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Capsaicin Inhibits *in Vitro* and *in Vivo* Angiogenesis

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ABSTRACT

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide), a natural product of Capsicum species, is known to induce excitation of nociceptive terminals involved in pain perception. Recent studies have also shown that capsaicin not only has chemopreventive properties against certain carcinogens and mutagens but also exerts anticancer activity. Here, we demonstrated the antiangiogenic activity of capsaicin using *in vitro* and *in vivo* assay systems. *In vitro*, capsaicin inhibited vascular endothelial growth factor (VEGF) -induced proliferation, DNA synthesis, chemotactic motility, and capillary-like tube formation of primary cultured human endothelial cells. Capsaicin inhibited both VEGF-induced vessel sprouting in rat aortic ring assay and VEGF-induced vessel formation in the mouse Matrigel plug assay. Moreover, capsaicin was able to suppress tumor-induced angiogenesis in chick chorioallantoic membrane assay. Capsaicin caused G_1 arrest in endothelial cells. This effect correlated with the down-regulation of the expression of cyclin D1 that led to inhibition of cyclin-dependent kinase 4-mediated phosphorylation of retinoblastoma protein. Signaling experiments show that capsaicin inhibits VEGF-induced p38 mitogen-activated protein kinase, $p125^{FAK}$, and AKT activation, but its molecular target is distinct from the VEGF receptor KDR/Flk-1. Taken together, these results demonstrate that capsaicin is a novel inhibitor of angiogenesis and suggest that it may be valuable to develop pharmaceutical drugs for treatment of angiogenesis-dependent human diseases such as tumors.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing endothelium, is a fundamental step in a variety of physiological and pathological conditions including wound healing, embryonic development, chronic inflammation, and tumor progression and metastasis (1–5). Complex and diverse cellular actions are implicated in angiogenesis, such as extracellular matrix degradation, proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes (6). The angiogenic process is tightly controlled by a wide variety of positive or negative regulators, which are composed of growth factors, cytokines, lipid metabolites, and cryptic fragments of hemostatic proteins (6), and many of these factors are initially characterized in other biological activities. Among these molecules, vascular endothelial growth factor (VEGF), a soluble angiogenic factor produced by many tumor and normal cells, plays a key role in regulating normal and abnormal angiogenesis (7).

A wide array of phenolic substances, particularly those present in dietary and medicinal plants, have been reported to possess substantial anticarcinogenic and antimutagenic activities (8, 9). Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide), the major pungent ingredient in red pepper, has long been used in food additives and drugs (10, 11). The compound has attracted considerable attention recently because of its chemoprotective properties against certain carcinogens and mutagens (12). Capsaicin was reported to preferentially repress the growth of various immortalized or malignant cell lines via induction of apoptosis (13–15). However, its biological and biochemical activities have not been elucidated in endothelial cells. In this report, we investigated whether capsaicin modulates angiogenesis *in vitro* and *in vivo*. Capsaicin inhibited VEGF-induced proliferation, DNA synthesis, migration, and tube formation of endothelial cells *in vitro*. Moreover, the compound markedly inhibited tumor- or VEGF-induced new blood vessel formation in chick chorioallantoic membrane and *in vivo* Matrigel plug assays. Capsaicin also caused G_1 arrest of endothelial cells through down-regulation of cyclin D1 and inhibited VEGF-induced angiogenic signaling pathways. Taken together, these results suggest that capsaicin may inhibit tumor growth via its antiangiogenic activity.

MATERIALS AND METHODS

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment as described previously (16) and used in passages 2–7. The cells were grown in M199 medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml basic fibroblast growth factor (Upstate Biotechnology, Lake Placid, NY), and 5 units/ml heparin at 37°C under a humidified 95%–5% (v/v) mixture of air and CO_2. Human dermal microvascular endothelial cells (HDMECs; Clonetics, Walkersville, MD) were grown in tissue culture plates coated with 0.1% gelatin and maintained in EBM-2 medium supplemented with EGM-2 MV growth factor mixture (Clonetics). Human fibrosarcoma (HT1080) cells were grown in RPMI 1640 containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Endothelial Cell Proliferation Assay. [^{3}H]Thymidine incorporation assay was carried out as described previously (17). Briefly, HUVECs were seeded at a density of 2×10^4 cells/well in gelatin-coated 24-well plates. After 24 h, cells were washed twice with M199 and incubated for 6 h in M199 containing 1% FBS. Cells were preincubated for 30 min with various concentrations of capsaicin (Sigma, St. Louis, MO) and stimulated by the addition of 10 ng/ml VEGF (Upstate) for 30 h, followed by the addition of 0.5 μCi/ml of [^{3}H]thymidine (Amersham, Aylesbury, United Kingdom) for 6 h. High molecular weight DNAs were precipitated using 10% trichloroacetic acid at 4°C for 30 min. After two washes with ice-cold H_2O, ^{3}H-radioactivity was solubilized in 0.2 N NaOH/0.1% SDS and determined by liquid scintillation counter.

Endothelial Cell Migration Assay. Chemotactic motility of HUVECs was assayed using Transwell (Corning Costar, Cambridge, MA) with 6.5-mm diameter polycarbonate filters (8-μm pore size) as described previously (17). Briefly, the lower surface of the filter was coated with 10 μg of gelatin. The fresh M199 medium (1% FBS) containing VEGF was placed in the lower wells. Cells were trypsinized and suspended at a final concentration of 1×10^6 cells/ml in M199 containing 1% FBS. Various concentrations of capsaicin were given to the cells for 30 min at room temperature before seeding. One hundred μl of the cell suspension was loaded into each of the upper wells. The chamber was incubated at 37°C for 4 h. Cells were fixed and stained with H&E. Nonmigrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and chemotaxis was quantified by counting with an optical microscope (×200) the cells that migrated to the lower side of the filter. Ten fields were counted for each assay.

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HDMEC migration assay was carried out in 48-well microchemotaxis chambers (Neuro Probe Inc., Cabin John, MD) as described previously (18). Briefly, HDMECs were resuspended in RPMI 1640 containing 0.1% BSA, and the bottom chamber was loaded with 30,000 cells. Polyester membranes (12-μm pore size) coated with a 0.1% gelatin (Sigma) was laid over the cells and the chamber was inverted and incubated for 2 h. Upper wells were then loaded with RPMI 1640 containing 0.1% BSA and test samples. After a 2-h incubation, the membrane filters were fixed and stained using Diff-Quick (Baxter Healthcare Corp., McGraw Park, IL). The number of cells that migrated through the filter was determined as described.

Tube Formation Assay. Tube formation assay was performed as described previously (17). Briefly, 250 μl of growth factor-reduced Matrigel (Collaborative Biomedical Products, Bedford, MA) was pipetted into a 16-mm diameter tissue culture well and polymerized for 30 min at 37°C. HUVECs incubated in M199 containing 1% FBS for 6 h were harvested after trypsin treatment and suspended in M199 containing 1% FBS. Various concentrations of capsaicin were given to the cells for 30 min at room temperature before seeding and plated onto the layer of Matrigel at a density of 2×10^5 cells/well, and followed by the addition of 10 ng/ml VEGF. After 20 h, the cultures were photographed (×40). The area covered by the tube network was determined using an optical imaging technique in which pictures of the tubes were scanned in Adobe Photoshop and quantitated using Image-Pro Plus (Media Cybermetics).

Aortic Ring Assay. As described previously (19), aortas were harvested from Sprague Dawley rats 6 weeks of age. Plates (48-well) were coated with 120 μl of Matrigel; after gelling, the rings were placed in the wells and sealed in place with an overlay of 50 μl of Matrigel. VEGF with or without capsaicin was added to the wells in a final volume of 200 μl of human endothelial serum-free medium (Invitrogen). As controls, medium alone was assayed. On days 6, cells were fixed and stained with Diff-Quick. The assay was scored from 0 (least positive) to 5 (most positive) in a double-blinded manner. Each data point was assayed in sextuplets.

***In Vivo* Matrigel Plug Assay.** Matrigel plug assay was performed as described previously (20). Briefly, C57BL/6 mice were injected s.c. with 0.6 ml of Matrigel containing the indicated amount of capsaicin, 100 ng VEGF, and 10 units heparin. The injected Matrigel rapidly formed a single, solid gel plug. After 7 days, the skin of the mouse was easily pulled back to expose the Matrigel plug, which remained intact. Hemoglobin was measured using the Drabkin method and Drabkin reagent kit 525 (Sigma) for the quantification of blood vessel formation. The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel.

Tumor-induced Angiogenesis on Chick Chorioallantoic Membrane. To investigate the effect of capsaicin on tumor-induced angiogenesis, a modified chick chorioallantoic membrane assay was carried out (21). Briefly, human fibrosarcoma HT1080 cells were trypsinized, washed, and resuspended at 2×10^7 cells/ml in serum-free RPMI 1640, which contained 1.5 mg/ml rat tail type I collagen (Collaborative Biomedical Products) in the absence or presence of capsaicin. Five μl of the mixture were loaded onto 1/4 piece of 15-mm Thermonox discs (Nunc, Naperville, IL) and permitted to be polymerized. The disk was then applied to the chorioallantoic membrane of a 10-day-old embryo. After a 72-h incubation, the area around the loaded disk was photographed with a Nikon digital camera, and the number of newly formed vessels was counted by two observers in a double-blind manner. Assays for each test sample were carried out using 8–10 eggs.

Cell Cycle Analysis. HUVECs were seeded at a density of 3×10^5 cells in 60-mm plates. After 24 h, the cells were washed twice with M199 and incubated for 6 h in M199 containing 1% FBS. Cells were preincubated for 30 min with various concentrations of capsaicin and stimulated by the addition of 10 ng/ml VEGF. After a 20-h incubation, the cells were harvested and then fixed in ice-cold ethanol. Fixed cells were dehydrated at 4°C for 30 min in PBS containing 2% FBS and 0.1% Tween 20, and then centrifuged and resuspended in 0.5 ml of the same buffer. RNase digestion (5 μg/ml) was carried out at 37°C for 1 h, followed by staining with propidium iodide (5 μg/ml). The cells were analyzed using a FACScan BD PharMingen flow cytometer.

Immunoprecipitation. Confluent HUVECs were incubated for 6 h in M199 containing 1% FBS, and cells were stimulated by the addition of VEGF with or without capsaicin. After stimulation, cells were lysed in 1 ml of lysis buffer [20 mM Tris/HCl (pH 8.0), 2 mM EDTA, 137 mM NaCl, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 1% Triton X-100]. Lysates were clarified by centrifugation at 15,000 × *g* for 10 min, and the resulting supernatants were immunoprecipitated with either 1 μg/ml anti-KDR/Flk-1 antibody or anti-$p125^{FAK}$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 3 h followed by the addition of protein A-agarose beads (Upstate) at 4°C for 1 h. Immunoprecipitates were washed three times with lysis buffer, solubilized in SDS-PAGE sample buffer containing β-mercaptoethanol, and additionally analyzed by Western blotting.

Western Blotting. Cell lysates or immunoprecipitates from HUVECs were run in SDS-PAGE and transferred to polyvinylidene difluoride membrane. The blocked membranes were then incubated with the indicated antibodies, and the immunoreactive bands were visualized using a chemiluminescent substrate.

Statistical Analysis. The data are presented as means ± SE and statistical comparisons between groups were performed using 1-way ANOVA followed by Student's *t* test.

RESULTS

Capsaicin Inhibits VEGF-Induced Proliferation of HUVECs. To determine antiangiogenic activity of capsaicin *in vitro*, its inhibitory effect on VEGF-induced proliferation of endothelial cells was evaluated. HUVECs were pretreated for 30 min with various concentrations of capsaicin before exposure to VEGF (10 ng/ml) for 48 h. Capsaicin inhibited VEGF-induced HUVEC proliferation in a dose-dependent manner, with half maximal inhibition at 5 μM (Fig. 1*A*). These inhibitory effects were not due to cytotoxicity of capsaicin in endothelial cells, because capsaicin had no effect on normal growth of HUVECs devoid of VEGF stimulation up to 25 μM (data not shown). In addition, the compound did not show any cytotoxic effect on other cell types tested such as follicular dendritic cell-like fibroblast (HK cells) and RAW 264.7 cells up to 50 μM (data not shown). The effect of capsaicin on DNA synthesis of HUVECs was monitored by [^{3}H]thymidine incorporation assay. VEGF (10 ng/ml) significantly increased DNA synthesis of HUVECs, and this effect was completely blocked by capsaicin (Fig. 1*B*).

The Antiproliferative Activity of Capsaicin Is Not Related to the Vanilloid VR1 Receptor. Capsaicin is known to exert its physiological functions in sensory neurons through its intracellular binding to the vanilloid VR1 receptor (22). To determine the involvement of the vanilloid VR1 receptor in the antiangiogenic activity of capsaicin, the effects of VR1 antagonist or agonist on VEGF-induced endothelial cell proliferation were tested. The antiproliferative activity of capsaicin was not affected by treatment of the competitive capsaicin antagonist capsazepine (Fig. 2). Moreover, resiniferatoxin, a potent agonist of capsaicin receptor, had no effect on VEGF-induced proliferation of HUVECs (Fig. 2). These results indicate that antiangiogenic

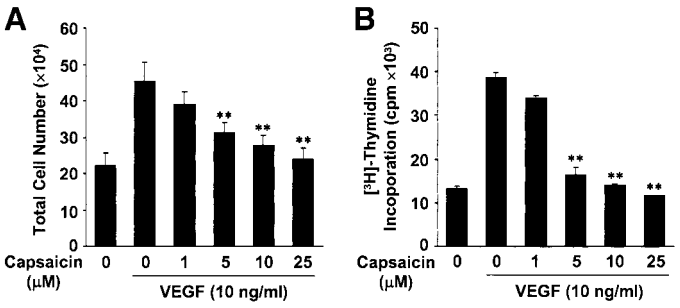

Fig. 1. Capsaicin inhibits vascular endothelial growth factor (*VEGF*) -induced proliferation and DNA sysnthesis of HUVECs. *A*, HUVECs were pretreated for 30 min with various concentrations (1, 5, 10, or 25 μM) of capsaicin before exposure to VEGF (10 ng/ml). After 48 h, the number of cells was counted under the microscope. *B*, HUVECs were treated the same as in *A* except for 36 h stimulation. [^{3}H]Thymidine was present during the last 6 h of incubation. Incorporated [^{3}H]thymidine into the cells was quantitated by scintillation counting. All data are presented as mean from three different experiments with duplicate (**, $P < 0.01$ *versus* VEGF alone); *bars*, ±SE.

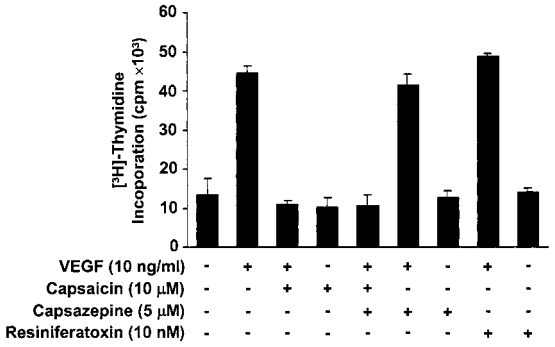

Fig. 2. The antiproliferative activity of capsaicin is not related to the vanilloid VR1 receptor. Human umbilical vein endothelial cells (HUVECs) were pretreated for 30 min with 10 μM capsaicin, 10 nM resiniferatoxin, 5 μM capsazepine, or 5 μM capsazepine plus 10 μM capsaicin before exposure to VEGF. Then, cells were stimulated with 10 ng/ml vascular endothelial growth factor (*VEGF*) and allowed to proliferate for 36 h. [^{3}H]Thymidine was present during the last 6 h of incubation. Incorporated [^{3}H]Thymidine into the cells was quantitated by scintillation counting; *bars,* ±SE.

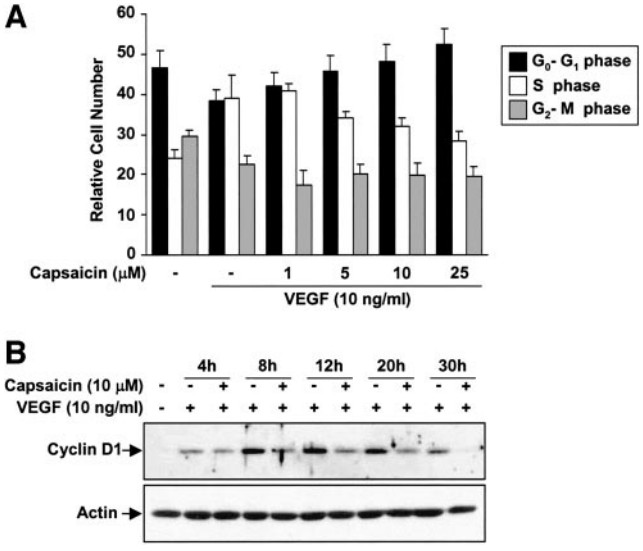

Fig. 3. Capsaicin causes G_1 arrest of endothelial cells through down-regulation of cyclin D1. *A,* human umbilical vein endothelial cells (HUVECs) were pretreated for 30 min with various concentrations (1, 5, 10, or 25 μM) of capsaicin before exposure to vascular endothelial growth factor (*VEGF;* 10 ng/ml). After 20 h, cell cycle analysis was performed as described in "Materials and Methods." *B,* HUVECs were pretreated for 30 min with 10 μM capsaicin and stimulated with VEGF (10 ng/ml) for the indicated times. At the indicated time points, cells were harvested, and Western blotting was performed; *bars,* ±SE.

activity of capsaicin is distinct from its binding to the vanilloid VR1 receptor.

Capsaicin Causes G_1 Arrest of Endothelial Cells through Down-Regulation of Cyclin D1. We additionally examined the effect of capsaicin on cell cycle progression using fluorescence-activated cell sorter analysis. After treatment of HUVECs with 10 ng/ml VEGF with or without various concentrations of capsaicin for 20 h, the percentage of cells in G_0/G_1, S, and G_2-M phase was monitored. VEGF induced HUVECs into S phase, whereas addition of capsaicin markedly reduced S phase entry in a dose-dependent manner (Fig. 3*A*). The half-maximal effect was noted at ~5 μM capsaicin. Thus, these results indicate that capsaicin affects the transition of cells from G_1 to S.

The transition of cells from G_1 to S is in part regulated by cyclin D1. Whether capsaicin manifests its antiproliferative effects through the regulation of cyclin D1 was examined. When HUVECs were treated with VEGF for various time periods, the expression of cyclin D1 was increased as early as 4 h in protein levels. These effects were significantly blocked by 10 μM capsaicin in all of the time periods tested (Fig. 3*B*). In addition, HUVECs were treated with VEGF in the presence or absence of various concentrations of capsaicin for 12 h and then examined for expression of cyclin D1 by Western blot analysis. Capsaicin significantly down-regulated VEGF-induced cyclin D1 expression in a dose-dependent manner, achieving half maximal effect at ~5 μM capsaicin (Fig. 4).

Cyclin D1 is known to regulate the activity of cyclin-dependent kinase 4, which is known to phosphorylate retinoblastoma (Rb) protein. Therefore, we next examined the phosphorylation state of Rb in endothelial cells. Capsaicin inhibited the hyper-phosphorylation of Rb induced by VEGF (Fig. 4). We also examined the protein expression level of cyclin E and cyclin A. The cyclin E and cyclin A protein levels in HUVECs were increased by treatment with VEGF, and these up-regulations were inhibited by capsaicin in a dose-dependent manner (Fig. 4). Taken together, these results suggest that cyclin D1 may be a crucial capsaicin target in mediating its effects on the cell cycle.

Capsaicin Inhibits VEGF-Induced Migration and Tube Formation of Endothelial Cells. The effect of capsaicin on chemotactic motility of HUVECs and HDMECs were measured by using either Transwell or microchemotaxis chamber assay. Capsaicin inhibited VEGF-induced migration of both HUVECs and HDMECs in a concentration-dependent manner (Fig. 5, *A* and *B*). Capsaicin alone had no significant effect on basal migration of these endothelial cells. Next, the effect of capsaicin on morphological differentiation of endothelial cells was investigated using two-dimensional Matrigel. When HUVECs were placed on growth factor-reduced Matrigel in the presence of VEGF, VEGF led to the formation of elongated and robust tube-like structures, which were organized by much larger number of cells compared with the control. Capsaicin effectively abrogated the width and the length of endothelial tubes induced by VEGF in a concentration-dependent manner (Fig. 5, *C* and *D*). Half maximal inhibition was seen at a concentration of 5 μM. These results demonstrate that capsaicin has an ability to block VEGF-induced *in vitro* angiogenesis.

Capsaicin Inhibits VEGF-Induced p38 Mitogen-Activated Protein Kinase and p125^{FAK} Activation Without Affecting Tyrosine Phosphorylation of KDR/Flk-1. VEGF induces proliferation and migration through activation of its cell surface receptor, KDR/Flk-1 (23). To understand the molecular mechanism by which capsaicin inhibits VEGF-induced angiogenesis, we investigated the effects of capsaicin on VEGF-induced KDR/Flk-1 autophosphorylation as well as activation of extracellular signal-regulated kinase, p38 mitogen-

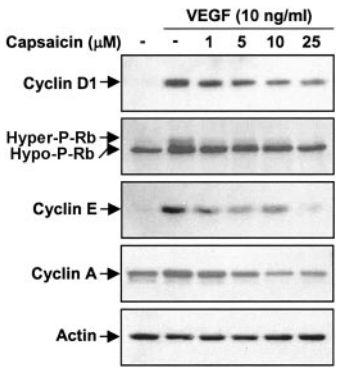

Fig. 4. Capsaicin inhibits hyperphosphorylation of retinoblastoma (*Rb*) induced by vascular endothelial growth factor (*VEGF*). Human umbilical vein endothelial cells were pretreated for 30 min with various concentrations (1, 5, 10, or 25 μM) of capsaicin before exposure to VEGF (10 ng/ml). After 12 h, the cells were harvested, and Western blotting was performed.

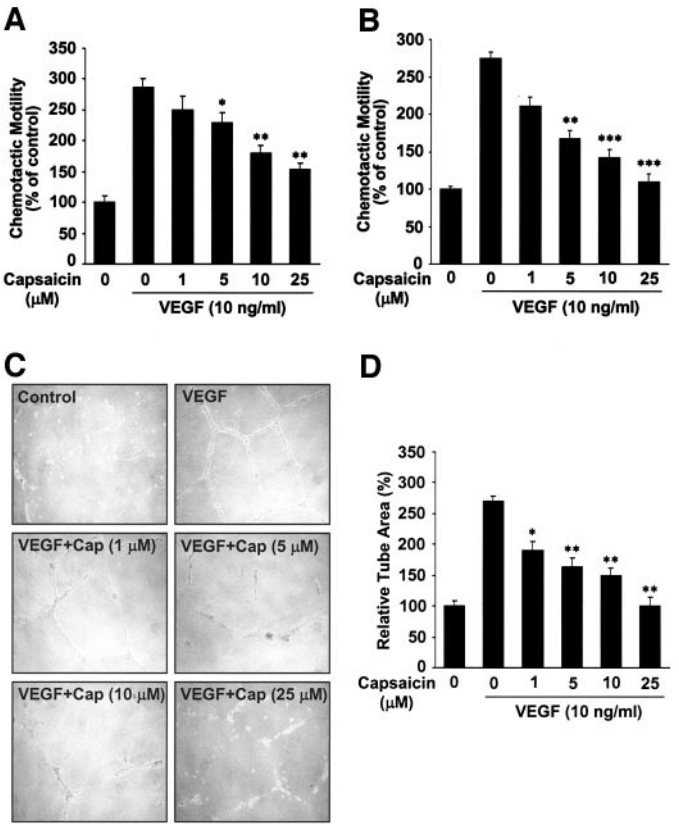

Fig. 5. Capsaicin inhibits vascular endothelial growth factor (*VEGF*) -induced migration and tube formation of human endothelial cells. *A*, Human umbilical vein endothelial cells (HUVECs) were pretreated for 30 min with various concentrations (1, 5, 10, or 25 μM) of capsaicin before treatment with 10 ng/ml VEGF. After a 4-h incubation, chemotaxis was quantified by counting the cells that migrated to the lower side of the filter with optical microscopy at $\times$200 magnification. The basal migration in the absence of VEGF was 100 $\pm$ 10 cells/field. *B*, human dermal microvascular endothelial cell (HDMEC) migration assays were carried out in 48-well microchemotaxis chambers. HDMECs were treated with VEGF in the absence or presence of capsaicin for 2 h. After fixation and staining, the number of cells that migrated through the filter was determined. The basal migration in the absence of VEGF was 57 $\pm$ 2 cells/field. In *C* and *D*, HUVECs were preincubated for 30 min with various concentrations of capsaicin (1, 5, 10, or 25 μM). Cells were collected and replated on Matrigel-coated plates at a density of 2×10^5 cells/well and then incubated in the absence (*control*) or presence of 10 ng/ml VEGF. After 20 h, microphotographs were taken ($\times$40). *C*, representative endothelial tubes were shown. *D*, the area covered by the tube network was quantitated using Image-Pro Plus software. Experiments were repeated twice and values are means of triplicates (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *versus* VEGF alone); *bars*, $\pm$SE.

activated protein kinase, and $p125^{FAK}$, which are downstream signaling molecules of KDR/Flk-1. Capsaicin had no effect on VEGF-induced KDR/Flk-1 autophosphorylation up to 25 μM (Fig. 6*A*). Nevertheless, VEGF-induced p38 mitogen-activated protein kinase and $p125^{FAK}$ activation was inhibited significantly by capsaicin in a dose-dependent manner (Fig. 6, *B* and *C*). In contrast, capsaicin (up to 25 μM) exhibited no influence on VEGF-induced extracellular signal-regulated kinase activation (Fig. 6*C*).

It has been established that the AKT/NO pathway is critical for VEGF-induced endothelial cell migration, proliferation, and tube formation *in vitro* (24, 25). VEGF induces phosphorylation of AKT (Ser-473) and endothelial NO synthase (eNOS; Ser-1177) that plays a key role in VEGF-stimulated angiogenesis (26, 27). Therefore, the effects of capsaicin on VEGF-induced AKT and eNOS phosphorylation were evaluated by antibodies directed against the phosphorylated forms of AKT (Ser-473) and eNOS (Ser-1177), respectively. As shown in Fig. 6*D*, capsaicin significantly diminished VEGF-induced AKT and eNOS phosphorylation in a dose-dependent manner, with half maximal effect at $\sim$5 μM. These data suggest that capsaicin is able to block VEGF-induced AKT/eNOS signaling pathways in endothelial cells.

Capsaicin Inhibits VEGF-Induced Vessel Sprouting *ex Vivo*. The sprouting of vessels from aortic rings was investigated to determine whether capsaicin inhibits VEGF-induced angiogenesis *ex vivo*. VEGF (20 ng/ml) significantly stimulated vessel sprouting above medium alone (Fig. 7). The presence of capsaicin resulted in a significant reduction of VEGF-induced vessel sprouting, and its inhibitory activity was dose-dependent.

Capsaicin Inhibits VEGF-Induced Angiogenesis *in Vivo*. To determine whether capsaicin is capable of blocking on VEGF-induced angiogenesis *in vivo*, an established *in vivo* angiogenesis model, the mouse Matrigel plug assay, was performed. Matrigel containing VEGF (100 ng) with or without capsaicin (20 or 60 μg, respectively) was s.c. injected into C57BL/6 mice, and 7 days later, the formed Matrigel plugs in mice were excised and photographed. Plugs with VEGF alone appeared dark-red color. In contrast, plugs with Matrigel alone and mixed capsaicin were pale in their color indicating no or less blood vessel formation (Fig. 8*A*). The vessels were abundantly filled with intact RBCs, which indicates the formation of a functional vasculature inside the Matrigel and blood circulation in newly formed vessels by angiogenesis induced by VEGF. The hemoglobin content inside the Matrigel plugs was measured to quantify the angiogenesis inhibited by capsaicin. Whereas hemoglobin in VEGF was nearly 8.5 g/dl, capsaicin markedly inhibited the hemoglobin quantity to $\sim$2.4 g/dl (Fig. 8*B*). These results indicate that capsaicin is capable of inhibiting VEGF-induced neovessel formation *in vivo*.

Capsaicin Inhibits Tumor-Induced Angiogenesis *in Vivo*. The ability of capsaicin to inhibit *in vivo* tumor-induced angiogenesis was next examined using the chick chorioallantoic membrane assay. HT1080 cells entrapped in type I collagen gels caused detectable infiltration of blood vessels into collagen sponges when compared with that of medium alone (Fig. 9*A*). Capsaicin inhibited the tumor-induced angiogenesis without any visible effect on the pre-existing blood vessels. Quantitative analysis revealed that tumor cells caused 2.8-fold increases in the number of newly formed blood vessels

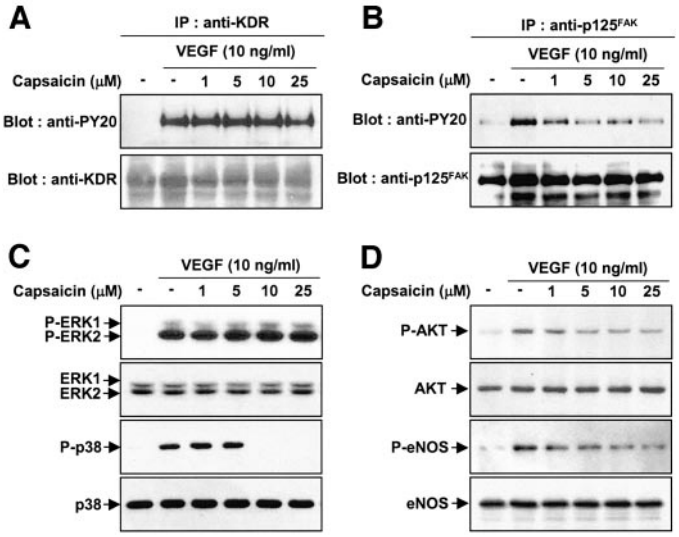

Fig. 6. Capsaicin inhibits vascular endothelial growth factor (VEGF) -induced p38 mitogen-activated protein kinase and $p125^{FAK}$ activation without affecting tyrosine phosphorylation of KDR/Flk-1. Human umbilical vein endothelial cells were pretreated for 30 min with various concentrations (1, 5, 10, or 25 μM) of capsaicin and stimulated with 10 ng/ml VEGF for 10 min. *A* and *B*, anti-KDR/Flk-1 and anti-$p125^{FAK}$ immunoprecipitates (*IP*) were analyzed by SDS-PAGE. Immunoblot analysis was performed with anti-phosphotyrosine antibody (*anti-PY20*). *C* and *D*, activation of extracellular signal-regulated kinase, p38 mitogen-activated protein kinase, AKT, and endothelial NO synthase by VEGF were determined by Western blotting.

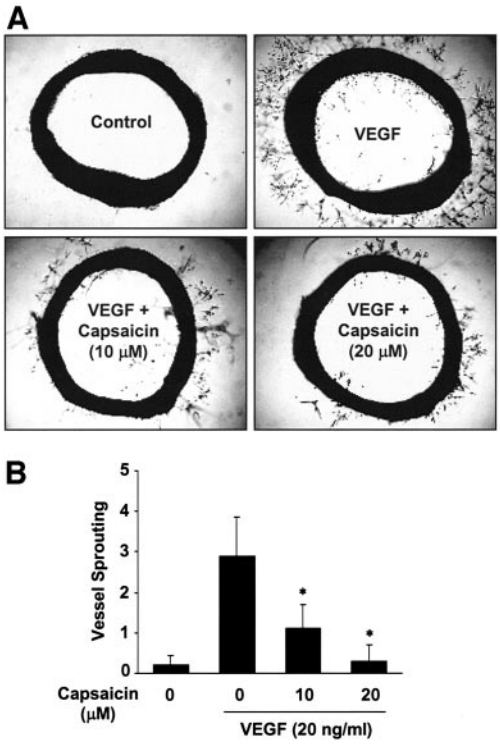

Fig. 7. Capsaicin inhibits vascular endothelial growth factor (*VEGF*) -induced vessel sprouting *ex vivo*. Aortas in Matrigel were treated with VEGF (20 ng/ml) in the absence or presence of capsaicin and stained with Diff-Quick on day 6. *A*, representative aortic rings were photographed. *B*, capsaicin blocked VEGF-induced vessel sprouting. *, $P < 0.05$ *versus* VEGF alone. The assay was scored from 0 (least positive) to 5 (most positive) and the data are presented as mean ($n = 5$); *bars*, ±SE.

compared with that of medium alone (Fig. 9; $P < 0.001$). The presence of 0.75, 1.5, 3, and 6 μg/egg caused 25%, 38%, 55%, and 87% reduction in the infiltration of blood vessels, respectively. These results clearly indicate that capsaicin is a potent antiangiogenic molecule *in vivo*.

DISCUSSION

Capsaicin is a major pungent ingredient present in hot green and red peppers of Capsicum family, among the most commonly and frequently used spices throughout the world. Because of its analgesic and anti-inflammatory activities, topical application of capsaicin has been used in clinical practice for the treatment of a variety of neuropathic pain conditions (28). Recently, capsaicin is also recognized for its pharmacological and toxicologic properties for the treatment of cancer in humans. A number of independent studies have demonstrated that capsaicin can promote or prevent carcinogenic and mutagenic processes *in vitro* and *in vivo* (29). In epidemiological observations, it is suggested that capsaicin consumption may reduce the risk of colon cancer in humans. On the contrary, high capsaicin pepper use in some ethnic-cultural groups is considered to be a risk factor of developing stomach cancer (30). Therefore, despite of a substantial body of data in cultured cells and in animal models showing the chemopreventive or chemoprotective activities of capsaicin, clinical or epidemiological data eliciting the precise role of capsaicin in human cancer development are still limited and inconclusive.

In the present study, we for the first time provide direct evidences that capsaicin has a potent antiangiogenic activity *in vitro* and *in vivo* that can support the tumor-preventive action of capsaicin. Because VEGF, generated from a variety of tumors, is the most important angiogenic factor associated closely with induction and maintenance of the neovasculature in human tumors (31, 32), the effects of capsaicin on VEGF-induced angiogenesis were investigated. On the cellular level, capsaicin almost completely suppressed the stimulatory effect of VEGF on endothelial cell proliferation, migration, and tube formation. Half maximal inhibition was <5 μM. Interestingly, the concentration of capsaicin that sufficiently inhibits the specific endothelial cell response to VEGF is much lower than those observed in the inhibition of normal cell growth of both endothelial cells and nonendothelial cells tested in this study. Previous studies have also shown that treatment of high concentrations of capsaicin over 50 μM results in growth inhibition or apoptosis of tumor cells including HeLa, ovarian carcinoma, mammary adenocarcinoma, adult T-cell leukemia, and promyelocytic leukemia (HL-60) cells in culture (13, 33). It has been shown that capsaicin treatment at a concentration >50 μM results in enhanced VEGF protein secretion in A375P melanoma cells (34). However, capsaicin at those concentrations significantly blocked melanoma cell growth. Thus, the growth-inhibitory action of capsaicin at low concentration is considered to be a more specific response to endothelial cells stimulated by angiogenic growth factors. Consistent with this notion, it is also observed that capsaicin inhibits basic fibroblast growth factor-induced proliferation of endothelial cells at a similar concentration used for VEGF (data not shown). Therefore, it is suggested that capsaicin may possess novel molecular properties that interfere with common angiogenic signaling pathways triggered on growth factor stimulation in endothelial cells.

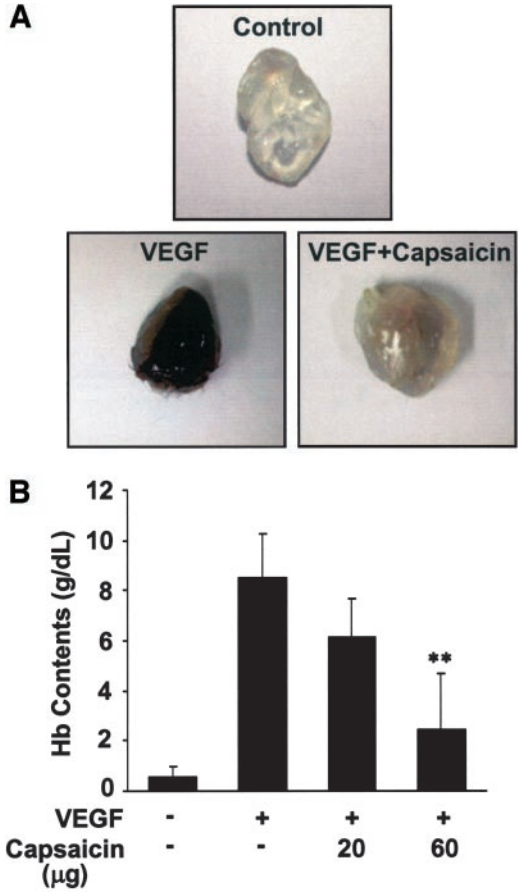

Fig. 8. Capsaicin inhibits VEGF-induced angiogenesis *in vivo*. C57BL/6 mice were injected with 0.6 ml of Matrigel containing vascular endothelial growth factor (*VEGF;* 100 ng) and capsaicin (20 or 60 μg) indicated. After 7 days, mice were killed and Matrigel plugs were excised. *A*, representative Matrigel plugs that contained no VEGF (*Control*), VEGF alone, or VEGF plus capsaicin (60 μg) were photographed. *B*, quantification of neovessel formation by measurement of hemoglobin in the Matrigel. Five mice were used as a group, and the experiment was repeated twice. The data are presented as mean; **, $P < 0.01$ *versus* VEGF alone; *bars*, ±SE.

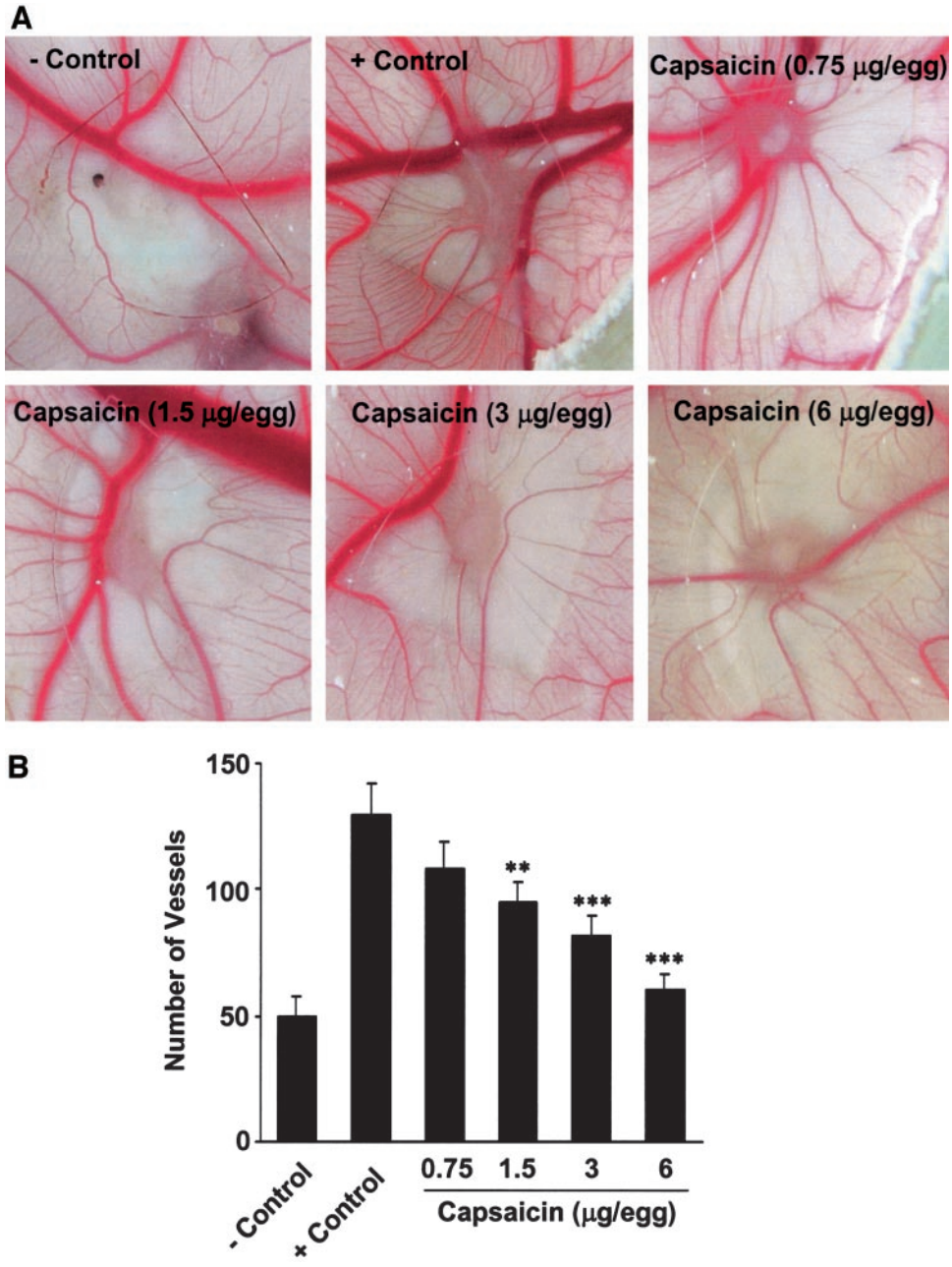

Fig. 9. Capsaicin inhibits tumor-induced angiogenesis *in vivo*. HT1080 cells entrapped in type I collagen gels were loaded on the chick chorioallantoic membranes (CAMs) of day 10 chick embryos. After a 72-h incubation, a fat emulsion was injected under the CAMs for better visualization of the vessels. Disks and surrounding CAMs were photographed. *A*, representative photographs of − control, + control, and capsaicin. *B*, quantification of newly formed blood vessels (**, $P < 0.01$; ***, $P < 0.001$ *versus* + control). Eight to 10 eggs were used for each data point, and mean is shown (− Control, no cells; + control, cells only; capsaicin, cells with capsaicin); *bars*, ±SE.

The present study demonstrates possible molecular mechanisms by which capsaicin inhibits VEGF-induced angiogenesis *in vitro*. Unlikely to its analgesic action in sensory neurons, the antiangiogenic activity of capsaicin was unrelated to the vanilloid VR1 receptor. Our data show that the VR1 agonist resiniferatoxin and the VR1 antagonist capsazepine, which are structurally related to capsaicin, are unable to functionally substitute antiproliferative activity of capsaicin in VEGF-stimulated endothelial cells, suggesting that the precise structural moiety of capsaicin is required for its antiangiogenic activity. Our results also reveal that antiproliferative effect of capsaicin correlates with G_1 arrest of endothelial cells. It is possible that this effect is mediated through either inhibition of cyclin D1 expression or induction of a cyclin-dependent kinase inhibitor, p21. The cyclin D1 is required for the activity of cyclin-dependent kinase 4, which phosphorylates Rb, thus releasing E2F to mediate the G_1 to S transition, in turn leading to DNA synthesis and cell cycle progression (35). Our data clearly show that capsaicin significantly diminishes VEGF-induced cyclin D1 expression (Fig. 3*B;* Fig. 4). However, capsaicin (up to 25 μM) did not induce p21 and its transcription factor p53 in endothelial cells (data not shown). Thus, capsaicin is most likely to induce G_1 arrest of endothelial cells through the down-regulation of cyclin D1. It is notable that other antiangiogenic molecules such as endostatin and curcumin also suppress Rb phosphorylation and DNA synthesis of endothelial cells through down-regulation of cyclin D1 (36, 37).

Our results also provide possible antiangiogenic signaling mechanisms of capsaicin in endothelial cells. Capsaicin could not interfere with VEGF-induced activation of its receptor KDR/Flk-1 that plays a crucial role in proliferation and migration of endothelial cells in response to VEGF (38, 39). Nevertheless, capsaicin was capable of blocking the downstream events of VEGF-induced KDR/Flk-1 signaling, such as activation of p38 mitogen-activated protein kinase and $p125^{FAK}$ tyrosine phosphorylation, that are requisite for the motogenic activity of VEGF in endothelial cells. Unlikely to these

events, the extracellular signal-regulated kinase signaling that also lies downstream of KDR/Flk-1 was not affected by capsaicin (Fig. 6*A*). Recent studies have established the importance of the AKT/NO pathway in VEGF-induced angiogenesis. Both molecular and pharmacological approaches have revealed that inhibition of eNOS results in abrogating VEGF-induced endothelial cell migration, proliferation, and tube formation *in vitro*, as well as VEGF-induced angiogenesis *in vivo* (24). It is also known that VEGF induces eNOS activation and increases NO production in endothelial cells via the AKT-dependent phosphorylation of Ser-1177 eNOS (40). Indeed, capsaicin significantly blocked VEGF-induced phosphorylation of AKT (Ser-473) and eNOS (Ser-1177) in the micromolar range. This working concentration was at a level similar to those shown in VEGF-induced angiogenesis *in vitro*. Taken together, these data indicate that capsaicin is able to inhibit several angiogenic signaling pathways induced by VEGF, but its molecular target is distinct from the VEGF receptor KDR/Flk-1. These inhibitory effects of capsaicin on VEGF signaling pathways should be at least in part correlated with its potent antiangiogenic activities *in vitro* and *in vivo*. Additional elucidation of detailed molecular mechanisms and the precise molecular target associated with antiangiogenic activities of capsaicin is the subject of ongoing investigation.

Previous studies have demonstrated that capsaicin abrogates tumor growth in animal models. Direct injection of capsaicin into the B16 mouse melanoma transplanted in C57BL/6 mice significantly blunted the growth of tumors (41). Moreover, dietary exposure of capsaicin was found to reduce the incidence of colonic adenocarcinoma in male F344 rats (42). Considering that angiogenesis is essential for tumor growth, the antitumor effects of capsaicin may be correlated with its antiangiogenic activity. By using *ex vivo* and *in vivo* angiogenesis models, the antiangiogenic activities of capsaicin were evaluated. Capsaicin remarkably suppressed sprouting of endothelial cells in rat aorta and induction of new blood formation in Matrigel plug implanted in C57BL/6 mice in response to VEGF. Moreover, capsaicin inhibited *in vivo* tumor-induced angiogenesis in the chick chorioallantoic membrane assay. In this experiment, it is notable that capsaicin selectively inhibited tumor-induced new blood vessel formation without any visible effect on the preexisting blood vessels. These antiangiogenic activities of capsaicin *in vivo* may be explained by its inhibitory action on proliferation, migration, and differentiation of endothelial cells in response to angiogenic growth factors such as VEGF.

Notably, in contrast to antiangiogenic action of capsaicin in our system, capsaicin is reported recently to promote the neurogenic enhancement of angiogenesis in rat knee synovitis (43). Indeed, it was shown that intra-articular injection with high doses of capsaicin indirectly increases endothelial cell proliferation via release of endogenous substance P, an angiogenic neuropeptide, from sensory nerves. In our system, capsaicin alone was incapable of stimulating angiogenesis. Therefore, antiangiogenic action mechanism of capsaicin should be distinct from that observed in neurogenic angiogenesis.

In conclusion, our present data demonstrate a possible role of capsaicin in preventing cancer from becoming malignant, presumably via selective curb of neovessel formation in the tumor site. Capsaicin may have a potential to develop pharmaceutical drugs for treatment of angiogenesis-dependent human diseases such as tumors.

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