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Novel angiogenesis inhibitory activity in cinnamon extract blocks VEGFR2 kinase and downstream signaling

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As a critical factor in the induction of angiogenesis, vascular endothelial growth factor (VEGF) has become an attractive target for anti-angiogenesis treatment. However, the side effects associated with most anti-VEGF agents limit their chronic use. Identification of naturally occurring VEGF inhibitors derived from diet is a potential alternative approach, with the advantage of known safety. To isolate natural inhibitors of VEGF, we established an *in vitro* tyrosine kinase assay to screen for diet-based agents that suppress VEGFR2 kinase activity. We found that a water-based extract from cinnamon (cinnamon extract, CE), one of the oldest and most popular spices, was a potent inhibitor of VEGFR2 kinase activity, directly inhibiting kinase activity of purified VEGFR2 as well as mitogen-activated protein kinase- and Stat3-mediated signaling pathway in endothelial cells. As a result, CE inhibited VEGF-induced endothelial cell proliferation, migration and tube formation *in vitro*, sprout formation from aortic ring *ex vivo* and tumor-induced blood vessel formation *in vivo*. Depletion of polyphenol from CE with polyvinylpyrrolidone abolished its anti-angiogenesis activity. While cinnamaldehyde, a component responsible for CE aroma, had little effect on VEGFR2 kinase activity, high-performance liquid chromatography-purified components of CE, procyanidin type A trimer (molecular weight, 864) and a tetramer (molecular weight, 1152) were found to inhibit kinase activity of purified VEGFR2 and VEGFR2 signaling, implicating procyanidin oligomers as active components in CE that inhibit angiogenesis. Our data revealed a novel activity in cinnamon and identified a natural VEGF inhibitor that could potentially be useful in cancer prevention and/or treatment.

Introduction

Angiogenesis, the formation of new vessels from preexisting vasculature, is an important mechanism used by tumors to promote growth and metastasis (1) and is tightly regulated by an intricate balance between stimulators and inhibitors (2). Vascular endothelial growth factor (VEGF) is one of the most critical and specific angiogenesis factors regulating normal physiological and tumor angiogenesis (3). It induces angiogenesis via binding to its two receptor tyrosine kinases expressed on endothelial cells, namely, vascular endothelial growth factor receptor 1 (VEGFR1) (Flt-1), required mainly for mitogenic and chemotactic responses, and VEGFR2 (KDR/Flk-1), which contributes to endothelial cell morphogenesis (3). By binding to and activating VEGFR, VEGF initiates a signal transduction cascade that affects numerous processes required for the formation of blood capillaries (4), including endothelial cell proliferation, migration, survival, tube formation, incorporation of endothelial cell precursors, recruitment of pericyte and smooth muscle cells and extracellular matrix remodeling.

VEGF is viewed as an attractive therapeutic target for the development of novel anticancer agents (5), and a variety of approaches to inhibit VEGF activity are currently being assessed in preclinical and clinical trials. These include monoclonal antibodies targeting VEGF ligands or VEGFRs (6), soluble receptors that sequester ligands (7) and small molecule inhibitors that inhibit kinase activity (8). Three drugs developed for their anti-angiogenic actions, bevacizumab (Avastin®), sunitinib malate (Sutent®, SU11248) and sorafenib (Nexavar®, BAY 43-9006), have been approved by the United States Food and Drug Administration for treatment of patients with specific types of cancer—all three inhibit VEGF signaling by blocking VEGF ligand or VEGFR (9).

However, serious side effects, such as hypertension, bleeding and gastrointestinal perforation, have been associated with currently available anti-VEGF agents, limiting their chronic use (9). There has, consequently, been a renewed interest in identifying natural food sources (diet-based approach) potentially rich in anti-VEGF agents, given the advantage of proven safety for human use (10–13). Additionally, consumption of a plant-based diet has been implicated in the prevention of cancer development and progression (14,15).

It has been well-established that polyphenols, especially flavonoids, are beneficial active components found in natural food products (14,15). They are abundant in a variety of foods including tea, coffee, fruits, vegetables, beans (soy), grains, seeds and spices. Recently, polyphenols extracted from various plants, including soy, berry, pomegranate, grape seed extract and green tea, have been found to be potent inhibitors of angiogenesis (16–26). However, the inhibitory activity of dietary components on VEGFR has not been fully elucidated (27–30).

To identify natural inhibitors of VEGF signaling, we have established an *in vitro* tyrosine kinase assay to screen for diet-based agents that suppress VEGFR2 kinase activity. We found that a water-based extract from cinnamon was a potent natural inhibitor of VEGFR2 kinase activity.

Abbreviations: BrdU, bromodeoxyuridine; EBM, endothelial basal medium; ECGS, endothelial cell growth supplement; FCS, fetal calf serum; HUVEC, human umbilical vascular endothelial cell; PVPP, polyvinylpyrrolidone; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Materials and methods

Cinnamon extract

Ground cinnamon (*Cinnamomum zeylanicum*) powder, obtained from Frontier Natural Products Co-op in Norway, IA, was dissolved in water (70°C, 1 h). The solution was centrifuged (13 000 r.p.m., 10 min) to remove insoluble ingredients and the supernatant was then passed through a 0.22 mm filter and used for this study. A water-based cinnamon (*C.zeylanicum*) prepared by Dr John Lew's group (University of California, Santa Barbara), was also used to confirm some of the results obtained (Figures 1A and B and 6A and B) (31).

In vitro kinase assay

In vitro VEGFR2 tyrosine kinase activity was assayed using an enzyme-linked immunosorbent assay kit (Sigma, St Louis, MO) as described previously (30). Briefly, cinnamon extract (CE) was incubated with VEGFR2 (Upstate) in kinase reaction buffer in 96-well plates coated with a poly-Glu-Tyr substrate. Substrate phosphorylation was monitored using a phosphotyrosine-specific monoclonal antibody-conjugated to horseradish peroxidase. Results were expressed as percent of control (not treated with CE); IC50 values were defined as the drug concentration that resulted in 50% inhibition of enzyme activity.

Immunoblot

Human umbilical vascular endothelial cells (HUVECs; Lonza, Basel, Switzerland) were cultured (24 h) in endothelial basal medium (EBM)-2 containing 2% fetal calf serum (FCS), incubated (30 min) with various concentrations of CE and then stimulated with VEGF (100 ng/ml). Total cell extracts were prepared in Laemmli sample buffer. Proteins were resolved by electrophoresis on sodium dodecyl sulfate gels then transferred to polyvinylidene difluoride membranes. The membrane was incubated with primary antibodies anti-VEGFR2, anti-phospho-VEGFR2, anti-p44/42 MAPK, anti-phospho-p44/42

mitogen-activated protein kinase (MAPK), anti-Stat3, anti-phospho-Stat3, anti-Jak2, anti-phospho-Jak2, anti-Src and anti-phospho-Src (Cell Signaling, Boston, MA) followed by horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate (Thermo Scientific, Rockford, IL).

Cell proliferation

HUVEC and bovine capillary endothelial cells (a generous gift from Catherine Butterfield and Dr Judah Folkman) were used for endothelial cell proliferation assay, as described previously (30). Endothelial cells were plated onto gelatinized 24-well culture plate in 0.5 ml EBM-2 (from Lonza for HUVEC) containing 10% FCS or Dulbecco's modified Eagle's medium (from Cellgro, Manassas,VA for bovine capillary endothelial cells) containing 10% bovine calf serum, incubated (24 h) and then treated with various concentrations of CE in the absence or presence of basic fibroblast growth factor (1 ng/ml) and VEGF (100 ng/ml; PeproTech, Rockyhill, NJ) or cocktail containing various growth factors (SingleQuo® kit; Lonza). After 48 h incubation, cells were trypsinized and counted with a Coulter counter.

For non-endothelial cell proliferation, cells were plated on to 24-well culture plate in 0.5 ml RPMI1640 culture medium (Cellgro; for MDA-MB-231 human breast cancer cells), McCoy 5A culture medium (Irvine Scientific, Santa Ana, CA; for human colon cancer cells) and MEM culture medium (Cellgro; for MCF-10A) containing 10% FCS, incubated (24 h) and then treated with various concentrations of CE. Cell numbers were determined by using a Coulter counter after 48 h incubation.

BrdU incorporation

DNA synthesis was measured by bromodeoxyuridine (BrdU) cell proliferation enzyme-linked immunosorbent assay (Roche, Indianapolis, IN). Briefly, 3000 HUVECs per well in 0.1 ml EBM-2 (from Lonza) containing 10% FCS were plated in 96-well tissue culture grade flat bottom plates, incubated for 24 hr and then treated (30 min) with various concentrations of CE in EBM-2 containing 2% FCS, followed by stimulation with VEGF (100 ng/ml). After 24 h, cells were incubated with BrdU labeling solution (37°C, 4 h), fixed and incubated with anti-BrdU antibody. Color intensity of substrate reaction was measured by an enzyme-linked immunosorbent assay reader at 405 nM.

Annexin V staining

Apoptosis was measured by annexin V apoptosis assay kit (Invitrogen). Briefly, HUVECs were cultured in endothelial cell growth medium-2 (Lonza) containing 10% FCS. At ~40% confluency, cells were treated with varying concentrations

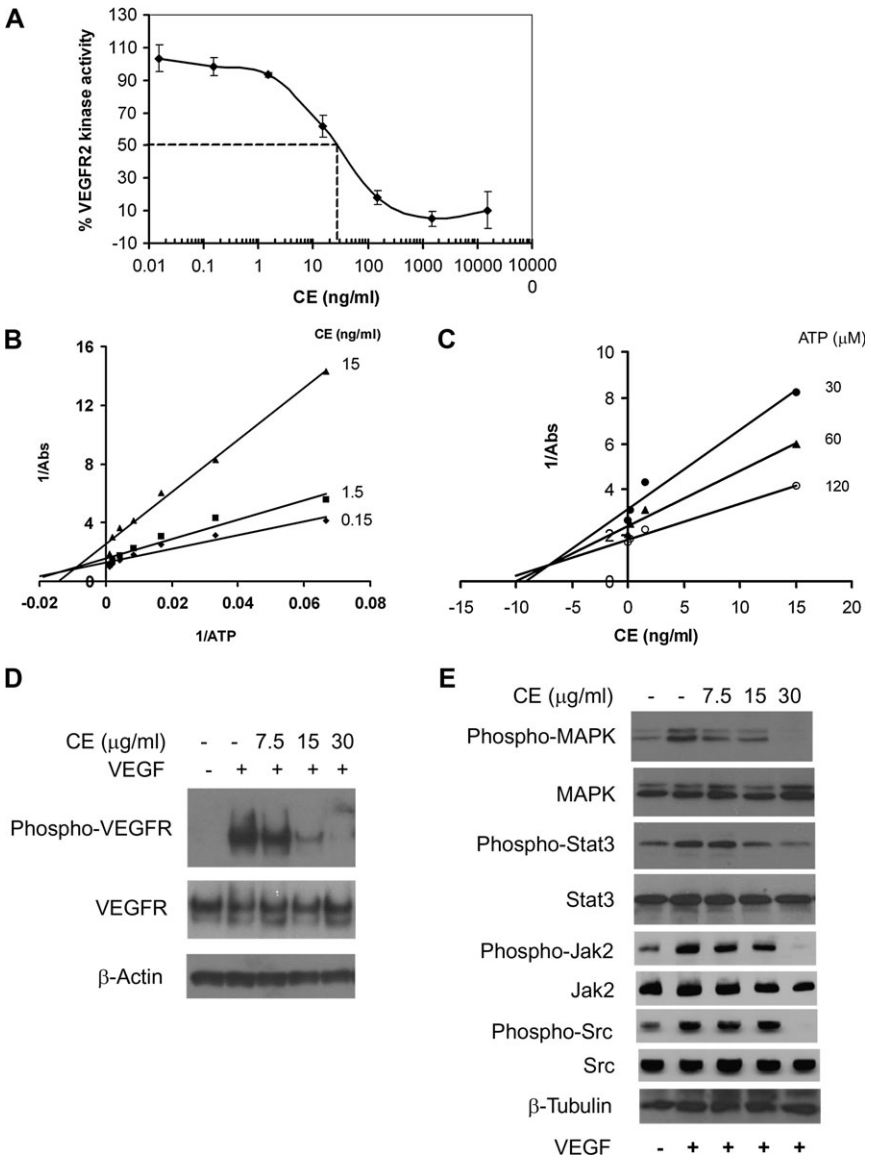

Fig. 1. CE inhibits VEGFR2 kinase activity. (**A**) VEGFR2 was incubated with various concentrations of CE and substrate phosphorylation was monitored by enzyme-linked immunosorbent assay. Data are represented as percentage of control (not treated with CE) and are mean ± SD from four experiments. (**B**) Lineweaver–Burk plot and (**C**) Dixon plot of the inhibition of VEGFR2 by CE. Increased concentrations of adenosine triphosphate (ATP) were incubated with VEGFR2 and various concentration of CE. (**D** and **E**) CE inhibits VEGFR2 signaling. Quiescent HUVECs were incubated in the presence or absence of CE followed by stimulation with VEGF for another 5 min (for VEGFR2) or 2 h (for MAPK, Stat3, Jak2 and Src). Phosphorylation of VEGFR2, MAPK, Stat3, Jak2 and Src was assessed by western blot. β-Actin/β-tubulin level was used as a loading control. Results are representative of two to four experiments.

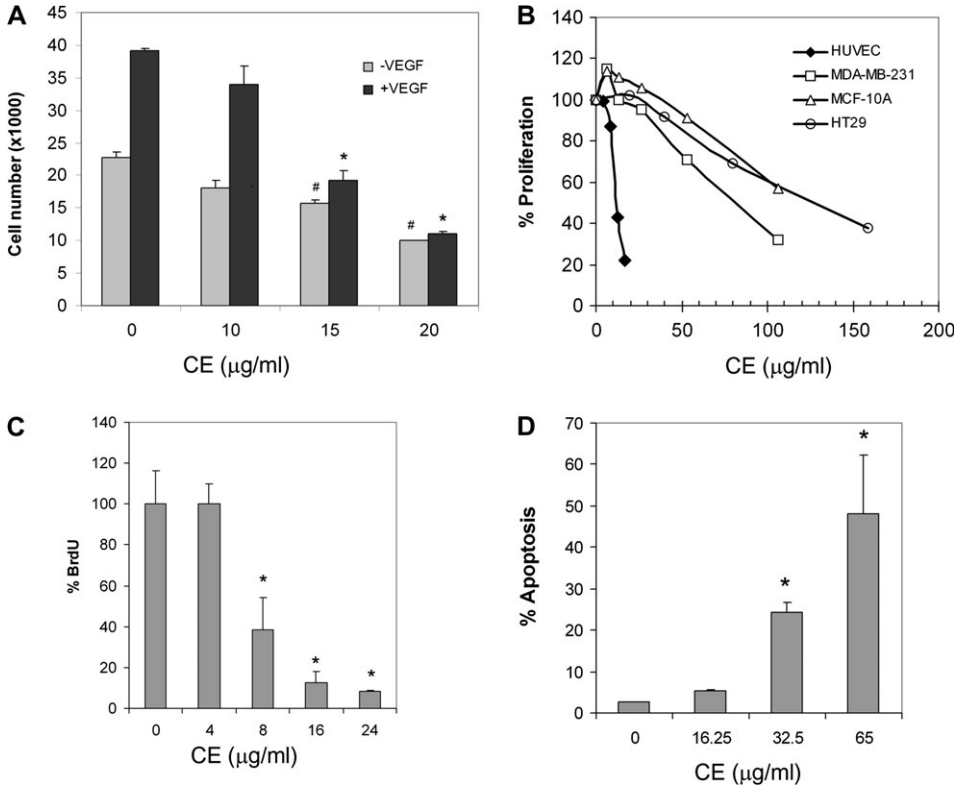

Fig. 2. CE inhibits endothelial cell proliferation. (**A**) HUVECs were treated with various concentrations of CE, stimulated with VEGF then cell number was assessed 48 h later. Data are mean ± SD ($n = 3$). $^{*}P < 0.05$ versus control with VEGF and $^{\#}P < 0.05$ versus control without VEGF. (**B**) MDA-MB-231, HT29 and MCF-10A cells were treated with various concentrations of CE and cells counted 48 h later. Data are represented as percentage of vehicle-treated control. (**C**) HUVEC were treated with various concentrations of CE and then labeled with BrdU. DNA synthesis was measured by BrdU cell proliferation enzyme-linked immunosorbent assay. Data are represented as percentage of vehicle control and are mean ± SD ($n = 3$). $^{*}P < 0.05$ versus control. (**D**) CE-induced endothelial cell apoptosis. HUVECs were treated with various concentration of CE then labeled with annexin V. Data represent percentage of apoptotic cells and are mean ± SD ($n = 3$). $^{*}P < 0.05$ versus control.

(0, 16.25, 32.5 and 65 mg/ml) of CE. After 24 h, both floating and attached cells were collected and stained with annexin V-conjugated with Alexa Fluor 488 dye and propidium iodide. The signals were then detected by fluorescence-activated cell sorting analysis.

Cell migration

Endothelial cell migration was assessed using a modified Boyden chamber assay, as described previously (30). Transwells (8 mm pore; Corning, Lowell, MA) pre-coated with 200 mg/ml Matrigel (BD Bioscience, Franklin Lake, NJ) were used for the assay. HUVECs (1.5×10^5) were plated in EBM-2 containing 0.05% FCS in the upper chamber of the transwell in the presence of various concentration of CE for 30 min at 37°C. EBM-2 containing 0.05% FCS and VEGF (50 ng/ml) was then added to the lower chamber. After 5 h incubation, cells were stained and non-migrated cells (on the top side of membrane) were removed by cotton swap. The number of migrated cells was quantified by counting the cells at ×40 objectives.

Tube formation

HUVECs (50 000 cells per well) were harvested and seeded in EBM-2 (2% fetal bovine serum) containing VEGF (100 ng/ml) onto 24-well culture plates coated with 250 μl Matrigel (BD Bioscience). Various concentrations of CE were added at plating then incubated overnight (16 h). Cells were photographed using phase-contrast microscopy.

Chick aortic ring assay

The aortic arch was dissected from day 12 to 14 chick embryos, cut into rings and embedded into Matrigel in four-well plates (NUNC, Rochester, NY), as described previously (30). After incubation (37°C, 10 min), aortic rings were fed with MCDB-131 serum-free medium (GIBCO; Invitrogen) containing various concentrations of CE with or without VEGF (100 ng/ml) or endothelial cell growth supplement (ECGS; Sigma) (50 mg/ml). Growing sprouts were photographed with an Olympus inverted IX81 at ×40 magnification.

Matrigel plug assay

MDA-MB-231 cells (5×10^6 cells) were mixed with phenol red-free Matrigel and injected into both flanks of severely combined immunodeficient mice (SCID). For cinnamon-treated group, Matrigel was mixed with cells in the presence of CE (15 or 75 μg/ml). Matrigel mixed with medium alone (500 ml) was used as a negative control; 10 days after implantation, Matrigel plugs were removed and the surrounding tissues trimmed. Hemoglobin content of the gel plug was measured using a Drabkin's reagent kit (Sigma) according to the manufacturer's instructions. The concentration of hemoglobin was calculated based on a set of hemoglobin standards. Blood vessels in the Matrigel were visualized with antibody against CD31, as described previously (30).

Polyvinylpyrrolidone depletion

CE was incubated (room temperature, 30 min) with polyvinylpyrrolidone (PVPP) to deplete polyphenols. PVPP was then removed by centrifugation (13 000 r.p.m., 10 min). The absorbance of the supernatant was measured (280 nm) to ensure complete removal of polyphenol.

High-performance liquid chromatography

Trimeric and tetrameric procyanidins were isolated from CE using high-performance liquid chromatography as described previously (32).

Statistical analysis

Data were expressed as mean ± SD. Analysis of variance test followed by Dunnett's *post-hoc* test was used to compare the means for the multiple groups. Each assay was repeated two to four times. $^{*}P < 0.05$ was considered statistically significant.

Results

Effect of CE on VEGFR2 kinase activity

In vitro tyrosine kinase assay was used to screen plant-based diets for new inhibitors of angiogenesis. CE, which is enriched in polyphenols,

was found to effectively inhibit kinase activity of purified VEGFR2 with an IC50 of ∼30 ng/ml (Figure 1A). SU5416, a known inhibitor of VEGFR2, was used as a positive control and showed inhibition of kinase activity with an IC50 of 1 μM (data not shown), in agreement with previous studies (33).

To understand the biochemical mechanism of CE inhibition of VEGFR2, we assayed VEGFR2 kinase activity at various concentrations of adenosine triphosphate in the presence of increasing concentrations of CE. A Lineweaver–Burk plot analysis revealed that increasing concentrations of CE affected apparent 1/Vmax (*y*-axis intersection), indicating CE behaved mainly as a non-competitive inhibitor for adenosine triphosphate binding (Figure 1B). The Ki for the inhibition of CE was ∼7.5 ng/ml, as calculated by Dixon plot (Figure 1C).

Given the observed inhibition of VEGFR2 kinase activity, we further examined the effects of CE on phosphorylation of VEGFR2 to determine whether it inhibited VEGFR2-mediated signaling pathways in endothelial cells. We found that VEGFR2 was phosphorylated by the addition of exogenous VEGF to HUVECs (Figure 1D). Pretreatment of cells with CE significantly blocked VEGF-induced phosphorylation of VEGFR2, without affecting overall VEGFR2 expression levels.

In order to identify the downstream signaling pathway targeted by CE treatment, we examined the expression and phosphorylation of MAPK, one of the key signaling pathway components supporting endothelial cell proliferation (34). While treatment of HUVEC with CE inhibited VEGF-dependent phosphorylation of MAPK in a dose-dependent manner, total MAPK levels were unaffected (Figure 1D). Another critical signaling pathway activated by VEGF in endothelial cells is Stat3 (35–37). We found that the phosphorylation of Stat3, as well as Jak2 and Src kinase, two main kinases that phosphorylate Stat3, was also significantly decreased in the presence of CE (Figure 1E). Higher doses of CE appeared to be required to achieve effective inhibitory effects on VEGFR2 expressed by endothelial cells compared with the dose used to inhibit kinase activity of purified VEGFR2 *in vitro*. This may have been due to the possibility that the active components in CE were not stable in culture or were not able to efficiently penetrate cells to exert an effect. Taken together, our data implicated CE as a potent angiogenesis inhibitor by inhibiting VEGFR2-mediated signaling pathway.

Effect of CE on endothelial cell proliferation, migration and tube formation

To characterize the anti-angiogenesis activity of CE, we first determined whether CE inhibited growth factor-induced endothelial cell proliferation. HUVEC (Figure 2A) and bovine capillary endothelial cells (data not shown) were incubated with CE then stimulated with VEGF (100 ng/ml), basic fibroblast growth factor (1 ng/ml) or growth medium containing various growth factors; cell count was assessed 48 h later.

While HUVEC proliferation was significantly increased in response to VEGF treatment in the absence of CE, it was markedly suppressed in the presence of CE (Figure 2A). In contrast to endothelial cells, much higher concentrations of CE were needed to inhibit the proliferation of non-endothelial cells, such as HT29 human colon cancer cells, MDA-MB-231 human breast cancer cells and MCF-10A mammary epithelial cells (Figure 2B), suggesting that CE had greater specificity as an inhibitor for VEGF-induced endothelial cell proliferation.

To clarify whether the observed reduction in cell number in HUVEC resulted from reduced cell growth and/or increased cell death, we studied the effects of CE on DNA synthesis and apoptosis by measuring BrdU incorporation and annexin V staining, respectively. Our data suggested that CE effectively inhibited DNA synthesis and induced apoptosis in these cells (Figure 2C and D).

We next studied the effects of CE on VEGF-induced cell migration using a modified Boyden chamber assay (30,38). In the absence of CE, a large number of HUVECs migrated to the lower side of the filter in the transwell chamber following stimulation with VEGF (50 ng/ml) (Figure 3A). However, in the presence of CE, the number of migratory cells was significantly reduced in response to VEGF, in a concentration-dependent manner.

To further characterize its anti-angiogenesis activity, we examined the effects of CE on VEGF-induced tube formation by HUVEC on Matrigel, a well-established angiogenesis assay. HUVECs formed tube-like networks within 8 h, which might, in part, reflect the process of angiogenesis. At a concentration of 32 μg/ml, CE effectively abrogated endothelial tube formation, reducing the tube-like structure both in width and in length. Endothelial cells rounded up and rendered network structures incomplete and broken in the presence of CE (Figure 3B).

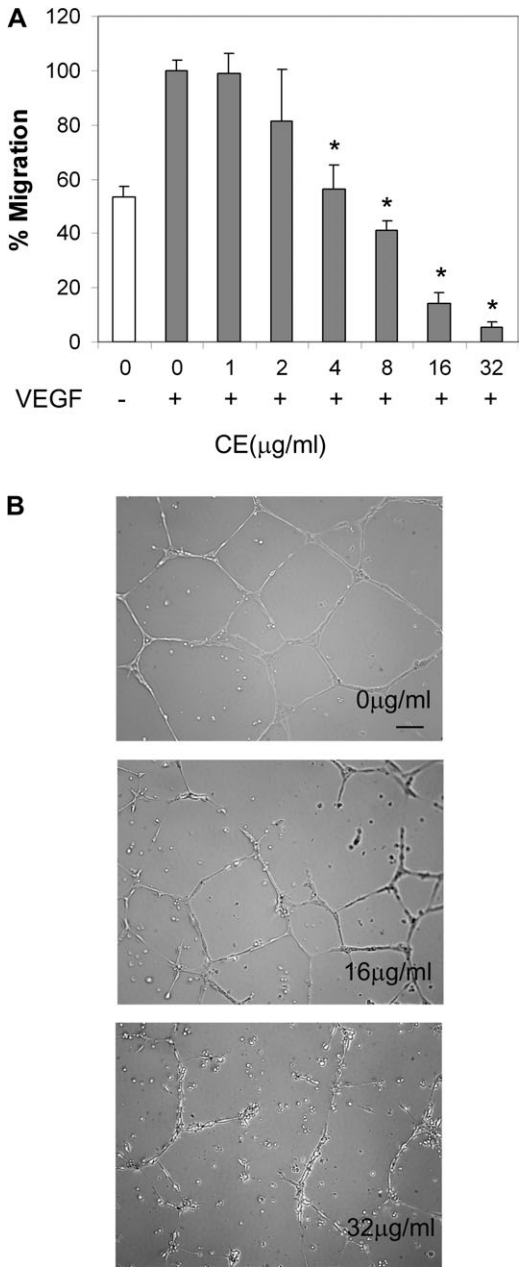

Fig. 3. CE inhibits endothelial cell migration and tube formation. (**A**) CE inhibits endothelial cell migration. Migration assay of HUVEC treated with various concentrations of CE then treated with VEGF; percentage of migrating cells was examined by microscopy. Data represent percentage of control (without CE + VEGF) and are mean ± SD ($n = 3$). $^*P < 0.05$ versus control (without CE treatment) with VEGF present. (**B**) CE inhibits endothelial tube formation. HUVECs were plated on Matrigel in the presence of VEGF with or without CE and photographed under phase-contrast after 16 h. Results are representative of three preparations. Scale bar, 100 μm.

Effect of CE on vessel sprout formation from aorta

The effect of CE on microvessel sprout formation (39) was determined by aortic ring sprout formation assay, a widely used *ex vivo* anti-angiogenesis assay that mimics several stages of angiogenesis, including endothelial cell proliferation, migration and tube formation. Chicken aortic rings were embedded in Matrigel and fed with medium containing different concentrations of CE. The rings were then stimulated with 100 ng/ml VEGF or 50 μg/ml ECGS and sprout formation was examined by microscopy.

In the absence of CE, sprouts emerged from the aortic ring and grew outward after 3 days in culture with either VEGF or ECGS. Treatment with CE resulted in a dramatic dose-dependent decrease in sprout length and density induced by VEGF (data not shown) and ECGS (Figure 4A). To test whether the inhibitory effect of CE was reversible, aortic rings were first incubated with ECGS and CE for 48 h; CE was then removed from the culture and rings were further incubated with ECGS for an additional 48 h. Removal of CE resulted in renewed vessel sprout formation (Figure 4B), suggesting that inhibition of vessel sprout formation by CE was reversible and unlikely due to cytotoxicity.

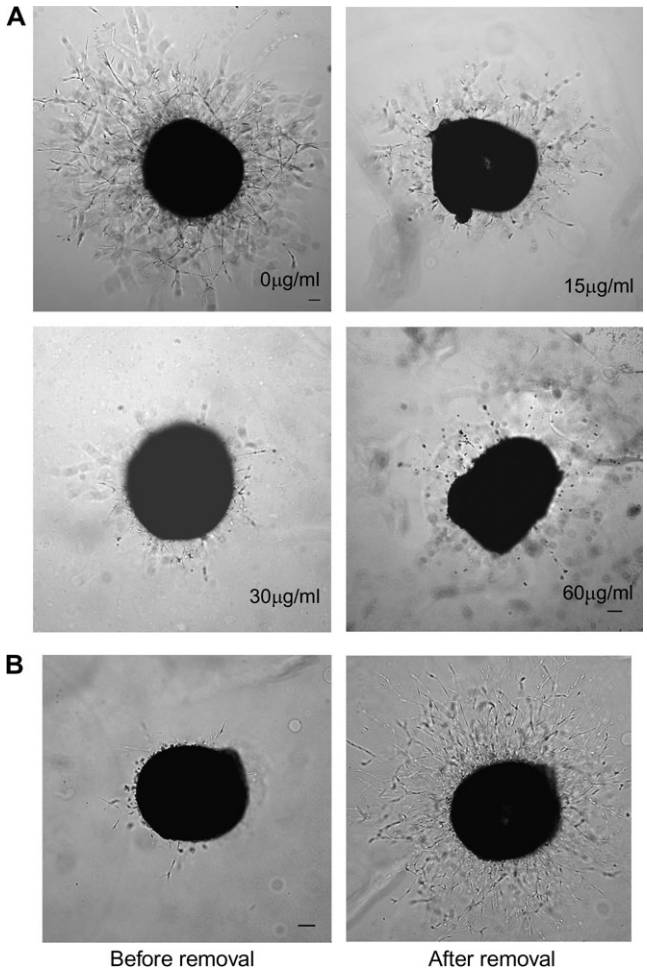

Fig. 4. CE inhibits sprout formation from chick aorta. (**A**) Chick aortic rings were placed in Matrigel and treated with various concentrations of CE in the presence or absence of ECGS. The effect of CE on formation of vessel sprout from various aorta samples was examined on day 3. (**B**) Inhibitory effect of CE on vessel sprout formation is reversible. Aortic rings were first incubated with ECGS and CE (30 μg/ml) for 48 hr (left panel) and continued to culture with ECGS for another 48 hr after cinnamon was removed (right panel). Scale bar, 100 μm. The images shown here are representative of four experiments.

Effect of CE on blood vessel formation in mice

We next tested whether CE was able to inhibit tumor-induced angiogenesis in mice using a Matrigel plug assay. MDA-MB-231 human breast cancer cells were mixed with Matrigel and injected into the flanks of mice; gel plugs were harvested 10 days after implantation and angiogenesis was assayed by hemoglobin content of the plugs. While MDA-MB-231 cells greatly induced blood vessel formation in the plug (Figure 5A), blood vessel formation induced by MDA-MB-231 was significantly reduced in the presence of CE. To further evaluate the effects of CE, blood vessels in the plug were visualized with antibody against CD31 (Figure 5B). Compared with controls, CE treatment led to a significant reduction in blood vessel density indicated by reduced expression of CD31. These data indicated that CE is an angiogenesis inhibitor *in vivo*.

Effect of oligomeric procyanidins derived from CE on angiogenesis

CE contains a large amount of polyphenols, including procyanidins and cinnamaldehyde, a phenylpropanoid (32,40). To test whether polyphenols are responsible for CE anti-angiogenesis activity, we used cross-linked PVPP to deplete polyphenol from CE. PVPP forms hydrogen bonds with phenolic compounds, yielding a PVPP-phenolic precipitate, which can be removed by centrifugation (31,41). Following PVPP depletion, the supernatant was subjected to a VEGFR2 kinase assay. PVPP-treated CE lost the inhibitory effect on *in vitro* kinase activity of VEGFR2 (Figure 6A), phosphorylation of VEGFR2 (Figure 6B) and sprout formation from aortic ring (Figure 6C), implicating the water-soluble polyphenol in CE in the inhibition of VEGFR2 kinase activity and angiogenesis.

Further testing of cinnamaldehyde, a component characteristic of the aroma of CE, found it had no inhibitory effects on VEGFR2 kinase activity (Figure 6D). The activity of high-performance liquid chromatography fractions of CE containing type A trimeric (molecular weight, 864) or tetrameric (molecular weight, 1152) procyanidins

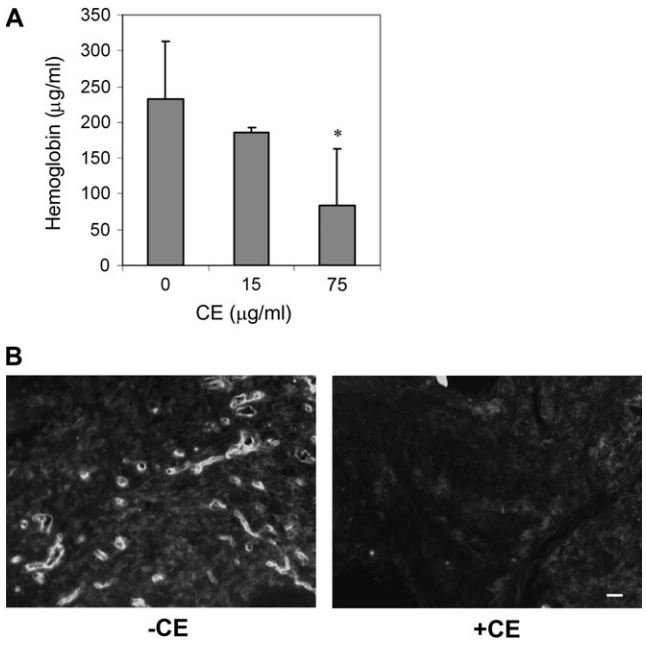

Fig. 5. CE inhibits tumor-induced blood vessel formation in mice. (**A**) MDA-MB-231 cells were mixed with Matrigel in the presence and absence of CE and injected into both flank sites of male severely combined immunodeficient mice (SCID). Hemoglobin levels in the Matrigel plug were quantified after 10 days. Data are mean ± SD ($n = 10$). $^{*}P < 0.05$ versus control treatment. (**B**) Blood vessels in the Matrigel plugs, either without CE (left panel) or with CE (75 μg/ml, right panel), were stained with CD31 antibody. The images shown here are representative of five samples for each condition. Scale bar, 20 μm.

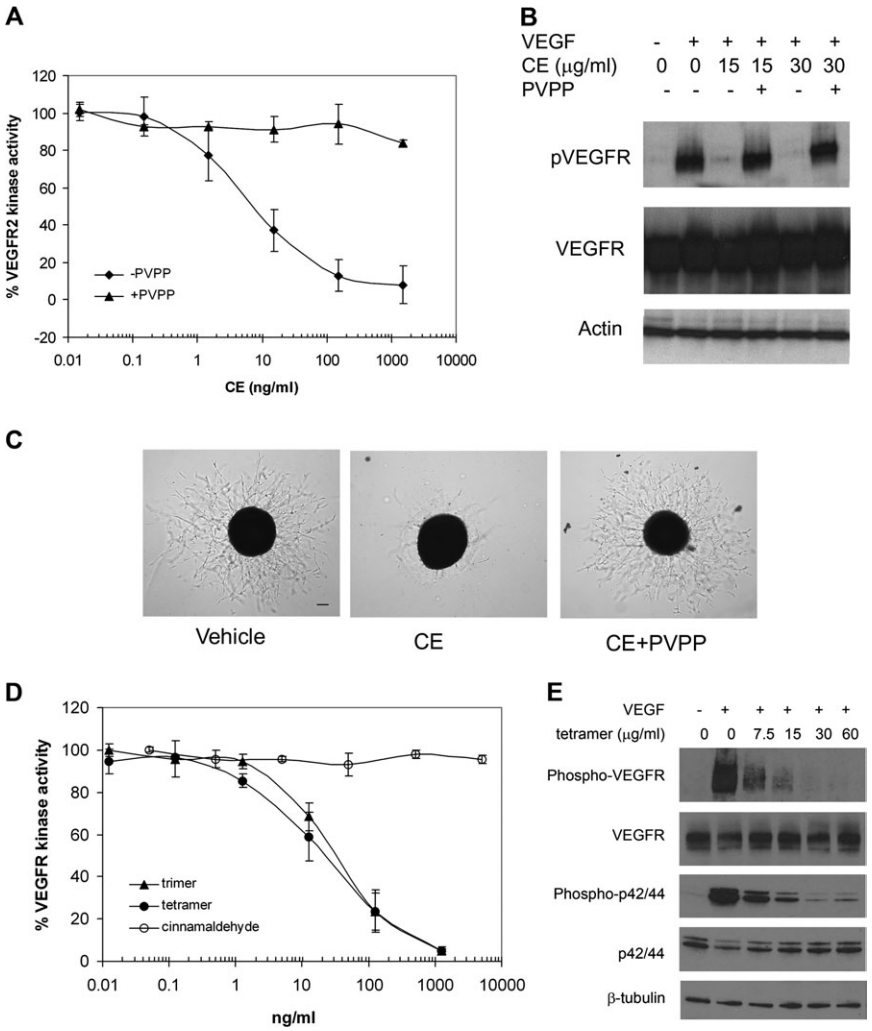

Fig. 6. Oligomeric procynanidins inhibit VEGFR2 kinase activity. (A–C) PVPP treatment depletes anti-angiogenesis activity in CE. PVPP depletion of procynanidins from CE (see Materials and Methods) resulted in (**A**) inhibited kinase activity of purified VEGFR2, and data are mean ± SD ($n = 3$), (**B**) phosphorylation of VEGFR2 in HUVEC and (**C**) sprout formation from aortic ring. Scale bar, 100 μm. Experiments were repeated at least twice. (**D**) Oligomeric procyanidins suppressed kinase activity of purified VEGFR2. Cinnamaldehyde, trimer or tetramer of procyanidins, was incubated with VEGFR2. Data represent the percentage of kinase control (without CE treatment) and are mean ± SD ($n = 3$). (**E**) Oligomeric procyanidins inhibited VEGFR2 signaling. HUVECs were incubated in the presence or absence of trimer and tetramer of procyanidins, followed by VEGF stimulation. Phosphorylation of VEGFR-2 or MAPK was assessed by western blot using anti-phospho-VEGFR2 antibody, anti-phospho-MAPK antibody, as well as anti-total VEGFR2 and anti-total MAPK antibody. Experiments were repeated twice.

(32) was also investigated. Oligomeric procyanidin is one of the major components in CE. Our data demonstrated that these components inhibited kinase activity of purified VEGFR2 in a dose-dependent manner (Figure 6D) and were also able to suppress the phosphorylation of VEGFR2 and MAPK induced by VEGF in HUVEC. Taken together, these results identified oligomeric procyanidins as active components in CE that inhibited VEGFR2 kinase activity and angiogenesis.

Discussion

Human tumors can remain dormant for years due to the balance between cell proliferation and apoptosis. Blockade of angiogenesis is therefore an important approach for cancer treatment and prevention, given that systemic concentrations of angiogenesis inhibitors, exceeding those of stimulators, could potentially prevent tumors from growing and spreading to other organs. While VEGF, a critical factor in angiogenesis induction, has emerged as an attractive target for anti-angiogenesis treatment, the chronic therapeutic use of anti-VEGF agents is limited due to side effects. The present study investigates naturally occurring VEGF inhibitors in cinnamon as a diet-derived source of anti-VEGF agents. Diet and nutrition have been shown to play an important role in the development of cancer (14,15). Additionally, certain plant-derived dietary groups may contain phytochemicals that exert antitumor and anti-angiogenesis activity, thereby offering anticancer protection (11,13).

We found that CE and specific characterized CE components, type A procyanidin trimer and tetrameric procyanidins, effectively inhibited VEGFR2 kinase activity as well as VEGF signaling in endothelial cells. CE also inhibited various aspects of angiogenesis, including endothelial cell proliferation, migration and tube formation *in vitro*, sprout formation *ex vivo*, as well as tumor-induced blood vessel formation in mice. Our data are in agreement with other studies in which several natural products were shown to inhibit VEGFR2 signaling, including catechins from green tea extract, delphinidin, ellagic acid, as well as grape seed extract (27–30).

Cinnamon, the dry bark and twig of *Cinnamomum* spp., is one of the most popular and oldest spices. The bark and leaves of CE are often added to food preparations to improve taste and aroma. In addition, this herb has been found to possess potent antioxidant,

antimicrobial and antipyretic properties and has been used in traditional Chinese medicine (42–44). Several recent studies have found that CE also contains anticancer activity. CE was shown to inhibit hematologic cancer cell proliferation *in vitro*, melanoma tumor growth in mice and VEGF expression in melanoma (45,46). In recent years, much attention has been paid to the influence of cinnamon on insulin action, which may provide benefits for diabetic patients (32,42,43,47,48). More recently, a water-based CE was found to inhibit Tau aggregation and filament formation associated with Alzheimer's disease (31).

Cinnamon contains the highest levels of procyanidins among all common foods (40). The majority of procyanidins in cinnamon is in the A-type oligomeric form. Procyanidins are of great interest in nutrition and medicine because of their potent antioxidant capacity and possible protective effects on human health (49). Although procyanidins have been suggested to account for a significant fraction of polyphenols ingested, as they are found in a variety of food sources (49), little is known about the mechanism underlying the health benefits of procyanidins.

Here we provide evidence that the high-performance liquid chromatography fraction of cinnamon containing oligomeric procyanidins inhibited VEGFR2 kinase activity, whereas no effects were observed by other constituents of CE, such as cinnamaldehyde and methylhydroxychalcone (data not shown). While a previous study suggested that cinnamaldehyde inhibited angiogenesis in a chick chorioallantoic membrane assay (50), the mechanism involved in the inhibition remained to be defined. Further research is needed to determine whether oligomeric procyanidins are the major active components responsible for CE anti-angiogenic activity as well as to identify other potential components involved.

Taken together, our data revealed a novel biological function of CE and suggested a possible molecular mechanism for its action. Further study is required to address whether CE inhibits tumor angiogenesis and tumor growth in mice through suppressing VEGFR2 signaling. As a natural inhibitor of VEGFR2, CE has the potential to be routine diet-based strategy for cancer prevention or treatment.

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Conflict of Interest Statement: None declared.

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