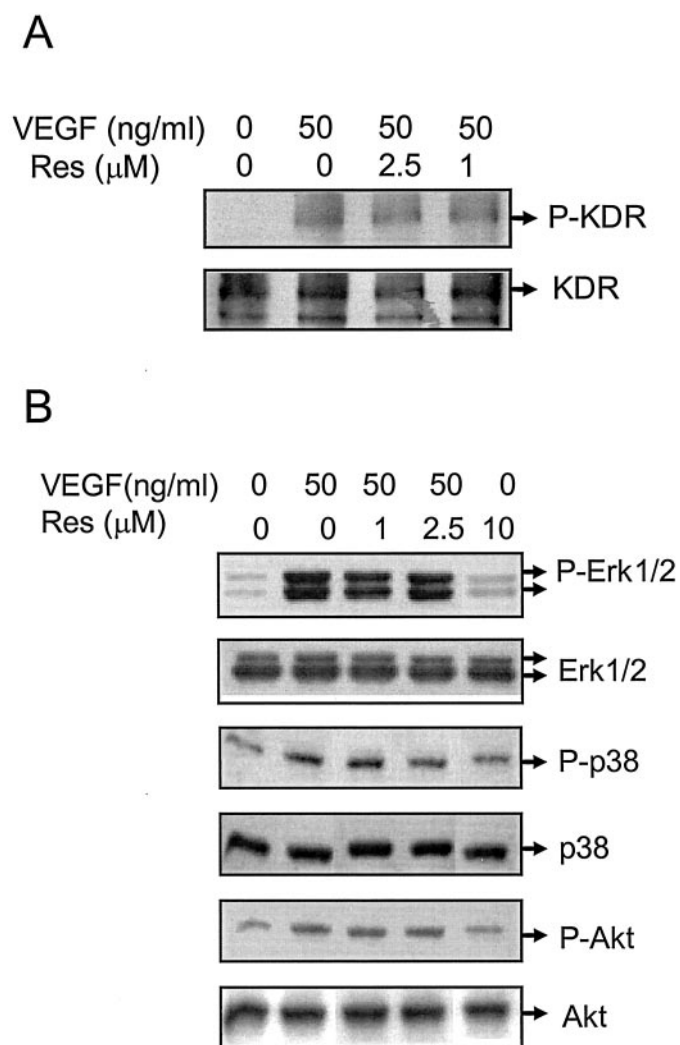
**Fig. 4.** Effect of resveratrol on VEGF-stimulated tyrosine phosphorylation of the adheren junction proteins. Confluent cultures of HUVECs were starved (1% serum) for 6 h and incubated with 50 ng/ml VEGF and/or 1 to 2.5  $\mu\text{M}$  resveratrol (Res) for a further 1 h. Cell extracts were subjected to immunoprecipitation with antibodies against VE-cadherin (A) or  $\beta$ -catenin (B). Precipitated proteins were analyzed by SDS-PAGE followed by immunoblotting with antibody to phosphotyrosine (A and B, top). The same blots were subsequently reprobred with antibodies of VE-cadherin (A, bottom) or  $\beta$ -catenin (B, bottom).

above-mentioned observations indicate that inhibition of VEGF-stimulated VE-cadherin tyrosine phosphorylation by resveratrol leads to the stabilization of EC cell-cell contacts.

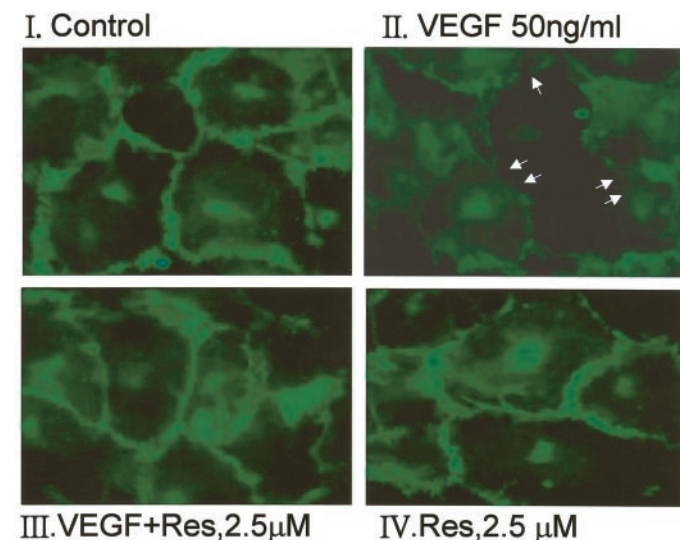
**The Role of Src Kinase in Resveratrol Inhibition of the Tyrosine Phosphorylation of VE-Cadherin as Well as Tube Formation.** Src tyrosine kinase has been shown to possibly mediate the tyrosine phosphorylation signal from VEGFR-2 to VE-cadherin (Lampugnani et al., 1997; Zanetti et al., 2002). Here, we test the possibility that resveratrol could affect VEGF-induced Src kinase activity in HUVECs. Src immunoprecipitates from VEGF- or VEGF plus resveratrol-treated HUVEC lysates were subjected to kinase assay using FAK-GST as substrate. A 3- to 4-fold increase in Src kinase activity was observed in cells treated with VEGF for 1 h. The increase in Src activity was alleviated by 45 and 80%

for 1 and 2.5  $\mu$ M resveratrol, respectively (Fig. 7A). Csk is a tyrosine kinase that specifically phosphorylates the carboxy-terminal tyrosine of Src kinases and returns them to an inactive form (Murphy et al., 1993). Overexpression of Csk can be used to block Src family kinases and test whether Src worked upstream of VE-cadherin. Transfection of HUVECs with Csk vector diminished VEGF-stimulated Src kinase activity (Fig. 7B, bottom) and the tyrosine phosphorylation of VE-cadherin (Fig. 7B, top). Furthermore, HUVECs were transfected with the v-Src expression vector, an active form of Src, and then were treated with VEGF and/or resveratrol. Cell lysates were subjected to Src kinase and tyrosine phosphorylation of VE-cadherin assays. The results show that overexpression of v-Src could counteract resveratrol-mediated inhibition of VEGF-stimulated Src kinase activity (Fig. 7C, bottom) and tyrosine phosphorylation of VE-cadherin (Fig. 7C, top). Together, these data strongly suggest that resveratrol inhibited VEGF-induced tyrosine phosphorylation of VE-cadherin through abolition of Src activation. To assess whether resveratrol directly targeted the Src molecules, Src kinase was immunoprecipitated from HUVECs and then incubated with resveratrol using poly-(Glu:Tyr 4:1) peptide as the substrate. Figure 7D shows that 1 and 2.5  $\mu$ M resveratrol failed to directly inhibit Src kinase activity, whereas 5  $\mu$ M resveratrol had a minor inhibitory effect. The above-mentioned data indicate that Src kinase may not be a direct target for resveratrol.

Recently, ROS has been reported to be involved in VE-cadherin tyrosine phosphorylation and its related functions (Watering et al., 2002). Here, we tested whether ROS acted as an upstream activator for Src kinase and the subsequent VE-cadherin phosphorylation and whether resveratrol could affect the ROS level. As Fig. 8A reveals, treatment of HUVECs with VEGF for 30 min stimulated a 1.5-fold elevation of peroxide as demonstrated by a net increase in cell fluorescence after cell loading with the peroxide-sensitive fluorescent dye DCF-DA. One and 2.5  $\mu$ M resveratrol caused



**Fig. 5.** Effect of resveratrol on VEGF-stimulated activation of VEGF-2 and related signaling pathways. A, confluent HUVECs were starved for 6 h and incubated with 50 ng/ml VEGF and/or 1 to 2.5  $\mu$ M resveratrol (Res) for a further 30 min. Cell extracts were subjected to immunoprecipitation with antibodies against VEGFR-2 (KDR) and precipitated proteins were analyzed by SDS-PAGE followed by immunoblotting with an antibody to phosphotyrosine (P-KDR). Confluent HUVECs were starved for 6 h and treated with 50 ng/ml VEGF and various concentrations of Res, as indicated, for 2 h. Cell lysates were obtained and subjected to SDS-PAGE followed by immunoblotting with various antibodies such as anti-phospho-Erk1/2, anti-phospho-p38, anti-phospho-Akt, anti-Erk1/2, anti-p38, and anti-Akt.



**Fig. 6.** Effect of resveratrol on VEGF-induced alteration in location of VE-cadherin at cell-cell contacts. Confluent HUVECs were starved for 6 h and treated with solvent control (I), 50 ng/ml VEGF (II), VEGF plus Res (III), and Res alone (IV) for 6 h. Cells were fixed, permeabilized, and then immunoblotted with anti-VE-cadherin and stained with fluorescein isothiocyanate-conjugated secondary antibody.

50 and 85% reduction of VEGF-stimulated peroxide production. Antioxidant NAC nearly completely abolished the generation of peroxide. Furthermore, pretreatment of cells with NAC strongly attenuated VEGF-mediated Src activation (Fig. 8B, top) and VE-cadherin tyrosine phosphorylation (Fig. 8B, bottom). Based on these observations, we suggest that resveratrol inhibited VEGF-mediated Src kinase activation and subsequent VE-cadherin phosphorylation by preventing ROS generation.

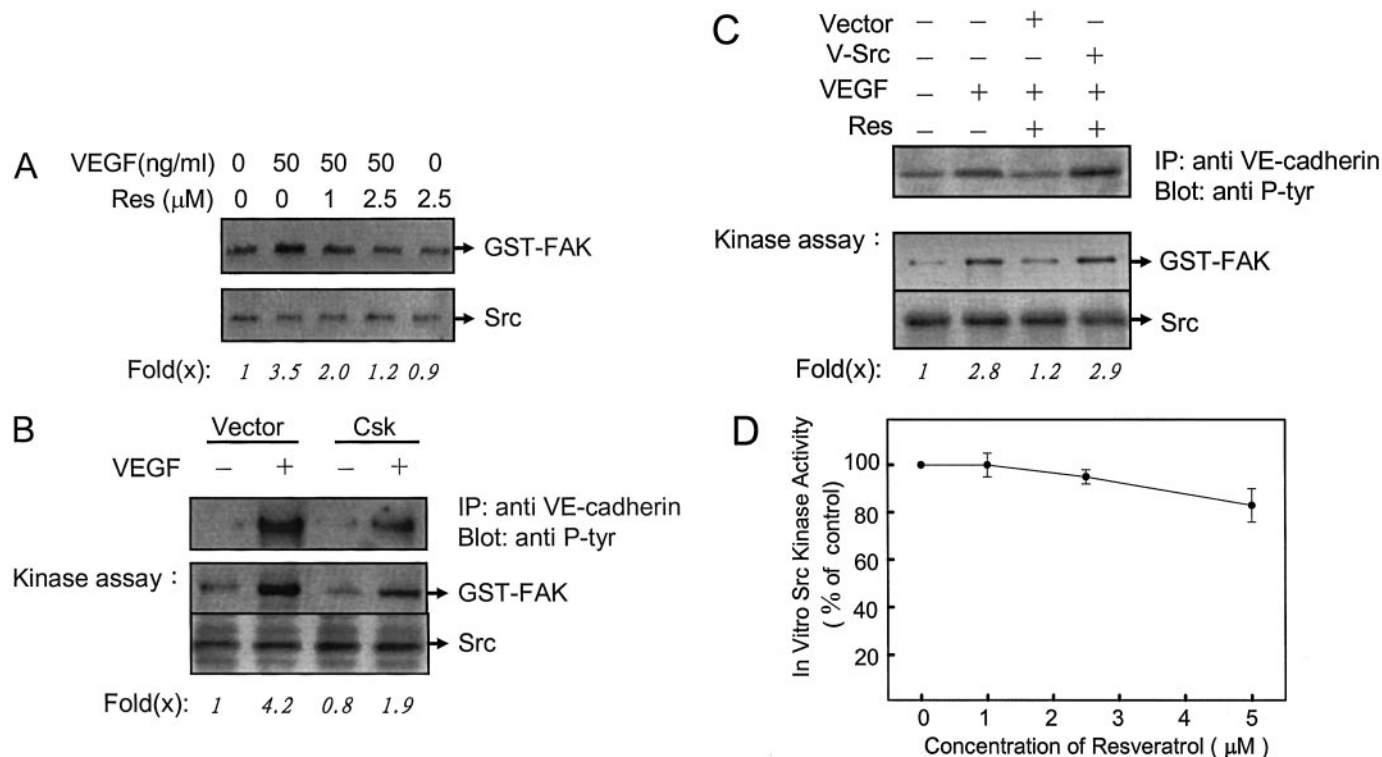
To examine whether modulation of Src activity would affect angiogenesis inhibition by resveratrol, we used the tube formation assay. Inhibition of VEGF-activated Src activity by Csk overexpression in HUVECs strongly disrupted their tube formation (Fig. 9; VEGF versus VEGF + Csk,  $P < 0.001$ ). In contrast, resveratrol-mediated inhibition on tube formation was strongly blocked by overexpression of v-Src (Fig. 9; VEGF + RES versus VEGF + Res + V-src,  $P < 0.05$ ). Interestingly, the antioxidant NAC also effectively blocked VEGF-induced HUVEC tube formation. These data strongly suggest that ROS-dependent Src kinase activation is a critical mechanism in VEGF-induced angiogenesis and is also inhibited by resveratrol.

## Discussion

A correlation between tyrosine phosphorylation of cadherin-catenin complexes and changes in the stability of in-

terendothelial cell contacts has been well documented (Daniel and Reynolds, 1997). In a VEGF-stimulated HUVEC model, numerous investigators pointed out that tyrosine phosphorylation of VE-cadherin is involved in the loosening of cell-cell contacts in established vessels to allow vessel sprouting and EC migration during VEGF-induced angiogenesis (Esser et al., 1998; Wright et al., 2002). Although targeting the VE-cadherin molecule is a relevant strategy for angiogenesis inhibition, no chemical or drug able to do so has been identified to date. Our present study, for the first time, demonstrates that a natural product, resveratrol, inhibited VEGF-induced angiogenesis through blocking Src kinase-dependent tyrosine phosphorylation of VE-cadherin. The data obtained here provide a novel mechanism to account for the inhibition of resveratrol on VEGF-mediated angiogenesis.

As mentioned above, the tyrosine phosphorylation of VE-cadherin is a critical step for VEGF-induced angiogenesis. However, the question of which molecule(s) is involved in regulating the phosphorylation event is as yet unclear. The involvement of Src tyrosine kinase in angiogenesis has been demonstrated by using dominant-negative Src or Csk to block VEGF-, but not basic fibroblast growth factor-induced blood vessel formation in mice (Eliceiri et al., 1999). This suggests that Src kinase functions as component of the VEGF-receptor signaling pathway (Waltenberger et al.,

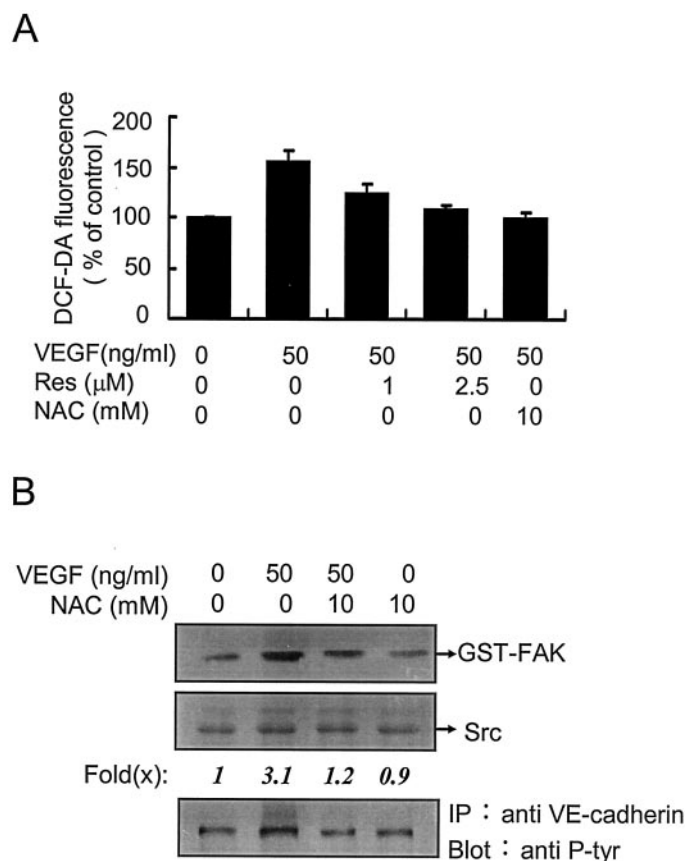


**Fig. 7.** A, inhibition of resveratrol on VEGF-induced activation of endogenous Src kinase. HUVECs were starved for 6 h and treated with 50 ng/ml VEGF and/or 1 to 2.5  $\mu$ M resveratrol (Res) for further 1 h. Endogenous Src was immunoprecipitated from equivalent amounts of total proteins and subjected to an in vitro immune complex kinase assay with a FAK-GST fusion protein as a substrate, electrophoresed, and transferred to nitrocellulose. The relative fold increase in Src activity is indicated in italics. The above-mentioned kinase assay blot was probed with an anti-Src antibody as a loading control for equivalent Src and IgG content. B, transfection with Csk (1  $\mu$ g) blocks VEGF-induced tyrosine phosphorylation of VE-cadherin and Src activity. HUVECs were transfected with Csk plasmid as described under *Materials and Methods*. VE-cadherin tyrosine phosphorylation and Src kinase activity assays were performed on cell lysates. C, transfection with v-Src reverses resveratrol inhibition of VEGF-mediated VE-cadherin tyrosine phosphorylation and Src activity. D, effect of resveratrol on purified Src kinase activity. Immunopurified Src kinase was incubated with 1 to 5  $\mu$ M of resveratrol and then the in vitro kinase assay was performed as described under *Materials and Methods*. The experiment was repeated three times with similar results (values are means  $\pm$  S.E.M.,  $n = 3$ ).

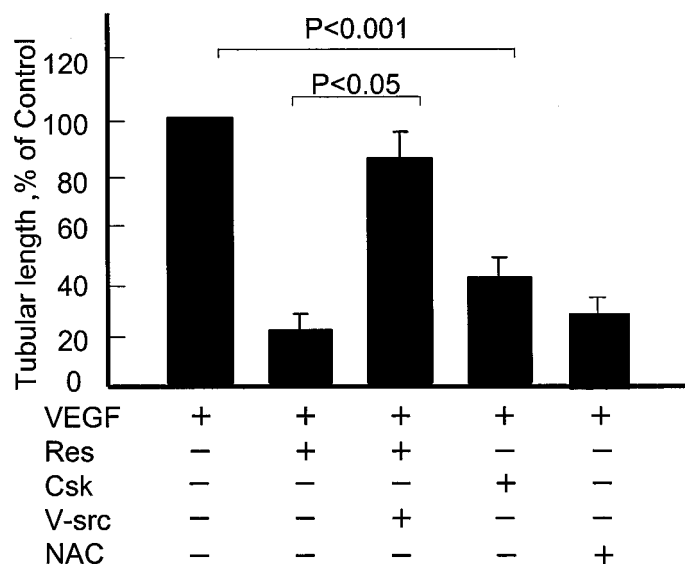
1994). Many downstream molecules, including integrin (Eliceri et al., 2002) and endothelial nitric-oxide synthase (He et al., 1999), have been found to be activated by Src during angiogenic activation of endothelial cells. However, at present there has been no evidence to demonstrate the linkage between Src and the tyrosine phosphorylation of VE-cadherin. Src has been shown to be enriched at cell-cell contacts and has been implicated in regulating the phosphorylation of cadherins and catenins (Papkoff, 1997). In fibroblasts, Src was capable of weakening E-cadherin-mediated cell contacts (Takeda et al., 1995). These observations strongly indicate a possible connection between Src and VE-cadherin. Indeed, our current data show that blockage of Src activity by resveratrol or expression of Csk (negative regulator of Src) would impair the VEGF-induced tyrosine phosphorylation of VE-cadherin and the subsequent endothelial cell migration and tube formation. In agreement with our finding, resveratrol has also been shown to inhibit phorbol ester and UV-induced activator protein-1 activation by blocking Src activity in HeLa cells (Yu et al., 2001). However, our data presented in Fig. 7D indicate that Src kinase is not a direct target for resveratrol. Such findings suggested that

some unidentified upstream events of Src are affected by resveratrol. Numerous studies have pointed out that ROS acts as an important mediator in VEGF-induced angiogenic signaling including VE-cadherin phosphorylation, endothelial cell migration and tube formation (Colavitti et al., 2002; Ushio-Fukai et al., 2002; Watering et al., 2002). In agreement with these studies, we found that ROS elevation is critical for VEGF-induced Src activation in HUVECs. In addition, the capacity of resveratrol in reducing ROS generation was equivalent to that of resveratrol in inhibiting Src kinase activity (compare Figs. 8A and 7A). Supportively, antioxidant NAC not only effectively reduced VEGF-induced ROS increase but also abolished Src activation and VE-cadherin phosphorylation as well as tube formation. Our data provide novel evidence that VEGF-mediated Src activation and VE-cadherin phosphorylation is ROS-dependent. More importantly, resveratrol has a profound effect in neutralizing VEGF-induced ROS increase.

In addition to Src kinase, many cellular targets such as activator protein-1, nuclear factor- $\kappa$ B, protein kinase C, and mitogen-activated protein kinase, have been found to be affected by resveratrol in varied cell lines (Garcia-Garcia et al., 1999; Manna et al., 2000). These cellular targets are functionally linked to cell proliferation, differentiation, and apoptosis. However, the effective doses of resveratrol used in inhibiting these cellular activities were always higher than 10  $\mu$ M. This concentration is hardly achieved by a reasonable red wine or other human food consumption (Wu et al., 2001). Our data, corroborating with other studies (Brakenhielm et al., 2002), showed that VEGF-induced endothelial cell tube formation and migration could be effectively inhibited by 1 to 2.5  $\mu$ M of resveratrol. These doses used in inhibiting endothelial cell activation are far lower than that used in other cell models. This may partly explain why the preventive effect of resveratrol on cardiovascular disease is predominantly observed.



**Fig. 8.** A, effect of resveratrol on VEGF-induced peroxide elevation. HUVECs were incubated with 50 ng/ml VEGF and 50  $\mu$ M fluorescent dye DCF-DA for 30 min in the presence or absence of resveratrol as indicated. The quantities of 2',7'-dichlorofluorescein fluorescence intensity were detected by using flow cytometry. Values are percent increases of mean cell fluorescence (average  $\pm$  S.E.M. of duplicate samples). B, antioxidant NAC blocked VEGF-induced Src activation and VE-cadherin phosphorylation. Cells were pretreated with 10 mM NAC for 1 h and then exposed to VEGF for further 1 h. Lysates were performed endogenous Src kinase assay (top) and VE-cadherin tyrosine (bottom) as described under *Materials and Methods*.



**Fig. 9.** Modulation of Src activity affects the inhibition of resveratrol on VEGF-induced tube formation. Cells were transfected with 1  $\mu$ g of v-Src or Csk expressing vectors as described under *Materials and Methods*. After transfection, transfectants were treated with VEGF (50 ng/ml), NAC (10 mM), and/or resveratrol (Res) (2.5  $\mu$ M) as indicated and then were subjected to tube formation assay. The experiment was repeated three times with similar results (values are means  $\pm$  S.E.M.,  $n = 3$ ).

Antibodies against VE-cadherin have been proposed as agents to block angiogenesis, but compared with resveratrol, have the disadvantages of large proteins (high cost of production, difficult to manufacture, and immunogenic potential). Our data strongly suggest that resveratrol could be used as a therapeutic modality to inhibit VEGF-mediated angiogenesis through preventing ROS-dependent Src activation and the subsequent tyrosine phosphorylation of VE-cadherin.

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