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Organosulfur Garlic Compounds Induce Neovasculogenesis in Human Endothelial Progenitor Cells through a Modulation of MicroRNA 221 and the PI3-K/Akt Signaling Pathways

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ABSTRACT: Human endothelial progenitor cells (EPCs) play crucial roles in the prevention of ischemic injury via neovasculogenesis. Frequent garlic consumption is reportedly associated with a low incidence of cardiovascular diseases (CVD). However, the molecular mechanisms by which garlic extracts, including diallyl disulfide (DADS) and diallyl trisulfide (DATS), exert an effect on neovasculogenesis have not been elucidated yet. The current study investigated the effects of these organosulfur compounds on neovasculogenesis by using vascular tube formation assay, Western blotting assay, real-time polymerase chain reaction (RT-PCR), and immunohistochemical (IHC) staining assays in both in vitro and in vivo models. The current study demonstrates that DADS and DATS dose-dependently enhance the neovasculogenesis of human EPCs in vitro. The mechanism of actions included the up-regulation of the c-kit protein, as well as the phosphorylation (i.e., activation) of the Akt and ERK 1/2 signaling molecules in human EPCs. Furthermore, DATS suppressed the expression of microRNA (miR) 221 in vitro. In a mouse xenograft model of neovasculogenesis, DATS consumption induced the formation of new blood vessels at a dosage of 10 mg/kg of body weight/day. It is suggested that garlic consumption enhances neovasculogenesis in human EPCs and thereby probably exerts a preventive effect against ischemic injuries.

KEYWORDS: *diallyl trisulfide, neovasculogenesis, c-kit, p-Akt, microRNA 221, human endothelial progenitor cells*

■ INTRODUCTION

The hemangioblast, an ancestral progenitor of both endothelial cells (ECs) and hematopoietic cells during embryogenesis, is responsible for the de novo development of blood vessels.[1] Adult circulating endothelial progenitor cells (EPCs) are derived from hemangioblasts and function as a cell source that contributes to neovascularization through a vasculogenic process.[2] These bone marrow-derived EPCs integrate naturally into the sites of blood vessels during an ischemic event.[3] Several studies have reported an inverse association between the levels of circulating EPCs and ischemic conditions.[4] Indeed, the formation of new blood vessels is necessary in a variety of pathological and physiological conditions, such as cardiovascular diseases (CVD), stroke, and tissue regeneration.[5,6]

Previous studies suggested that the number and function of EPCs are inversely correlated with certain risk factors and forms of CVD, such as coronary artery disease. EPCs have been implicated in re-endothelialization and neovascularization processes.[7] During neovascularization, multiple pathways related to the proliferation and migration of EPCs are involved.[8] Among them, stem cell factor (SCF) and its c-kit receptor signaling cascades have attracted considerable attention recently. Previous studies have indicated that the c-kit vasculogenic protein, a receptor tyrosine kinase (RTK) involved in neovasculogenesis, is a biomarker of human EPCs.[9] One study reported that mesenchymal stem cells exert a beneficial effect on c-kit up-regulation and myocardial regeneration through the activation of the phosphatidylinositide 3-kinase (PI3-K)/Akt signaling pathway.[10] Augmentation of c-kit expression was associated with the activation of the PI3-K/Akt signaling pathways in human EPCs.[11] Furthermore, SCF (a c-kit ligand) induces cell proliferation/survival and neovasculogenesis in EPCs through the activation of c-kit RTK protein and downstream multiple signaling pathways, including the PI3-K/Akt and mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) signaling cascades.[9,12] The activation of PI3-K/Akt leads to the phosphorylation downstream of the I-κB molecule and nuclear accumulation of the NF-κB (Rel A) protein. Previous study also indicated that the activation of Akt modulated the GSK-3β protein and stabilized its downstream targeted β-catenin protein through a suppression of the phosphorylated β-catenin level. Moreover, the Akt/GSK3-β /β-catenin cascades enhanced cell proliferation/survival as the result of increased levels of the nuclear cyclin D1 protein.[13] In addition to these regulatory pathways, microRNA has emerged as a class of small noncoding RNAs that negatively regulate gene expression

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through translational inhibition or degradation of the target mRNAs.[14] Several studies have indicated that miR down-regulates the translation of targeted mRNA by binding with sites in the 3′ untranslated regions.[14] A recent study indicated that *c-kit* gene expression is regulated in part through miR-221. miR-221 targeted the 3′ untranslated regions of c-kit mRNA, and this resulted in an incomplete translation of the c-kit protein.[15] Therefore, c-kit protein expression was partially suppressed by miR-221.[16] A clinical study also has reported that the expression of miR-221 was augmented in CVD patients and associated with the degree of ischemic injury.[17] Therefore, the modulation of EPC activity, cell number, and neovasculogenic regulation has become the target of several cardiovascular drug research efforts and attracted considerable interest from scientists. Although cardiovascular drugs leading to neovasculogenesis are the main therapeutic approach to treating CVD, other approaches, such as dietary ones targeted at increasing EPC activity and neovasculogenesis, have yet to be reported in any detail.

Epidemiological studies have demonstrated that dietary garlic consumption is inversely correlated with the incidence of CVD and stroke.[18] For many years, garlic consumption has been identified as a potentially beneficial approach to the prevention of CVD.[19] Studies have reported that garlic consumption prevented platelet aggregation and reduced the risk of ischemic attack.[20] Other studies also suggested beneficial effects of dietary garlic intake on the reduction of hypertension and hyperlipidemia.[21] These results suggest that garlic consumption has favorable effects on attenuating platelet aggregation and adhesion. The biological functions of garlic are attributed to several organosulfur compounds that are ingredients. The most important organosulfur compounds in garlic contain lipid-soluble and water-soluble components. The major lipid-soluble components include diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS). The water-soluble components include *S*-allylcysteine (SAC).[22] A recent study reported that DATS affords protection to ischemic myocardium and helps to prevent CVD.[23]

To our knowledge, to date no experimental evidence has been presented on the mechanisms underlying the beneficial effects of these garlic organosulfur components (DADS and DATS) on neovasculogenesis in human EPCs. Thus, the specific aims of the current study were to examine the molecular mechanisms of the DADS/DATS effect on neovasculogenesis both in vitro and in vivo.

MATERIALS AND METHODS

Reagents and Antibodies. Anti-phospho-Akt (p-Akt), anti-total-Akt (t-Akt), anti-phospho-ERK 1/2 (p-ERK 1/2), anti-total-ERK 1/2 (t-ERK 1/2), anti-phospho-GSK-3β (p-GSK-3β), anti-total-GSK-3β (t-GSK-3β), anti-phospho-IκBα (p-IκBα), anti-phospho-β-catenin (p-β-catenin), anti-total-β-catenin (t-β-catenin), anti-c-kit, anti-phospho-p65 Rel A (p-p65 Rel A), and anti-lamin A/C monoclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rat tail type I collagen, matrigels, and anti-cyclin D1 monoclonal antibody were purchased from BD Bioscience Inc. (San Jose, CA, USA). Anti-β-actin antibody, MEK specific inhibitor (PD098059), NF-κB specific inhibitor (Bay-11-7082), and PI3-K specific inhibitor (wortmannin) were purchased from Sigma (St. Louis, MO, USA). The NE-PER nuclear and cytoplasmic extraction reagent kit was purchased from Pierce Biotechnology Inc. (Rockford, IL, USA). DAS and DATS with a purity >99% were purchased from Sigma. DADS and DATS were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 400 mM and stored at −20 °C. Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA). Trizol reagent and Lipofectamine LTX with Plus Reagent were purchased from Invitrogen Inc. (Carlsbad, CA, USA). The two-step RT-PCR kit was purchased from Promega Inc. (Madison, WI, USA). The specific Taqman MicroRNA assays, including the primers for hsa-miR-221 and U6 snRNA, were purchased from Applied Biosystems (Carlsbad, CA, USA). EGM-2 media were purchased from Lonza, Inc. (Allendale, NJ, USA). The control vector and anti-miR-221 plasmids were purchased from System Biosciences Inc. (Mountain View, CA, USA).

Cell Culture. Human EPCs were purchased from ApproCell Inc. (Hayward, CA, USA). Briefly, human EPCs (passage 7−9) cultured in 10% FBS MCDB-131 with an EGM-2 growth kit in a collagen-coated tissue culture dish were lifted off by trypsinization, pelleted by centrifugation, and resuspended in 10% FBS MCDB-131 medium. The working concentration of type I collagen was 50 μg/mL (prepared in 0.02 N acetic acid). For the transfection procedure, 50% confluent human EPCs were transfected with an anti-miR-221 plasmid using lipofectamine LTX Plus Reagent according to the manufacturer's instructions.

Supplementation with DADS and DATS. The EPC monolayer was incubated with different concentrations (0, 0.1, 1, and 10 μM) of DADS and DATS (at 8 h time point), respectively. For efficient uptake of DADS and DATS by EPCs, DADS and DATS were incorporated into FBS for 30 min and mixed with MCDB-131 medium with 10% heat-inactivated FBS, 2 mM L-glutamine, and 1.5 g/L sodium bicarbonate in the absence of antibiotics.

Preparation of Protein Extraction. Cell nuclear and cytoplasmic fractions were prepared with a NE-PER nuclear and cytoplasmic extraction reagent kit containing protease and phosphatase inhibitors according to the manufacturer's instruction. After centrifugation for 10 min at 12000*g* to remove cell debris, the supernatants were retained as a cytoplasmic extract. Cross-contamination between the nuclear and cytoplasmic fractions was not observed (data not shown).

Western Blotting Analysis. Briefly, cytoplasmic proteins (100 μg) were fractioned using 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and blotted with an anti-c-kit monoclonal antibody according to the manufacturer's instructions. The blots were stripped and reprobed with β-actin antibody as the loading control. The levels of phosphorylated Akt, phosphorylated ERK 1/2, phosphorylated IκB-α, phosphorylated β-catenin, phosphorylated GSK-3β, total Akt, total ERK 1/2, and total GSK-3β in cell lysates were measured using the same procedure described above. The total Akt, total ERK 1/2, and total GSK-3β proteins were used as internal controls for phosphorylated Akt, phosphorylated ERK 1/2, and phosphorylated GSK-3β proteins, respectively. Nuclear proteins were also fractionated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-β-catenin monoclonal antibody. The blots were stripped and reprobed with Lamin A/C antibodies as the loading control. The protein levels of cyclinD1 and phosphorylated p65 (Rel A) were measured with the same procedure described above.

Neovasculogenesis (Vascular Tube Formation) Assay. EPCs were cultured in MCDB-131 supplemented medium that was changed every 2 days. For the vasculogenesis assay, a confluent monolayer of EPCs from passages 6−9 was cultured in MCDB-131 medium on 3-D matrigels. The matrigel was diluted to a concentration of 4 mg/mL, and the pH was neutralized by adding MCDB-131. Aliquots of 50 μL of matrigel were added to 96-well culture plates and incubated at 37 °C until gelatinization. EPCs were seeded on the matrigel-coated 96-well plates (1×10^4 cells/well) to form confluent monolayers using MCDB-131 (pH 7.4) containing 10% FBS. After seeding in the 96-well plates for 8 h, EPCs were fixed with 0.5 mL of a glutaraldehyde/paraformaldehyde mixture (2.5%) and stained with modified May−Gruenwald solution (0.25%). Tubular structure formations on the 3-D matrigels were visualized under inverted phase-contrast microscopy (40×), and photomicrographs were documented and analyzed with the Olympus DP-71 digital camera and imaging system (Tokyo, Japan).

Quantitative Real-Time PCR (qPCR). Total RNA samples from the EPCs were prepared, extracted from each group with Trizol reagent, and converted into cDNA for further analysis using a two-step RT-PCR kit. Briefly, cDNA samples were used in a PCR reaction containing the hsa-miR-221 specific primers, as described above. U6 snRNA was used as the internal control. Quantitative PCR experiments were performed using the real-time PCR detection system (Applied Biosystems, Carlsbad, CA, USA). The expression levels of miR-221 adjusted by the U6 snRNA in human EPCs are presented as a percentage of the corresponding untreated control group.

Xenograft Implantation of EPCs. To achieve the specific aims of this study, we used immunodeficient nude mice in which human EPCs were xenografted subcutaneously (sc). Briefly, human EPCs were maintained at 37 °C in a 5% CO_2 incubator and grown to subconfluency using 10% FBS and EGM-2 kit-supplemented MCDB-131 media. To produce a mouse xenograft model of neovasculogenesis, subconfluent cultures of EPCs were harvested by a brief treatment with 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed twice and resuspended in serum-free medium. Only single-cell suspensions with a viability of >90% were used for the injections.

Female adult (3−4 weeks old) BALB/C AnN-Foxn1 nude mice (18−21 g) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mice were maintained under specific pathogen-free conditions in facilities approved by the National Laboratory Animal Center in accordance with current regulations and standards (animal protocol no. 101-134-N). During the entire experimental period, the mice (n = 6 in each group) were fed a standard Lab 5010 diet (purchased from LabDiet Inc., St. Louis, MO, USA). Mice were anesthetized with an inhalation of isofluorane and placed in a supine position. The mice were sc injected with ~3 million human EPCs (3×10^6/0.1 mL medium) and matrigel on the right flank. A well-localized bleb was a sign of a technically satisfactory injection. After the inoculation, mice were divided into three subgroups (n = 6 in each group). The neovasculogenesis positive subgroup (n = 6) received injection of EPCs incubated with 20 ng/mL SCF in matrigel. In the negative control subgroup (n = 6), mice received an injection of EPCs and matrigel only. For the experimental subgroups, mice received DATS by gavage feeding (oral tube feeding) at a dosage of 10 mg/kg of BW/day after the injection of EPCs and matrigel. At the end of the experiment (second week), the mice were euthanized with CO_2 inhalation and sacrificed. The matrigel implanted for neovasculogenesis was removed from the experimental animals and frozen in liquid nitrogen immediately. For the subsequent investigation, matrigels were sectioned and probed with a specific c-kit antibody for the identification of this stem cell biomarker.

Immunohistochemical Staining of Neovasculogenesis. Frozen matrigel sections were treated with 0.3% hydrogen peroxide to block the endogenous peroxide activity. Nonspecific protein binding was blocked with 10% normal goat serum (NGS) for 1 h followed by incubation with an anti-c-kit primary antibody (1:300). Tissue sections were washed with 0.1 M PBS and incubated with biotinyated immunoglobin G (1:300 secondary antibody) at room temperature for 1 h. Tissue sections were stained with avidin−biotin complex (ABC), diaminobenzidine (DAB), and hydrogen peroxide. Imaging was performed at either 200× or 400× magnification. Images of the matrigel sections were acquired on an Olympus BX-51 microscope using an Olympus DP-71 digital camera and imaging system.

Statistical Analysis. The quantitative methodology was used to determine whether there were differences in vascular formation between the experimental (the DADS and DATS groups) and control groups of EPCs. In brief, statistical analyses of the differences in neovasculogenic capacity were performed in triplicate using SYSTAT software. Confirmation of a statistically significant difference in the neovasculogenic index requires rejection of the null hypothesis of no difference between the mean neovasculogenic indices obtained from replicate sets of experimental and control groups at the P = 0.05 level with one-way analysis of variance (ANOVA). The Bonferroni post hoc test was used to determine differences among the different groups. A significant difference in protein expression between experimental and control groups was examined using the Student t test at the P = 0.05 level.

RESULTS

Garlic Organosulfur Components Significantly Induced Neovasculogenesis of Human EPCs in Vitro. The effects of the garlic organosulfur components DADS and DATS on neovasculogenesis in human EPCs were investigated in vitro. SCF was adopted as the positive control group. As shown in Figure 1A, SCF (at concentrations of 0, 2, 10, and 20 ng/mL) significantly induced neovasculogenesis in a dose-dependent manner (P < 0.05). At a concentration of 20 ng/mL, SCF induced neovasculogenesis up to 1.4-fold that of the unstimulated control group. We further investigated the neovasculogenic effects of DADS and DATS using vascular tube formation assay. As shown in Figure 1B,C, DADS and DATS (at concentrations of 0, 0.1, 1, and 10 μM) dose-dependently induced neovasculogenesis of human EPCs. At concentrations of 0.1, 1, and 10 μM, DADS significantly induced neovasculogenesis by approximately 1.6-, 2.7-, and 3-

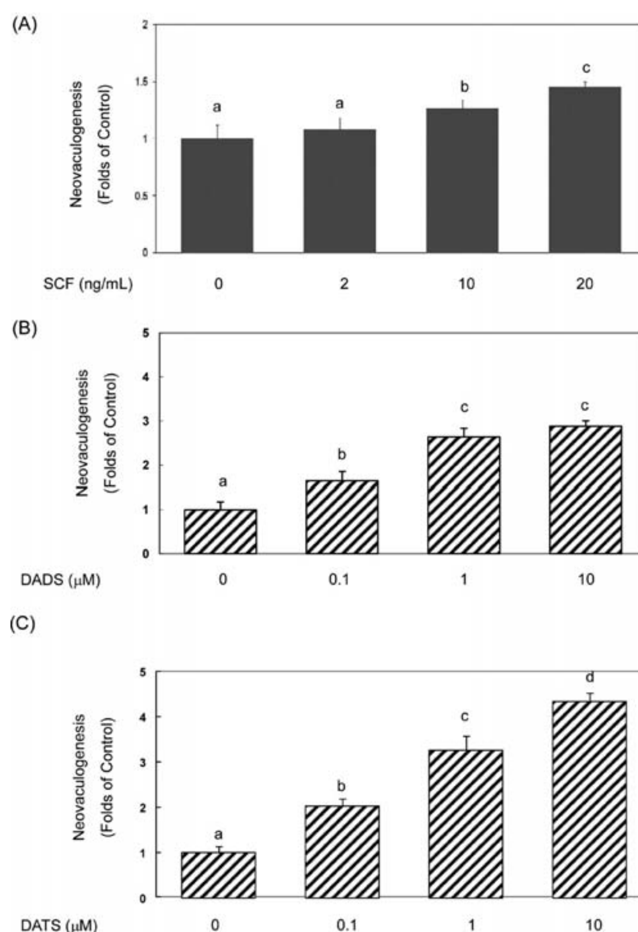
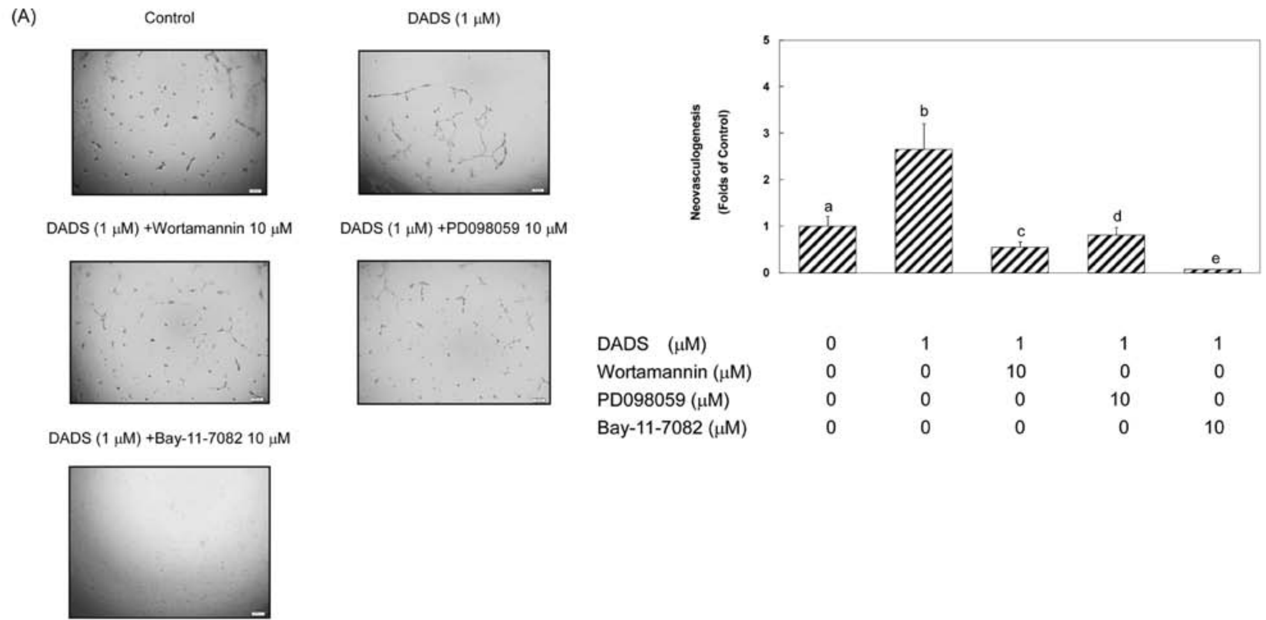
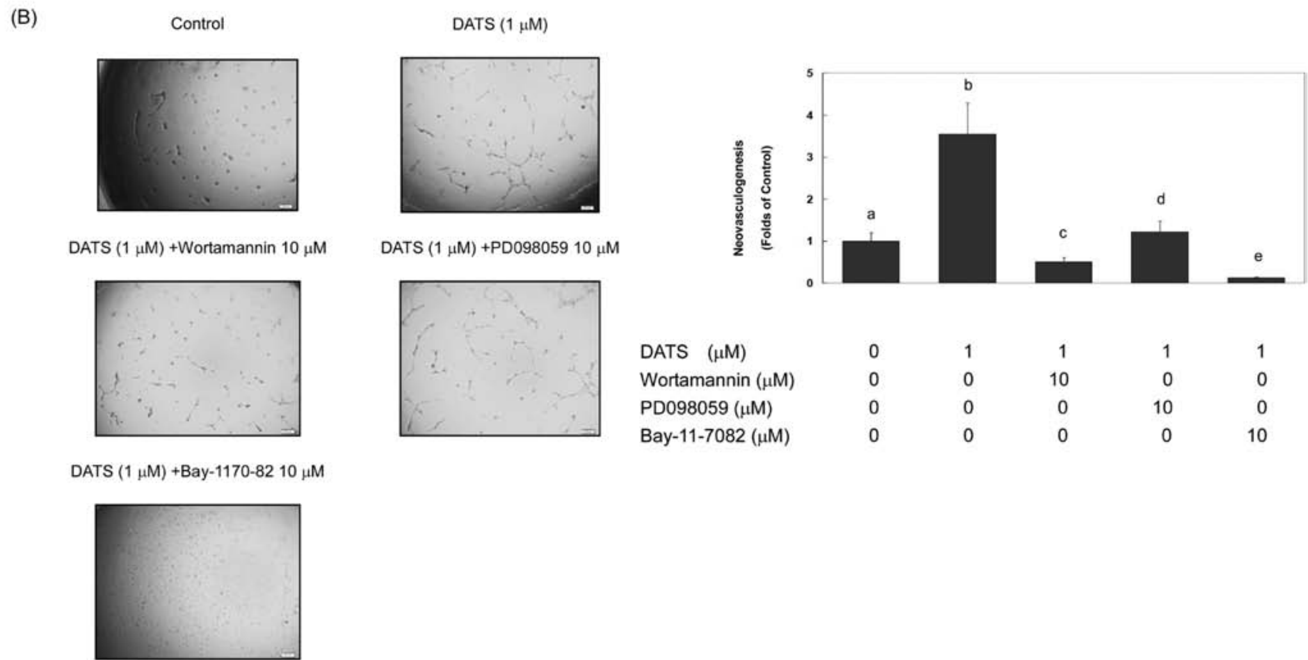


Figure 1. Garlic organosulfur components significantly induced neovasculogenesis of human EPCs in vitro. Neovasculogenesis of human EPCs was induced by (A) SCF (0, 2, 10, and 20 ng/mL) or (B) DADS or (C) DATS (at concentrations of 0.1, 1, and 10 μM) for 8 h until the measurement of tubular formation. The values are the mean ± SD of the total tube length in eight randomly selected fields in each culture dish, each carried out in triplicate and repeated twice. The different letters indicate a significant difference, P < 0.05.





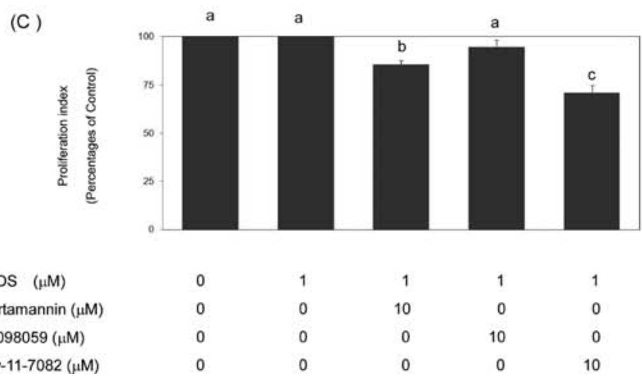


Figure 2. continued

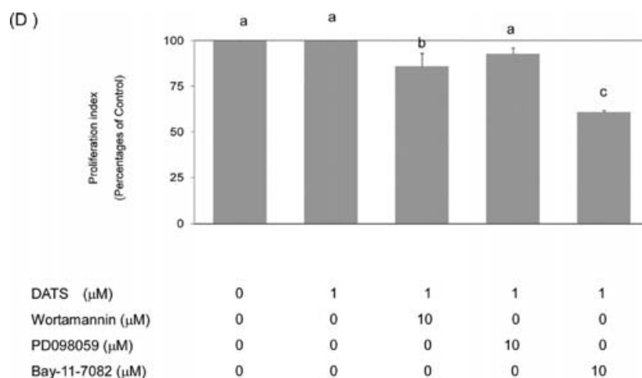


Figure 2. PI3-K/Akt/NF-κB and MAPK/ERK signaling pathways play an important role in DADS- and DATS-mediated neovasculogenesis in human EPCs. Neovasculogenesis of human EPCs incubated with wortmannin, PD098059, and Bay-11-7082 at a concentration of 10 μM in the presence of organosulfur compounds (A) 1 μM DADS or (B) 1 μM DATS was followed for 8 h until the measurement of tubular formation. The values are the mean ± SD of the total length of the tubes in eight randomly selected fields in each culture dish, each carried out in triplicate and repeated twice. Cell viability assays were carried out under the same conditions. Human EPCs were incubated with wortmannin, PD098059, and Bay-11-7082 at a concentration of 10 μM in the presence of organosulfur compounds (C) 1 μM DADS or (D) 1 μM DATS for 8 h until the measurement of cell viability. The proliferation index is represented as the mean ± SD. The different letters represent a statistically significant difference, $P < 0.05$.

fold ($P < 0.05$), respectively (Figure 1 B). Further analysis revealed that DATS at concentrations of 0.1, 1, and 10 μM significantly induced neovasculogenesis by approximately 2.1-, 3.2-, and 4.3-fold ($P < 0.05$), respectively (Figure 1C). These results indicate that DADS and DATS are able to induce neovasculogenesis of human EPCs in vitro.

PI3-K/Akt/NF-κB and MAPK/ERK Signaling Pathways Play an Important Role in DADS- and DATS-Mediated Neovasculogenesis in Human EPCs. To determine the molecular mechanisms underlying these actions, we further investigated the possible signaling pathways involved in DADS- or DATS-mediated neovasculogenesis. To achieve this aim, different specific inhibitors of the PI3-K, MEK/ERK, and NF-κB signaling cascades were utilized in this study. As shown in Figure 2A,B, treatment with wortmannin (a specific inhibitor of PI3-K), PD098059 (a specific inhibitor of MEK), or Bay 11-7082 (a specific inhibitor of NF-κB) significantly inhibited DADS-mediated (Figure 2A) and DATS-mediated (Figure 2B) neovasculogenesis in EPCs ($P < 0.05$). At the concentration of 10 μM, wortmannin significantly inhibited DADS- and DATS-mediated neovasculogenesis up to 79 and 81%, respectively. PD098059 (10 μM) significantly inhibited DADS- and DATS-mediated neovasculogenesis up to 69 and 54%, respectively. Bay 11-7082 (10 μM) significantly inhibited DADS- and DATS-mediated neovasculogenesis, even up to levels as high as 97 and 95%, respectively. However, wortmannin, PD098059, and Bay-11-7082 all had milder cytotoxic effects on human EPCs (Figure 2C,D). Bay 11-7082 (10 μM) inhibited both DADS- and DATS-mediated cell survival up to 39% (Figure 2C,D). Furthermore, wortmannin and PD098059 had milder cytotoxic effects on DADS- or DATS-mediated cell survival (Figure 2C,D). These results demonstrate that the inhibitory effects of wortmannin, PD098059, and Bay-11-7082 on neovasculogenesis were not exerted through their cytotoxic effects. In contrast, wortmannin, PD098059, and Bay-11-7082 played crucial roles in neovasculogenesis through an augmentation of vascular signaling pathways. These results suggest that the PI3-K/Akt/NF-κB and MEK/ERK signaling pathways are involved in DADS- and DATS-mediated neovasculogenesis.

DADS- and DATS-Mediated Neovasculogenesis Is Associated with Up-regulation of c-kit Protein as well as the Activation of the PI3-K/Akt/NF-κB and MAPK/ERK Pathways in Human EPCs. To verify the neovasculogenic effects, we further examined whether DADS and DATS would modulate the expression of c-kit vasculogenic protein and the phosphorylation levels of the signaling molecules ERK 1/2, Akt, and I-κB, as well as the downstream target GSK-3β and β-catenin proteins. As shown in Figure 3, DADS and DATS significantly induced the expression of c-kit protein ($P < 0.05$). DADS and DATS also significantly induced the phosphorylation of the Akt, GSK-3β, and I-κBα proteins in human EPCs ($P < 0.05$). In contrast, DADS and DATS further inhibited the phosphorylation of the β-catenin protein in human EPCs. The results suggest that DADS and DATS augmented the phosphorylation of the GSK-3β protein and stabilized its downstream target β-catenin protein through the suppression of the phosphorylated β-catenin level. Moreover, these results also suggest that DADS- and DATS-mediated phosphorylation of the I-κBα protein leads to the activation of the NF-κB pathway and accumulation of the nuclear NF-κB protein. The results demonstrated that DADS and DATS effectively enhanced the phosphorylation of ERK 1/2 molecules. It seems probable, therefore, that DADS and DATS effectively acted as neovasculogenic agents through the induction of c-kit expression as well as the activation of the PI3-K/Akt/NF-κB and MAPK/ERK pathways. Furthermore, these results indicate that DATS was more effective than DADS in terms of the transactivation of the Akt/NF-κB and ERK 1/2 signaling pathways.

DADS- and DATS-Mediated Neovasculogenesis Is Associated with Increased Levels of Nuclear β-Catenin and NF-κB Proteins in Human EPCs. To further test our hypothesis, we investigated and compared the neovasculogenic effects of DADS and DATS by analyzing the downstream nuclear proteins in human EPCs. As shown in Figure 4, DADS and DATS effectively modulated the phosphorylation of the nuclear NF-κB protein. Moreover, DADS and DATS significantly induced the levels of β-catenin and the cyclin D1 proteins in the nucleus of human EPCs. DATS is more effective than DADS in the induction of the nuclear β-catenin and cyclin

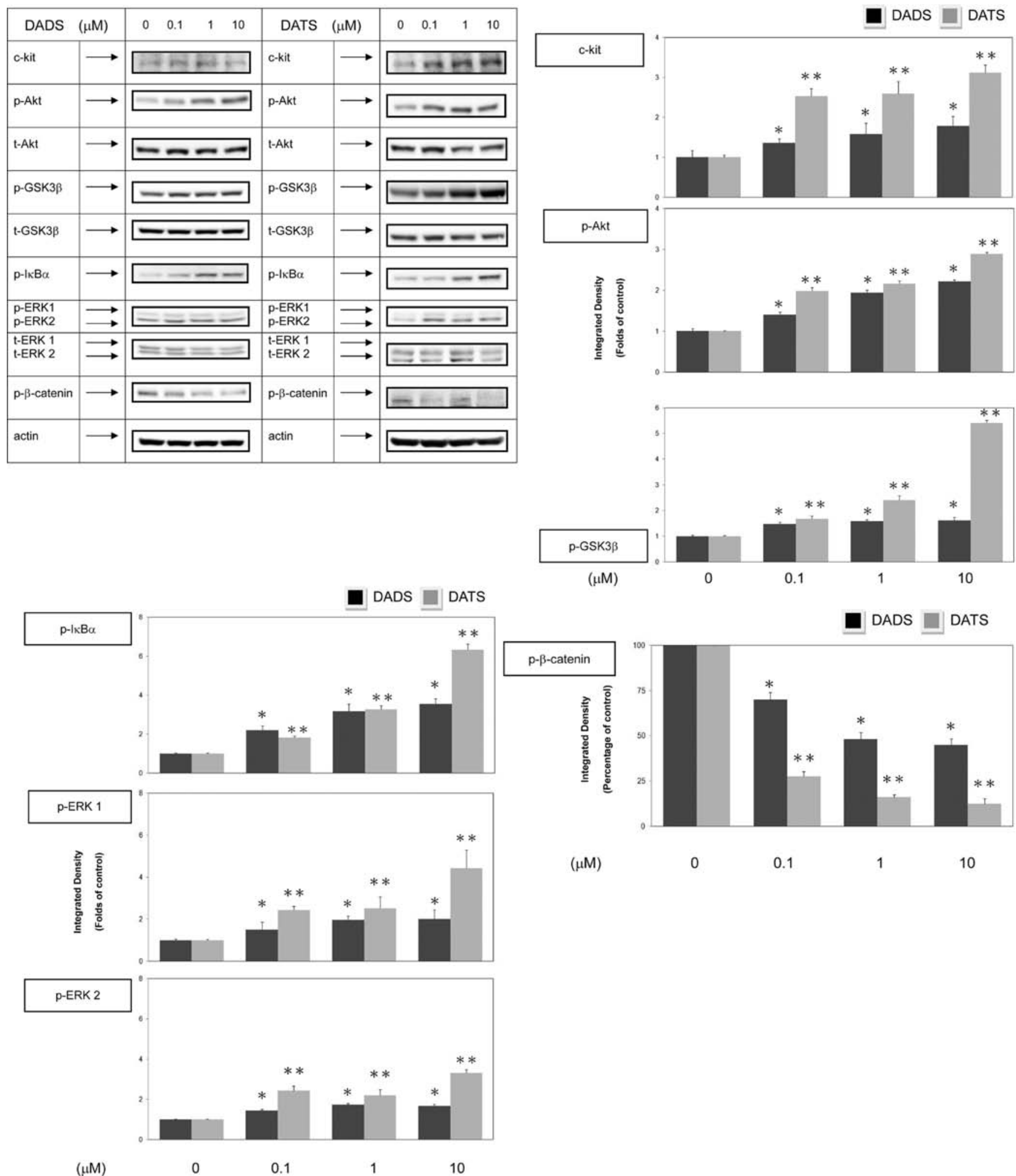


Figure 3. DADS- and DATS-mediated neovasculogenesis is associated with up-regulation of c-kit protein as well as the activation of the PI3-K/Akt/NF-κB and MAPK/ERK pathways in human EPCs. Human EPCs were treated with DADS or DATS (at concentrations of 0.1, 1, and 10 μM) in 10% FBS MCDB-131 for 8 h. Cytoplasmic proteins were prepared for Western blotting analysis using monoclonal antibodies against c-kit, p-Akt, t-Akt, p-GSK-3β, t-GSK-3β, p-ERK1/2,t-ERK 1/2, p-IκBα, p-β-catenin, and actin as described under Materials and Methods. The levels of detection represented the amount of c-kit, p-Akt, p-GSK-3β, p-ERK1/2, p-IκBα, and p-β-catenin in the cytoplasm of human EPCs. The results are representative of three different experiments. The immunoreactive bands are noted with an arrow. The integrated densities (mean ± SD) of these proteins adjusted with the corresponding control proteins (t-Akt, t-GSK-3β, t-ERK 1/2, or actin) are shown in the bottom panel. A single asterisk indicates a statistically significant difference compared to the DADS-untreated control group ($P < 0.05$). Double asterisks indicate a statistically significant difference compared to the DATS-untreated control group ($P < 0.05$).

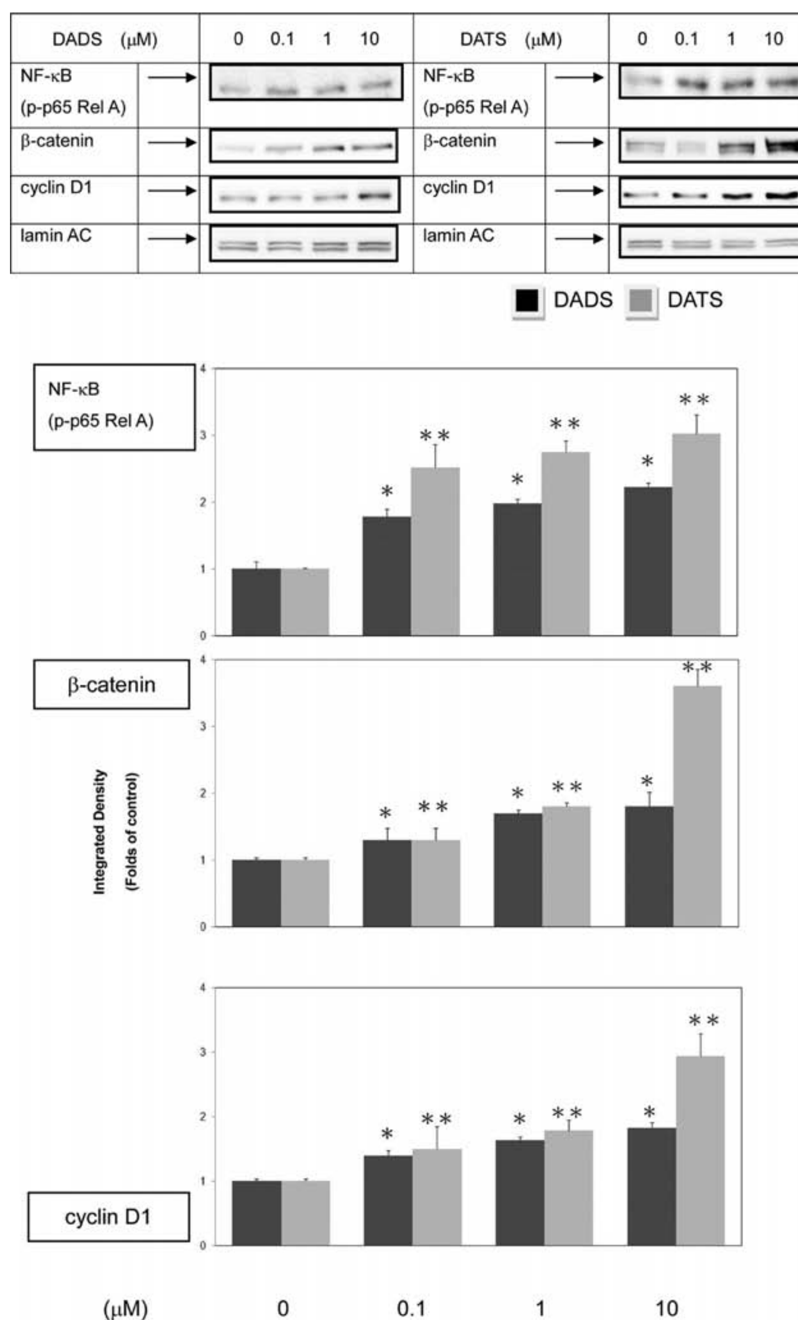


Figure 4. DADS- and DATS-mediated neovasculogenesis is associated with increased levels of nuclear β-catenin and NF-κB proteins in human EPCs. Human EPCs were treated with DADS or DATS (at concentrations of 0.1, 1, and 10 μM) in 10% FBS MCDB-131 for 8 h. Nuclear proteins were prepared for Western blotting analysis using monoclonal antibodies against t-β-catenin, cyclin D1, p-p65 Rel A (NF-κB), and lamin A/C as described under Materials and Methods. The levels of detection represent the amounts of cyclin D1, β-catenin, and NF-κB in the nuclei of human EPCs. The results are representative of three different experiments. The immunoreactive bands are noted with an arrow. The integrated densities (mean ± SD) of the β-catenin, cyclin D1, and NF-κB proteins adjusted with the internal control lamin A/C protein are shown in the bottom panel. A single asterisk indicates a significant difference compared to the DADS-untreated control group ($P < 0.05$). Double asterisks indicate a significant difference compared to the DATS-untreated control group ($P < 0.05$).

D1 proteins. These results suggested that DADS and DATS might function as neovasculogenic agents through the activation of multiple signaling pathways, including the PI3-K/Akt/NF-κB cascades and thus augment the expression of cyclin D1 protein.

DATS Induced the Up-regulation of c-kit and the Phosphorylation of Akt Molecules through the Suppression of Antivasculogenic miR-221 in Human EPCs. Previous studies indicated that miR-221 functions as an antivasculogenic miR targeting neovasculogenic c-kit mRNA by degradation and translational inhibition. To investigate whether DATS-mediated up-regulation of c-kit expression was in part through miR regulation, we examined the effects of DATS on the expression of miR-221 in human EPCs by using RT-PCR analysis. As shown in Figure 5A, DATS dose-dependently inhibited the expression of antivasculogenic miR-221 in human EPCs. Moreover, the results revealed that c-kit expression was restored through transfection of the anti-miR-

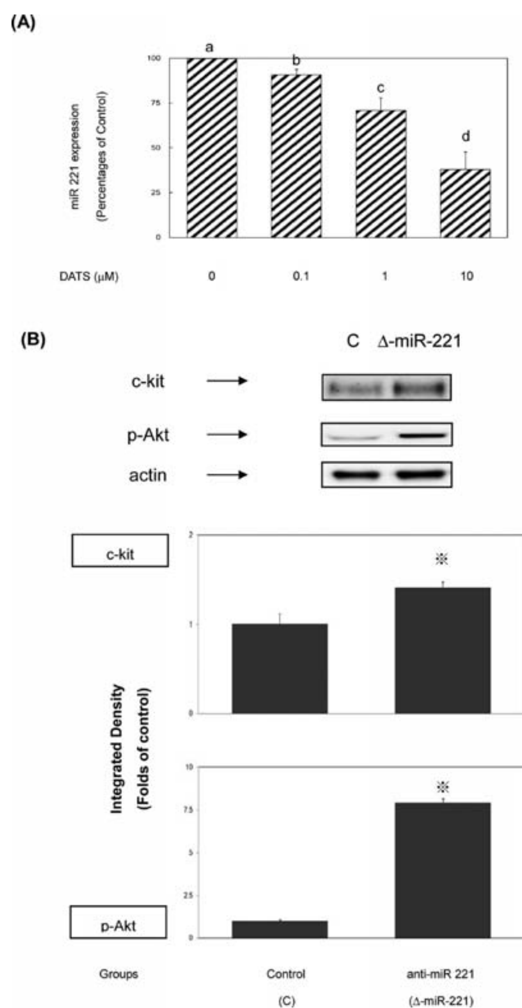


Figure 5. DATS induced the up-regulation of c-kit and the phosphorylation of Akt molecules through the suppression of antivasculogenic miR-221 in human EPCs. (A) Human EPCs were treated with DATS (at concentrations of 0.1, 1, and 10 μM) in 10% FBS MCDB-131 for 4 h. Total RNA was prepared from each group and then converted into cDNA for further analysis. Briefly, cDNA samples were used in a PCR reaction mix containing hsa-miR-221 specific primers, as described under Materials and Methods. U6 snRNA was used as the internal control. The expression level (mean ± SD) of miR-221 adjusted by the U6 snRNA is shown in the bottom panel. The different letters indicate a significant difference at $P < 0.05$. (B) Human EPCs cultured in 10% FBS MCDB-131 were transected with a control vector or anti-miR 221 plasmid (Δ-miR-221) for 8 h. Cytoplasmic proteins were prepared for Western blotting analysis using monoclonal antibodies against c-kit, p-Akt, and actin, as described under Materials and Methods. The levels of detection indicate the amount of c-kit or p-Akt of human EPCs. The results are representative of three different experiments. The immunoreactive bands are noted with an arrow. The integrated densities (mean ± SD) of these proteins adjusted by the internal control actin protein are shown in the bottom panel. The asterisk indicates a significant difference compared to the control group ($P < 0.05$).

221 plasmid (Δ-miR-221) (Figure 5B). The results also demonstrated that human EPC cells transfected with anti-miR 221 had increased levels of phosphorylated Akt molecules. It is thus plausible that suppression of miR-221 would lead to the increased c-kit expression and the phosphorylation (activation) of the Akt signaling cascade in human EPCs.

These results show that miR-221 inhibited the expression of c-kit protein in human EPCs. DATS acts as an effective agent to augment c-kit protein expression, Akt signaling cascades, and neovasculogenesis, in part through the suppression of miR-221, in human EPCs.

Consumption of DATS Induced Blood Vessel Formation and c-kit Protein Expression in a Mouse Xenograft Model of Neovasculogenesis. To further investigate these in vitro findings and the benefit of DATS, we examined whether DATS consumption would induce neovasculogenesis in a mouse xenograft model. Each mouse was given DATS (10 mg/kg of BW/day) for 2 weeks. SCF was adopted as a positive control group. As shown in Figure 6A, DATS consumption significantly induced the formation of new blood vessels in an EPC-transplanted mouse model of neovasculogenesis. It was shown that the neovasculogenic effect in the DATS group was significantly more than in SCF group. Moreover, DATS consumption significantly induced the

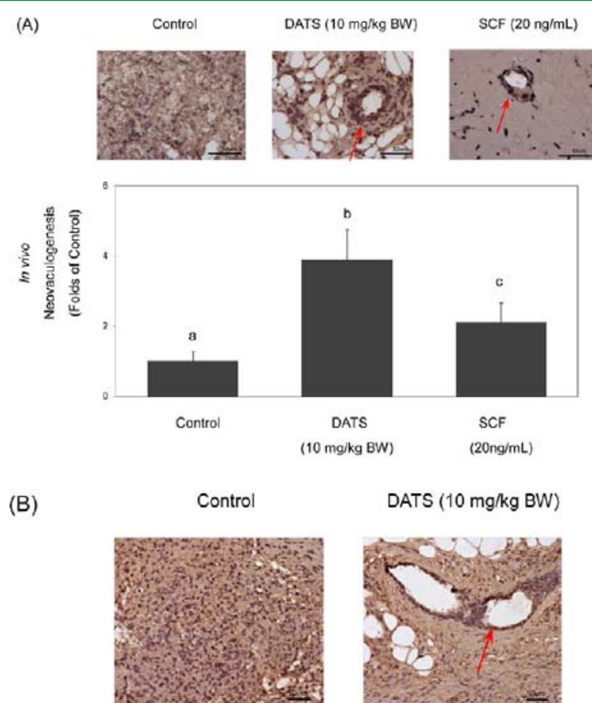


Figure 6. Consumption of DATS induced blood vessel formation and c-kit protein expression in a mouse xenograft model of neovasculogenesis. For the control group, human EPCs alone were transplanted into the experimental animals. For the SCF group, human EPCs were mixed with SCF (20 ng/mL) and sc transplanted into experimental animals. For the DATS group, experimental animals were inoculated with human EPCs and received DATS (10 mg/kg of BW/day) by gavage (oral tube feeding) for 2 weeks. (A) The matrigel samples from the experimental animals were frozen, sectioned, and documented at 400× magnification. Vascular vessel formation is indicated with a red arrow. The quantitative results (mean ± SD) of vascular tube formation are shown in the bottom panel. The different letters indicate a significant difference at $P < 0.05$. (B) The matrigel samples from experimental animals were frozen, sectioned, and subjected to an anti-c-kit antibody using IHC staining, as described under Materials and Methods. Imaging was documented at 200× magnification. Intense dark brown indicates the distribution and c-kit protein level during neovasculogenesis stained with the monoclonal antibody. Vascular vessel formation is indicated with a red arrow. The blue area represents the location of cell nuclei.

expression of the c-kit protein in these animals (Figure 6B). These results show that DATS consumption enhanced the expression of vasculogenic c-kit protein and the formation of new blood vessels in a mouse xenograft model. Taken together, it is clear that DATS has potential as an agent for augmenting the expression of neovasculogenic c-kit protein and thereby the formation of new blood vessels (neovasculogenesis) in both in vitro and in vivo models.

■ DISCUSSION

CVD, including both heart disease and stroke, are leading causes of death worldwide. Factors such as inadequate physical activity and other unhealthy lifestyle habits, such as excess caloric intake, have been shown to contribute to the increasing risk of this chronic disease. Therefore, revascularization in these ischemic tissues is a key step in the prevention and repair of the damage from stroke and heart disease. Adult angiogenesis occurs through the migration and proliferation of mature endothelial cells from pre-existing vessels.[24] However, these mature endothelial cells are terminally differentiated and normally have a low proliferative potential. Therefore, the capability of these mature endothelial cells to repair and/or replace damaged endothelial cells limited. Human EPCs from adult bone marrow possess an excellent repair capability as well as neovasculogenic characteristics. These human bone marrow-derived EPCs are similar to angioblasts and have the potential to differentiate into endothelial cells in a less-limited manner.

To the best of our knowledge, this is the first demonstration that the garlic organosulfur components DADS and DATS are effective agents in the induction of neovasculogenesis, and this result was shown in both in vitro and in vivo studies. The results show that, in vitro, DADS and DATS dose-dependently enhanced neovasculogenesis of human EPCs, in part through the up-regulation of c-kit protein. The molecular actions of these garlic organosulfur components were mediated by the activation of multiple signaling pathways, including the PI3-K/Akt/NF-κB and ERK 1/2 pathways (Figure 2A,B). At most there were only mild cytotoxic effects observed in the wortmannin-, PD-098059-, or Bay-11-7082-treated cells (Figure 2C,D). DATS induced the neovasculogenesis by modulation of the GSK-3β/β-catenin cascades and up-regulation of cyclin D1 expression. Moreover, DATS blocked the expression of miR-221 in human EPCs.

There are several scenarios in which garlic organosulfur components such as DADS and DATS would be able to induce neovasculogenesis. One is the augmented activation of the Akt/NF-κB and ERK 1/2 molecules in human EPCs. The activation of the Akt pathway and subsequent phosphorylation of GSK-3β would lead to the decreased phosphorylation of β-catenin. It is known that cyclin D1 expression is under the control of β-catenin. Akt activation would enhance the nuclear level of the β-catenin protein through a reduction in the phosphorylated β-catenin level in human EPCs. It would in turn lead to the up-regulation of cyclin D1 and increased cell proliferation and survival in human EPCs. Alternatively, the Akt activation might induce the phosphorylation of IκB and nuclear accumulation of the NF-κB protein in human EPCs. Previous study demonstrates that endothelial nitric oxide synthase (NOS)/nitric oxide (NO) may play an important role in the protection against cardiovascular disease. The activation of e-NOS is regulated by the activation of the PI3-K/Akt signaling pathway.[25] Inhibition of the PI3-K/Akt/eNOS pathway was reported to enhance endothelial cell apoptosis.[25] These studies have suggested that DATS protects the ischemic myocardium by the induction of nitric oxide in vascular tissues.[23] Previous study suggested that organosulfur compounds may function as hydrogen sulfide donors and modulate signaling pathways.[26] The results also demonstrated that DATS is even more effective than DADS in the augmentation of neovasculogenesis. These results are consistent with previous findings showing that DATS (containing three sulfurs) has a stronger effect than DADS (containing two sulfurs) on the induction of Akt activity.[27] Here, we report a novel role of DATS in EPC-mediated neovasculogenesis. In the current study, the results show that DATS up-regulated the expression of the neo-vasculogenic c-kit protein and activated the PI3-K/Akt/NF-κB signaling pathways. Suppression of Akt and NF-κB activities by specific inhibitors would result in reduced levels of neovasculogenesis in human EPCs. Therefore, maintaining the expression of c-kit and the activation of crucial signaling pathways such as Akt and NF-κB seems to be the mechanisms of DADS/DATS-mediated neovasculogenesis. The results also showed that human EPCs transfected with an anti-miR 221 plasmid were prone to augmentation of the c-kit protein. Furthermore, it was also demonstrated that DATS modulated the expression of the c-kit protein through the suppression of anti-neovasculogenic miR 221 expression. Previous studies had indicated that up-regulation of miRNA 221 suppressed the expression of vasculogenic c-kit protein, and this has an impact on angiogenesis in human endothelial cells.[15] The level of antivasculogenic miR 221 in the EPCs from CVD patients was shown to be significantly higher than the one from healthy subjects.[17] This report suggested that the expression of miR 221 was inversely associated with the proliferation of human endothelial cells and endothelial progenitor cells.[16,28] Here, our results also demonstrate that transfection with the anti-miR-221 plasmid significantly induced c-kit protein expression. Moreover, the results also indicate that human EPC cells transfected with anti-miR 221 exhibit augemented phosphorylation of the Akt molecule. A previous study showed that miR221 induced the activation of the Akt pathway so as to promote tumor progression in glioma cancer cells.[29] The evidence reported here demonstrates that suppression of miR-221 leads to the up-regulation of c-kit expression and the phosphorylation (activation) of the Akt signaling cascade in human EPCs. Moreover, DATS blocked the expression of miR-221 in human EPCs. These results suggest that DATS induced neovasculogenesis through the modulation of crucial signaling pathways and the suppression of miR 221.

To further examine these important in vitro findings, we performed an in vivo murine model of neovasculogenesis. As shown in Figure 6A, the results showed that consumption of DATS enhanced the formation of new blood vessel derived from human EPCs in a mouse xenograft model of neovasculogenesis. Furthermore, DATS consumption significantly augmented the expression of neovasculogenic c-kit protein in these experimental animals (Figure 6B). At a dosage of 10 mg/kg of BW/day, DATS significantly induced the formation of new blood vessels by up to 4.1-fold compared with the control group (Figure 6A). There was no hepatoxicity of DATS at 10 mg/kg of BW observed in this study (data not shown). DATS consumption at a dose of 10 mg/kg of BW/day in mice is equivalent to an average consumption of DATS at a dose of 0.8 mg/kg of BW/day for humans (or approximately 56 mg of DATS per day for humans with a 70 kg BW).[30] Previous studies indicated that the average intake of garlic consumption

for humans is highly variable, ranging from 0.5 to 6 g/day garlic in many countries.[20,31−33] The results from the current study show that DATS effectively enhances the functional activities and neovasculogenic potential of human EPCs both in vitro and in vivo. Previous studies have indicated that the migratory capacity of EPCs is mainly influenced by hypertension in coronary artery disease patients.[34] Moreover, garlic consumption is reported to significantly reduce blood pressure.[21] On the basis of our findings in these experimental animals, DATS has potential as an agent that is able to induce neovasculogenesis. Although we have not verified the beneficial effects of whole garlic extracts in vivo, these findings suggest that garlic extracts containing DATS would contribute to neovasculogenesis in human EPCs. In conclusion, DATS may have potential as a novel clinical agent that is able to induce neovasculogenesis.

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