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Piperine, a dietary phytochemical, inhibits angiogenesis

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Abstract

Angiogenesis plays an important role in tumor progression. Piperine, a major alkaloid constituent of black pepper, has diverse physiological actions including killing of cancer cells; however, the effect of piperine on angiogenesis is not known. Here we show that piperine inhibited the proliferation and G_1/S transition of human umbilical vein endothelial cells (HUVECs) without causing cell death. Piperine also inhibited HUVEC migration and tubule formation *in vitro*, as well as collagen-induced angiogenic activity by rat aorta explants and breast cancer cell-induced angiogenesis in chick embryos. Although piperine binds to and activates the cation channel transient receptor potential vanilloid 1 (TRPV1), its effects on endothelial cells did not involve TRPV1 since the antiproliferative effect of piperine was not affected by TRPV1-selective antagonists, nor did HUVECs express detectable TRPV1 mRNA. Importantly, piperine inhibited phosphorylation of Ser 473 and Thr 308 residues of Akt (protein kinase B), which is a key regulator of endothelial cell function and angiogenesis. Consistent with Akt inhibition as the basis of piperine's action on HUVECs, inhibition of the phosphoinositide-3 kinase/Akt signaling pathway with LY-294002 also inhibited HUVEC proliferation and collagen-induced angiogenesis. Taken together, these data support the further investigation of piperine as an angiogenesis inhibitor for use in cancer treatment.

Keywords: Angiogenesis; Piperine; Akt; Endothelial cell

1. Introduction

Angiogenesis is normally a tightly regulated and complex process that involves endothelial cell proliferation, migration, alignment and tubule formation in response to various growth factors [1]. Importantly, angiogenesis is essential for tumor progression since tumors cannot grow to a diameter greater than 1–2 mm before their oxygen and nutrient demands, as well as waste product elimination, can no longer be met by diffusion alone [2]. Lack of oxygen and nutrients in the tumor microenvironment causes tumor cells to express angiogenesis-promoting mediators such as vascular endothelial growth factor (VEGF). These proangiogenic factors initiate new blood vessel development from existing nearby endothelium. Because angiogenesis is the rate-limiting step in the growth of a solid tumor, the angiogenic process is considered to be an important target for cancer therapy [3]. Interestingly, antiangiogenic therapy, when timed correctly, greatly increases the effectiveness of conventional chemotherapy and radiotherapy by normalizing the tumor vasculature [4]. The resulting increase in tumor oxygenation and decreased interstitial fluid pressure enhance the sensitivity of tumor cells to ionizing radiation and facilitate tumor penetration by chemotherapeutic drugs, respectively.

There is currently widespread interest in developing new and less toxic anticancer agents from natural sources, including spices [5]. Well known for its pungency and diverse physiological effects, black pepper obtained from the dried fruits of the black pepper plant (*Piper nigrum*) has been used in traditional Indian and Chinese medicine to treat conditions ranging from gastrointestinal ailments to epilepsy [6]. The medicinal value of black pepper is attributed to the alkaloid piperine, which exerts anti-inflammatory [7,8], neuroprotective [9] and cardiovascular protective effects [10] and is used to increase the bioavailability of drugs and nutrients by increasing intestinal fluidity and the surface area of the intestinal microvilli [11], as well as by inhibiting drug-metabolizing enzymes and drug efflux pumps [12]. Recent studies show that piperine is cytotoxic for and prevents the *in vivo* growth of cancer cells, including B16-F10 melanoma cells [13], Dalton's lymphoma ascites cells and Ehrlich ascites carcinoma cells [14]. Piperine also inhibits HT-1080 fibrosarcoma cell expression of matrix metalloproteinase (MMP)-9, thereby interfering with tumor cell migration and invasion [15]. However, the effect of piperine on angiogenesis has not yet been investigated.

In this study, we determined the effect of piperine on individual aspects of the angiogenic process, including *in vitro* proliferation, migration and tubule formation by human umbilical vein endothelial cells (HUVECs), as well as the effect of piperine on collagen-induced

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angiogenic activity by rat aorta explants and breast cancer cell-induced angiogenesis in chick embryos. Piperine inhibited all aspects of the angiogenic process, as well as collagen-induced blood vessel outgrowth *ex vivo* and breast cancer cell-induced angiogenesis *in vivo*. The inhibitory effects of piperine on angiogenesis were not the result of piperine-mediated activation of the cation channel transient receptor potential vanilloid 1 (TRPV1) [16]; rather, piperine inhibited the phosphoinositide-3 kinase (PI3K)/Akt signaling cascade that promotes angiogenesis [17]. Our findings indicate that piperine warrants further study as a potential antiangiogenic agent for the prevention and/or treatment of cancer.

2. Materials and methods

2.1. Cell culture

All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Pooled HUVECs were purchased from Lonza Inc. (Walkersville, MD, USA) and were cultured in Clonetics EGM-2 Endothelial Cell Growth Medium (Lonza). EGM-2 medium, which contains various endothelial cell growth factors including VEGF, was used as the culture medium for all experiments unless otherwise noted. HUVECs from passages 2–6 were used in experiments. HMC-1 human mastocytoma cells (from Dr. Jean Marshall; Dalhousie University, Halifax, NS, Canada), were cultured in Iscove's Modified Dulbecco's Medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 5 mM HEPES (all from Invitrogen Canada Inc., Burlington, ON, Canada).

2.2. Reagents

RPMI-1640 medium, phosphate-buffered saline (PBS), SB-366791, and piperine were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). A 100-mM stock solution of piperine was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at −80°C. Antibodies (Abs) against phospho-Akt (Ser 473), phospho-Akt (Thr 308), total Akt and cyclin D3 were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Phycoerythrin (PE)-conjugated anti-VEGF receptor 1 (VEGFR1) Ab, PE-conjugated anti-VEGFR2 Ab and PE-conjugated mouse IgG1 were a generous gift from Dr. Alexander Easton (purchased from R&D Systems Inc., Minneapolis, MN, USA). Anti-actin, anti-MMP-2, anti-MMP-9, bovine anti-goat horseradish peroxidase (HRP) and donkey anti-rabbit HRP Abs were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). LY-294002, *N*-(4-*t*-butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide (BCTC) and capsazepine were purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). Paraformaldehyde (PFA) was from Bio-Shop Canada Inc. (Burlington, ON, Canada). All cell culture plastics were obtained from Sarstedt Inc. (Montreal, QC, Canada), unless otherwise noted.

2.3. Flow cytometric measurement of cell division

HUVECs were plated at 2.5×10^4 cells/well in six-well flat-bottom plates and allowed to adhere overnight. Medium was removed, and HUVEC monolayers were washed with warm PBS. Warm serum-free RPMI-1640 medium containing 5 μM CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) was added to each well, and cells were incubated for 45 min at 37°C. Staining medium was then removed, and wells were washed three times with warm RPMI-1640 medium containing 10% FCS. Warm EGM-2 was added to each well, and cells were incubated for 2–3 h at 37°C. Control cells were trypsinized, fixed with 1% PFA and stored at 4°C until analysis at the end of the experiment to provide baseline fluorescence. The remaining cells were treated as indicated and incubated for 72 h. At the end of the time point, cells were trypsinized and fixed with 1% PFA, and the mean channel fluorescence (MCF) of each sample was analyzed using a FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON, Canada). The number of cell divisions (n) was calculated as $MCF_{control}=2^n\times MCF_{sample}$.

2.4. Tritiated-thymidine incorporation assay

HUVECs were plated at 3×10^3 cells/well in 96-well flat-bottom plates and allowed to adhere overnight. Cells were treated with EGM-2 medium, vehicle (DMSO) or the indicated concentrations of piperine and incubated for 24 h. For the last 6 h of incubation, cells were pulsed with 0.2 μCi of methyl tritiated-thymidine ([^{3}H]TdR; MP Biomedicals, Irvine, CA, USA). Following incubation, cells underwent one freeze–thaw cycle prior to harvesting with a Titertek Cell Harvester (Skatron Instruments, Sterling, VA, USA). [^{3}H]TdR incorporation was measured using a Beckman LS6000IC liquid scintillation counter (Beckman Coulter Inc., Mississauga, ON, Canada).

2.5. Annexin-V staining

HUVECs were plated at 6×10^4 cells/well in a six-well flat-bottom plate and allowed to adhere overnight. Cells were treated as indicated, incubated for 24 h, then harvested by trypsinization, washed with PBS and resuspended in incubation buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$) containing 1:50 Annexin-V–FLUOS (Roche Diagnostics, Laval, QC, Canada). Samples were incubated at room temperature for 10–15 min, diluted 1:10 with additional incubation buffer and analyzed by flow cytometry. Percent viability was calculated as the percentage of cells that stained negative for Annexin-V–FLUOS.

2.6. Lactate dehydrogenase (LDH) assay

A CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) was used as per the manufacturer's instructions. In brief, HUVECs were plated at 5×10^3 cells/well in 96-well flat-bottom plates and allowed to adhere overnight. Cells were then treated as indicated and incubated for 24 h. The plate was centrifuged at 500*g* for 5 min, and 50 μl of the supernatant from each well was transferred to a new plate and stored at 4°C, with the exception of the positive control wells. The original plate containing the positive control wells was frozen at −80°C and thawed at 37°C three times to induce maximum cell lysis and LDH release. Following the final freeze–thaw cycle, the original plate was centrifuged at 500*g* for 5 min, and 50 μl of supernatant from the positive control wells was transferred to the new plate. Substrate was then added to each well. The plate was incubated in the dark for 30 min at room temperature, after which 50 μl of stop solution was added to each well. The spectrometric absorbance was read at 490 nm on an ELx800 microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Percent cytotoxicity was calculated as follows: percent cytotoxicity=100×([experimental LDH release−spontaneous LDH release]/[maximum LDH release−spontaneous LDH release]), where spontaneous LDH release was the average absorbance from the supernatants of the medium-treated cells and maximum LDH release was the average absorbance of the supernatants of the positive control cells.

2.7. Cell cycle analysis

HUVECs were plated at 2.5×10^4 cells/well in six-well flat-bottom plates and allowed to adhere overnight. Cells were treated with medium, vehicle (DMSO) or the indicated concentrations of piperine and incubated for 72 h, then harvested by trypsinization, resuspended in PBS and then fixed by the dropwise addition of ice-cold 70% ethanol while vortexing. Cells were frozen at −20°C for at least 24 h. Samples were then centrifuged, washed with ice-cold PBS and stained with 0.02 mg/ml propidium iodide (PI) in PBS containing 0.2 mg/ml DNase-free RNase A (Qiagen Inc., Mississauga, ON, Canada) and 0.1% (v/v) Triton X-100. Samples were stained in the dark at room temperature for 30 min prior to analysis by flow cytometry. BD CellQuest software (BD Biosciences) was used to determine the fluorescence intensity of 1×10^4 quantifiable cells from each sample. The percentage of cells in the various phases of the cell cycle was determined using ModFit LT software (Verity Software House, Topsham, ME USA).

2.8. Western blot analysis

HUVECs were plated at 3×10^5 cells/T75 flask and allowed to adhere overnight, then treated as indicated and incubated for 24 h. Cells were trypsinized and lysed with ice-cold lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 50 mM Na_2HPO_4, 0.25% sodium deoxycholate (w/v), 0.1% Nonidet P-40 (v/v), 5 mM (ethylenedinitrilo)-tetraacetic acid and 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetra-acetic acid] containing freshly added protease and phosphatase inhibitors (5 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 mM NaF, 1 mM phenylmethyl sulfonyl fluoride, 1 mM dithiothreitol, 100 μM Na_3VO_4, 10 μM phenylarsine oxide and 10 μg/ml aprotinin). Samples were incubated on ice for 30 min and clarified by centrifugation at 10,000*g* for 10 min. Total cell protein was collected and quantified by colorimetric assay using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories Inc., Mississauga, ON, Canada). Protein levels were equalized between samples, which were then denatured by the addition of sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample loading buffer [200 mM Tris–HCl (pH 6.8), 30% glycerol (v/v), 6% SDS (w/v), 15% β-mercaptoethanol (v/v) and 0.01% bromophenol blue (w/v)].

Each sample was then heated to 95°C for 5 min and stored at −80°C until use. Prestained protein markers (Bio-Rad Laboratories) and protein samples were resolved on Tris–HCl acrylamide gels [12% acrylamide resolving gel containing 375 mM Tris–HCl (pH 8.8), 0.1% SDS (w/v), 0.1% ammonium persulfate (APS, w/v) and 0.15% *N,N,N′,N′*-tetramethylethylenediamine (TEMED, v/v) with a 4% acrylamide stacking gel containing 125 mM Tris–HCl (pH 6.8), 0.1% SDS (w/v), 0.1% APS (w/v) and 0.3% TEMED (v/v)]. Gels were electrophoresed at 200 V for 1 h in SDS-PAGE running buffer [20 mM Tris–HCl (pH 8.3), 200 mM glycine and 0.1% SDS (v/v)] and then transferred to nitrocellulose membranes using the iBlot dry blotting system (Invitrogen). Nitrocellulose membranes were incubated in blocking solution containing Tris-buffered saline [20 mM Tris–HCl (pH 7.6), 200 mM NaCl] and 0.05% Tween-20 (v/v) (TBST) with 5% fat-free milk (w/v) for 1 h at room temperature or overnight at 4°C. Membranes were washed extensively with TBST and then incubated with the appropriate primary Ab for 1 h at room temperature or overnight at 4°C. Stock Abs were typically diluted 1:1000 in TBST containing either 5% fat-free milk or 5% bovine serum albumin (Sigma-Aldrich), as per manufacturer's instructions. Following extensive washing with TBST, membranes were incubated with the appropriate HRP-conjugated secondary Ab diluted 1:1000 in TBST with 5% fat-free milk for 1 h at room temperature. Membranes were

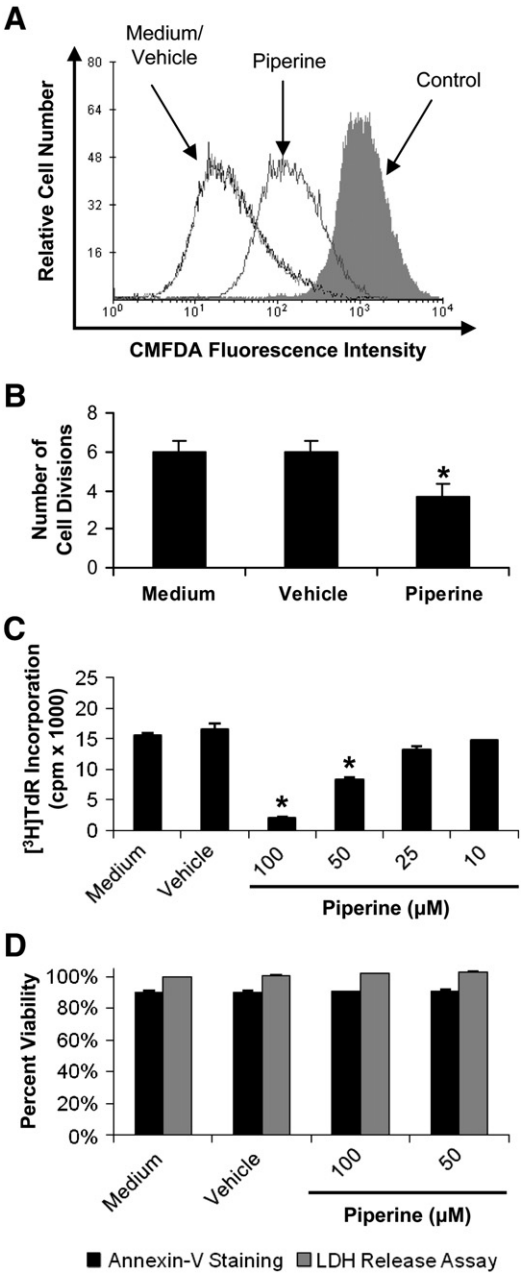

Fig. 1. Piperine is a noncytotoxic inhibitor of endothelial cell proliferation. (A) HUVECs were stained with CMFDA prior to treatment with medium, vehicle (DMSO) or 100 μM piperine for 72 h. Fluorescence was quantified by flow cytometry to measure cell proliferation. Data shown are from a representative experiment ($n=3$). (B) The number of cell divisions was calculated from the MCF of CMFDA-labeled HUVECs. Data shown are the mean of three independent experiments±standard error of the mean (S.E.M.); *$P<.05$ when compared to the vehicle control as determined by ANOVA with the Tukey–Kramer multiple-comparisons posttest. (C) HUVECs were treated with medium, vehicle (DMSO) or the indicated concentrations of piperine for 24 h. Cells were pulsed with [^{3}H]TdR for the last 6 h of incubation and harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of three independent experiments±S.E.M.; *$P<.05$ when compared to the vehicle control as determined by ANOVA with the Tukey–Kramer multiple-comparisons posttest. (D) HUVECs were incubated with medium, vehicle (DMSO) or the indicated concentrations of piperine for 24 h prior to Annexin-V staining and flow cytometry to determine phosphatidylserine translocation or detection of LDH release by colorimetric assay. Percent viability was calculated relative to a positive control. Data shown are the mean of three independent experiments±S.E.M.; $P>.05$ when compared to the vehicle control by ANOVA with the Tukey–Kramer multiple-comparisons posttest.

reacted with ECL reagents (GE Healthcare, Baie d'Urfe, QC, Canada) and exposed to X-ray film that was processed in a Kodak X-OMAT 1000A automated X-ray developer. To confirm equal protein loading, membranes were reprobed for actin expression. Protein bands were quantified by densitometry using AlphaEaseFC software (Cell Biosciences, Santa Clara, CA, USA).

2.9. Flow cytometric analysis of VEGFR1 and VEGFR2 expression

HUVECs were plated at 3×10^5 cells/T75 flask and allowed to adhere overnight, then treated as indicated and incubated for 24 h. Cells were harvested by trypsinization, washed with PBS, resuspended in immunofluorescence buffer (0.2% NaN_3, 1% BSA, in 1× PBS) and labeled on ice with PE-conjugated anti-VEGFR1 Ab, PE-conjugated anti-VEGFR2 Ab or PE-conjugated mouse IgG1 at a concentration of 0.5 μg Ab in 25 μl of immunofluorescence buffer for 45 min in the dark. Cells were then washed three times with immunofluorescence buffer, fixed in 1% PFA and analyzed by flow cytometry.

2.10. In vitro cell migration assay

HUVECS were plated at 2.5×10^5 cells/well in six-well flat-bottom plates and allowed to adhere overnight. As previously described [18], a p10 pipette tip was used to scratch the confluent monolayer of cells in each well; then wells were washed once with medium, treated as indicated and photographed (time 0). The migration of cells into the area that was devoid of cells was monitored for the next 24 h and photographed at various time points. HUVEC migration was quantified using National

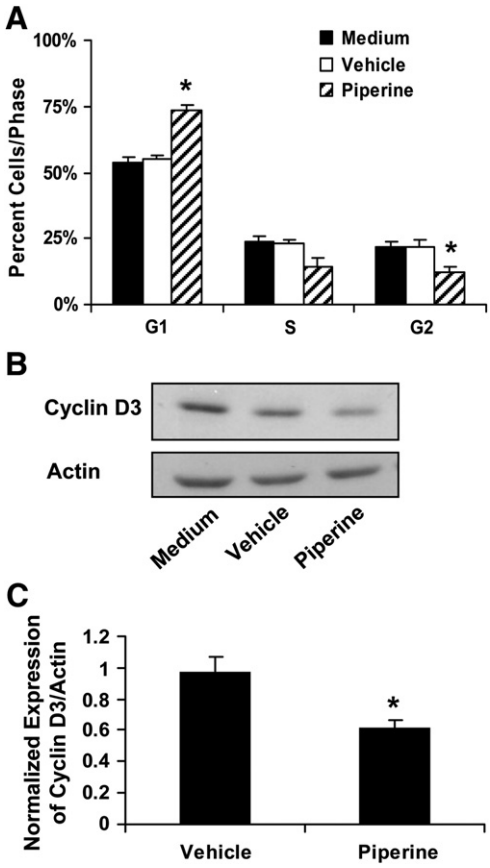

Fig. 2. Piperine inhibits endothelial cell entry into the S phase of the cell cycle. (A) Medium, vehicle (DMSO) or 100 μM piperine was added to HUVECs at 0 and 48 h. Cells were harvested after 72 h of culture, fixed with 70% ethanol for 24 h and then stained with PI for 30 min prior to analysis by flow cytometry. Data shown are the mean of four independent experiments±S.E.M.;*$P<.05$ when compared to the vehicle control as determined by ANOVA with the Tukey–Kramer multiple-comparisons posttest. (B) HUVECs cultured in the presence of medium, vehicle (DMSO) or 100 μM piperine for 24 h were lysed, and total protein was collected for Western blot analysis. Membranes were probed with anti-cyclin D3 Ab, then washed and probed with anti-actin Ab to confirm equal protein loading. Results from a representative experiment are shown ($n=3$). (C) Relative expression of cyclin D3 was calculated as the ratio of cyclin D3 to actin expression, as determined by densitometric analysis. Ratios were normalized to the medium control. Results shown are the mean±S.E.M. of three independent experiments; *$P<.05$ when compared to the vehicle control as determined by Student's t test.

Institutes of Health ImageJ software; percent migration equaled the percentage of the original void that was repopulated by migrating HUVECs.

2.11. In vitro angiogenesis assay

An *in vitro* angiogenesis assay kit (Chemicon International, Temecula, CA, USA) was used as per the manufacturer's instructions. In brief, thawed ECMatrix was diluted with 10× Diluent Buffer, and the liquefied gel solution was added (50 μl/well) to a 96-well flat-bottom plate and allowed to solidify for at least 1 h at 37°C. HUVECs were harvested by trypsinization and pretreated as indicated for 15 min before addition to the solidified gel matrix at 5×10^3 cells/well. Wells were photographed 18–24 h after the addition of the cells. The complexity of tubule formation was scored using the numerical grading scale provided by the manufacturer.

2.12. Ex vivo angiogenesis assay

As previously described [19], excised thoracic aortas from 3-month-old male Wistar rats were turned inside-out and cut into 1.5-mm segments. Each segment was placed in one well of a 12-well flat-bottom plate (BD Biosciences). Collagen matrix was prepared using a collagen cell culturing kit as per the manufacturer's instructions (Wako Chemicals USA, Inc., Richmond, VA, USA). In brief, aorta segments were covered with collagen solution (eight parts collagen, one part 10× Eagle's minimum essential medium, one part reconstitution buffer). Collagen solution was allowed to solidify for 20 min at 37°C. RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1% ITS + Premix (BD Biosciences) was added to wells, either alone or with vehicle (DMSO) or the indicated doses of piperine or LY-294002. Aortas were monitored and photographed over a period of 7 days to document the development of tubules.

2.13. TRPV1 mRNA expression

HUVECs and HMC-1 cells were lysed with TRIzol Reagent (Invitrogen), and total RNA was extracted according to the manufacturer's instructions. RNA concentrations were quantified by spectrophotometric analysis and equalized between samples. RNA was reverse transcribed as per the manufacturer's instructions using Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Invitrogen). cDNA was amplified by polymerase chain reaction according to the manufacturer's instructions with *Taq* DNA polymerase (Invitrogen). A 680-base-pair fragment of *TRPV1* was amplified as previously described [20] under the following conditions: 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 90 s, with a final elongation step of 10 min at 72°C. *TRPV1* primer sequences were 5′-AAGGCCCAGTGTTGACAGTG-3′ (F) and 5′-CTCCTACAACAGCCTGTAC-3′ (R; Invitrogen). A 238-base-pair fragment of *GAPDH* was also amplified as a control, using the same amplification conditions and the following primer sequences: 5′-GAGTCAACGGATTTGGTCGT-3′ (F) and 5′-TTGATTTTG-GAGGGATCTCG-3′ (R). Amplicons were run at 100 V for 1 h on a 1.5% agarose gel containing ethidium bromide, visualized by ultraviolet light and photographed by a Polaroid DS34 camera (Bio/Can Scientific Inc., Mississauga, ON, Canada).

2.14. In vivo chick embryo chorioallantoic membrane (CAM) assay

Innovascreen Inc. (Halifax, NS, Canada) performed the *in vivo* chick embryo CAM assays as described [21]. In brief, mesh onplants containing MDA-MB-231 breast cancer cells treated with the vehicle alone or 50 μM piperine were embedded in collagen and placed on 10-day-old chick embryos (4 onplants/embryo, 10 embryos per treatment). Following incubation for 72 h, onplants were photographed, and the presence or absence of blood vessels in each grid square of the mesh onplant was determined. Percent angiogenesis was calculated as the percentage of positive grid squares divided by the total number of grid squares per onplant.

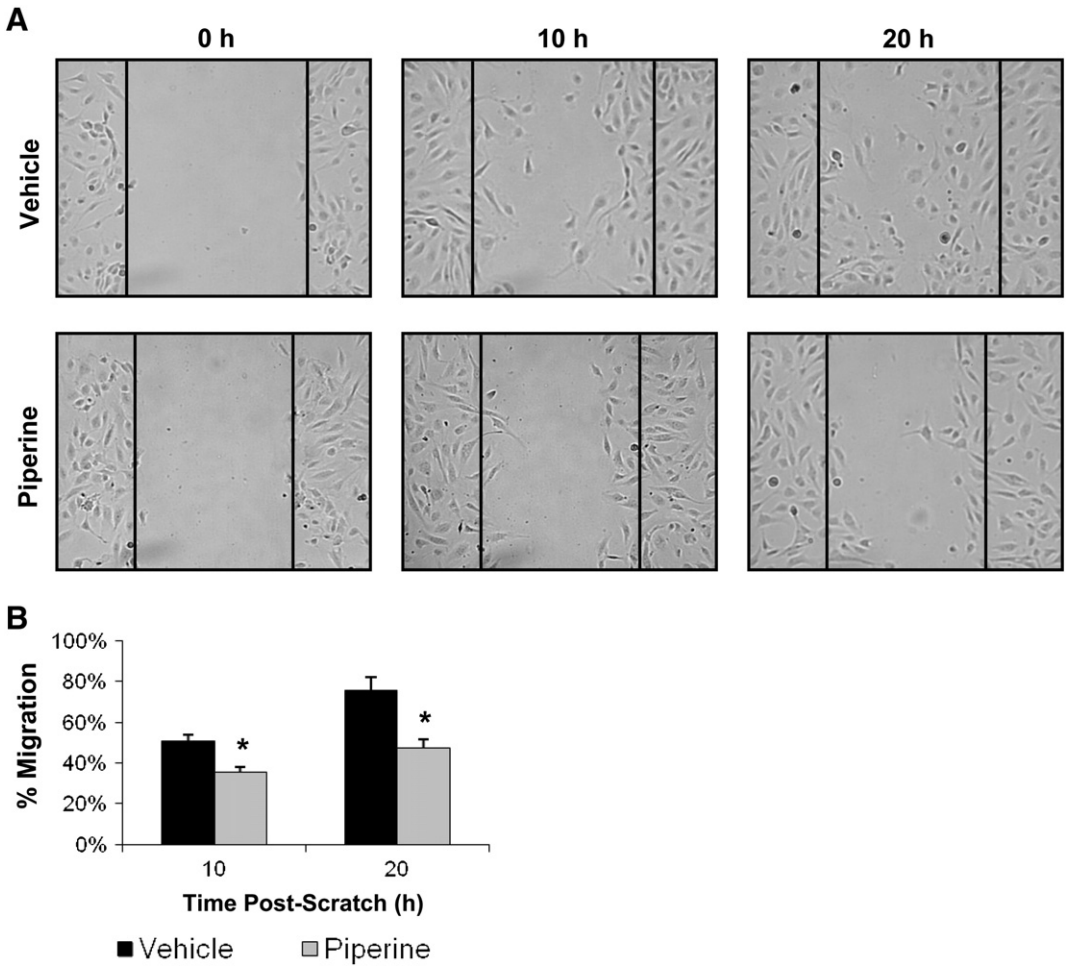

Fig. 3. Piperine attenuates endothelial cell migration. (A) A confluent monolayer of HUVECs was wounded with a p10 pipette tip, and then HUVEC cultures were washed with fresh medium, treated with vehicle (DMSO) or 100 μM piperine and photographed at 0 h under an inverted microscope (20× magnification). The wounded area was monitored for repopulation of the void and photographed at 10 h and 20 h postwounding. Vertical lines indicate the approximate boundary of the original wound. Data shown are from a representative experiment ($n=3$). (B) The percentage of the wounded area that was repopulated was calculated. Data shown are the mean of three independent experiments±S.E.M.; *$P<.05$ when compared to the vehicle control as determined by Student's *t* test.

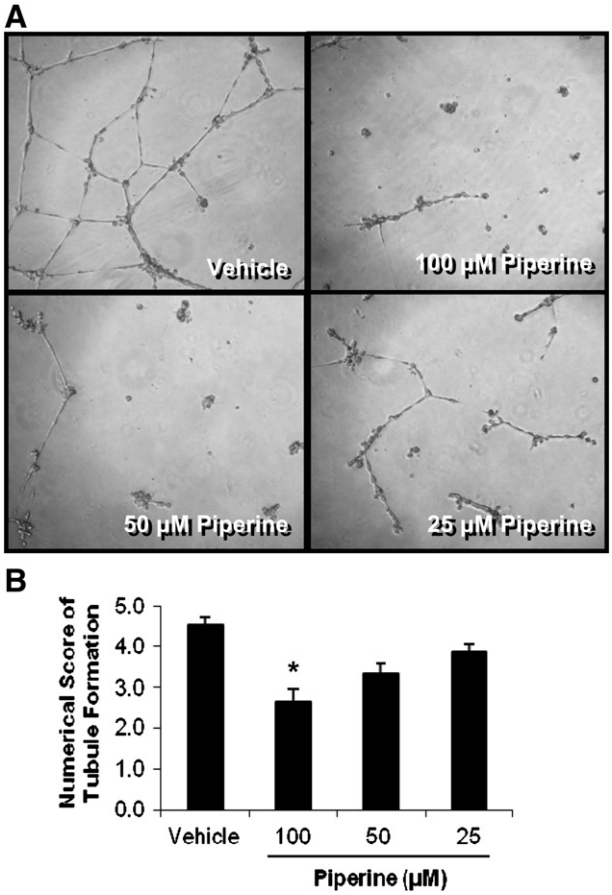

Fig. 4. Piperine inhibits tubule formation by endothelial cells. (A) HUVECs were treated with the vehicle (DMSO) or the indicated concentrations of piperine for 15 min, plated on top of solidified extracellular matrix, incubated for 18–24 h and photographed under an inverted microscope (4× magnification). Photographs are from a representative experiment ($n=3$). (B) Tubule formation was graded by numerical score. Data shown are the mean±S.E.M. of three independent experiments; *$P<.05$ when compared to the vehicle control as determined by the Kruskal–Wallis nonparametric ANOVA test with Dunn's multiple-comparisons test.

2.15. Statistical analysis

Student's *t* test, one-way analysis of variance (ANOVA) with the Tukey–Kramer multiple-comparisons posttest or Kruskal–Wallis nonparametric ANOVA with Dunn's multiple-comparisons posttest was employed, as appropriate, using InStat analysis software (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered statistically significant when the *P* value was less than .05.

3. Results

3.1. Piperine inhibits endothelial cell proliferation without causing cell death

We first determined the effect of piperine on the *in vitro* proliferation of HUVECs using the cell-permeable fluorescent dye, CMFDA, which can quantify cell proliferation as the dye's fluorescence is halved with each round of cell division. As shown in Fig. 1A, HUVECs treated with 100 μM piperine for 72 h underwent fewer rounds of proliferation compared to cells treated with medium or vehicle alone. MCF values indicated that the average number of cell divisions was 6.3±0.3 for both medium and vehicle treatments compared to 3.7±0.7 for HUVECs treated with 100 μM piperine (Fig. 1B; $P<.05$). To confirm the antiproliferative effect of piperine treatment, a [^{3}H]TdR incorporation assay was used to measure the amount of DNA synthesis occurring in piperine-treated HUVECS. As shown in Fig. 1C, exposure to 100 μM piperine for 24 h resulted in an 87% inhibition of DNA synthesis ($P<.05$). To determine whether piperine's antiproliferative effect was due to cytotoxicity, the viability of piperine-treated HUVECs was assessed by LDH release and Annexin-V staining. As shown in Fig. 1D, both assays indicated that piperine did not adversely affect HUVEC viability, indicating that the inhibitory effect of piperine on HUVEC proliferation was not due to cell death.

3.2. Piperine inhibits endothelial cell entry into the S phase of the cell cycle

The antiproliferative effect of piperine on HUVECs was further examined by cell cycle analysis using the DNA-intercalating dye PI. As shown in Fig. 2A, in comparison to vehicle-treated cells, the

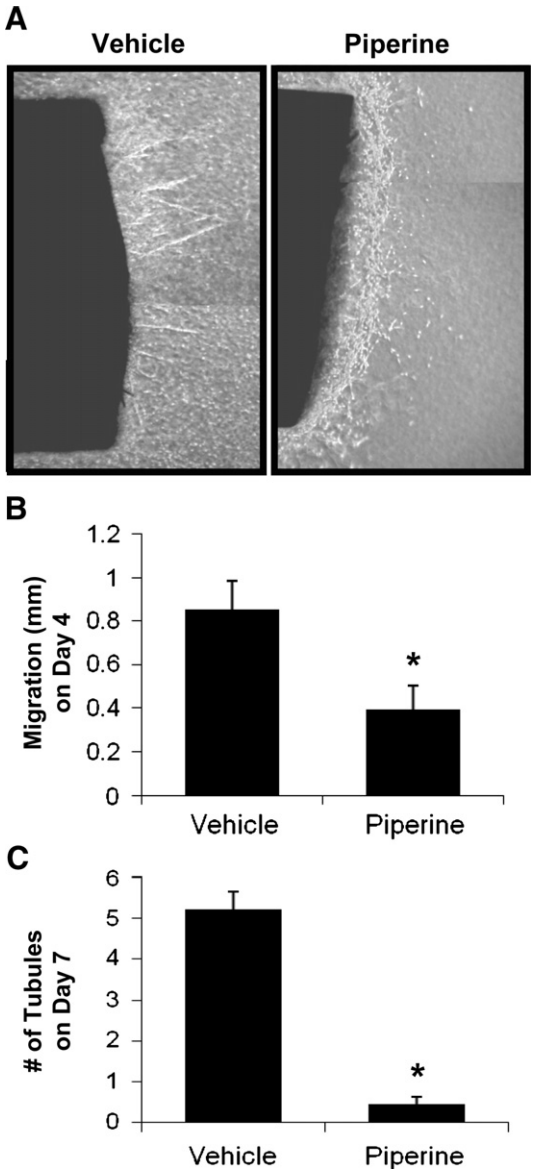

Fig. 5. Piperine inhibits *ex vivo* angiogenesis. Rat aortas were excised, inverted, cut into segments and incubated in a supplemented collagen matrix in the presence of the vehicle (DMSO) or 100 μM piperine. (A) Blood vessel development was monitored and photographed under an inverted microscope (4× magnification) after 7 days. Images of representative aortas ($n=4$) are shown. (B) Cell migration was measured on day 4. (C) Tubules were counted on day 7. Data shown are the mean of four independent experiments±S.E.M.; *$P<.05$ when compared to the vehicle control as determined by Student's *t* test.

percentage of HUVECs in the G_0/G_1 phase of the cell cycle was significantly increased following piperine treatment ($P<.05$), indicating that piperine caused a block at the G_0/G_1 phase of the cell cycle and prevented cells from entering the S phase. Additionally, Western blot analysis determined that piperine inhibited the expression of the cell cycle protein cyclin D3 (Fig. 2B). Densitometric analysis showed that cyclin D3 protein expression was reduced by 35% in piperine-treated HUVECs (Fig. 2C; $P<.05$).

3.3. Piperine attenuates endothelial cell migration

We next used an *in vitro* cell migration assay to determine the effect of piperine on endothelial cell motility. Following wounding of the cell monolayer, Fig. 3A shows that in the presence of 100 μM piperine, HUVEC migration was reduced by 30% and 38% at 10 h and 20 h, respectively (Fig. 3B; $P<.05$), compared to vehicle-treated cells. Since MMP-2 and MMP-9 are involved in HUVEC migration [22], we also examined the effect of piperine on MMP-2 and MMP-9 expression by HUVECs. Western blot analysis showed that 24-h treatment of HUVECs with 100 μM piperine did not affect MMP-2 or MMP-9 expression (data not shown).

3.4. Piperine inhibits in vitro tubule formation

The effect of piperine on tubule formation was investigated by culturing HUVECs on growth-factor-enriched gel matrix, which induces endothelial cell alignment, the development of offshoots between neighboring cells and the formation of tubules. As shown in Fig. 4A, piperine inhibited tubule formation by HUVECs in a dose-dependent manner. Only small clusters and offshoots were visible in piperine-treated cultures, while vehicle-treated cultures showed a complex mesh of tubules with the formation of numerous closed polygons. Semiquantitative scoring of tubule complexity revealed that 100 μM piperine significantly inhibited tubule formation (Fig. 4B; $P<.05$).

3.5. Piperine inhibits ex vivo angiogenesis

We next used the *ex vivo* rat aorta angiogenesis model to confirm the antiangiogenic activity of piperine. Fig. 5A shows that collagen-induced angiogenesis and tubule formation by the excised and inverted aortas were inhibited in the presence of 100 μM piperine. Compared to the vehicle control, there was significant reduction in the migration of piperine-treated cells from the aorta on day 4 (55% decrease, $P<.05$; Fig. 5B), as well as a near-complete absence of tubule formation on day 7 (93% decrease, $P<.05$; Fig. 5C).

3.6. TRPV1 is not involved in the antiangiogenic activity of piperine

Since piperine binds to and desensitizes the cation channel TRPV1 [16], we employed three different TRPV1 antagonists (SB-366791, capsazepine and BCTC) to determine whether TRPV1 mediated the antiangiogenic activity of piperine. Fig. 6 shows that none of the TRPV1 antagonists prevented piperine from inhibiting HUVEC proliferation (Figs. 6A–C). In addition, HUVECs failed to express TRPV1 mRNA, which was abundant in HMC-1 mastocytoma cells that were used as a positive control (Fig. 6D).

3.7. Piperine inhibits Akt phosphorylation in endothelial cells

We next determined the effect of piperine on the phosphorylation of Akt, which is an important proangiogenic signaling molecule [17]. Western blot analysis showed that treatment of HUVECs with 100 μM piperine resulted in a significant reduction in Akt phosphorylation at Ser 473 and Thr 308 residues (Fig. 7A and B). LY-294002, a selective inhibitor of PI3K, which is upstream of Akt in growth factor receptor

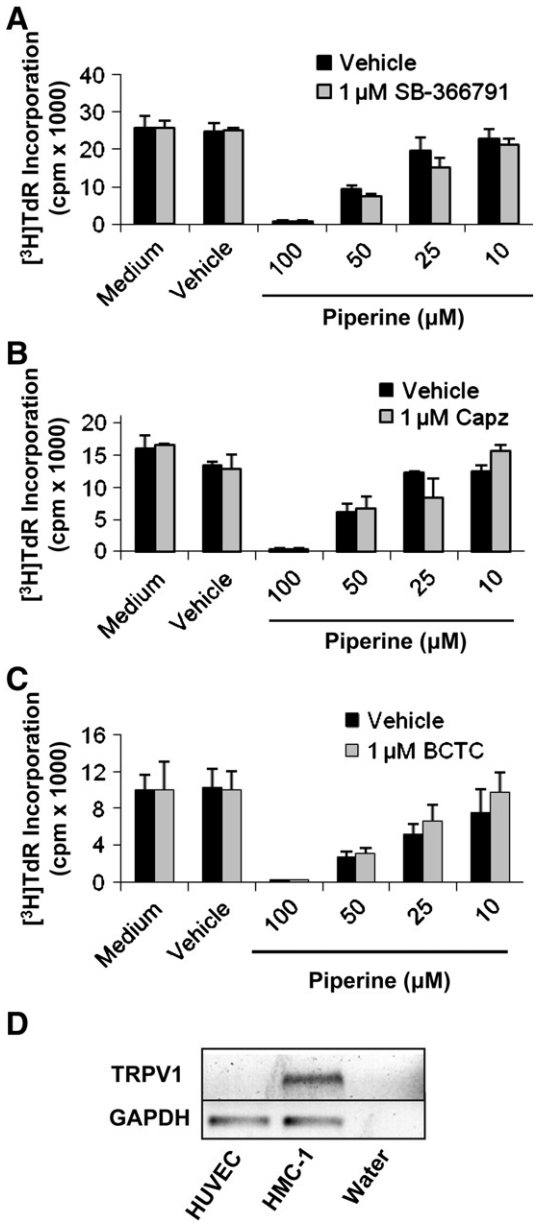

Fig. 6. TRPV1 does not mediate the antiangiogenic effect of piperine. HUVECs were exposed to (A) SB-366791, (B) capsazepine (capz) or (C) BCTC (all at 1 μM) for 30 min prior to the addition of medium, vehicle (DMSO) or the indicated concentrations of piperine. HUVECs were cultured for 24 h, then pulsed with [^{3}H]TdR for the last 6 h of culture and harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of three independent experiments±S.E.M.; $P>.05$ when comparing the antagonist vehicle control to the antagonist for each treatment, as determined by ANOVA with the Tukey–Kramer multiple-comparisons posttest. (D) RNA was extracted from HUVECs and HMC-1 cells, reverse transcribed into cDNA and then amplified for TRPV1 or GAPDH. Water was used instead of cDNA as a negative control.

signaling, was used to confirm the importance of the PI3K/Akt pathway in HUVEC proliferation. As with piperine, LY-294002 inhibited HUVEC proliferation (Fig. 7C) and also inhibited tubule development in the *ex vivo* rat aorta angiogenesis model (Fig. 7D).

We also determined the effect of piperine on VEGFR1 and VEGFR2 expression by HUVECs since both of these receptors are expressed by endothelial cells and are important in VEGF-induced angiogenesis [23]. Fig. 8 shows that piperine-treated HUVECs had increased expression of VEGFR1, whereas there was no change in VEGFR2 expression.

3.8. Piperine inhibits angiogenesis induced in vivo by MDA-MB-231 breast cancer cells

Piperine-mediated inhibition of various aspects of the angiogenic process *in vitro* and *ex vivo* led us to employ the chick embryo CAM assay to confirm the antiangiogenic activity of piperine *in vivo*. The MDA-MB-231 breast cancer cell line, which is a good source of proangiogenic factors, including VEGF [24], was used in the embryo onplants to stimulate angiogenesis. As shown in Fig. 9, treatment with 50 μM piperine caused a greater than 50% reduction in MDA-MB-231-induced angiogenesis in the CAM compared to the vehicle control ($P<.05$). The concentration of piperine used in this experiment did not affect the proliferation or viability of MDA-MB-231 cells (data not shown).

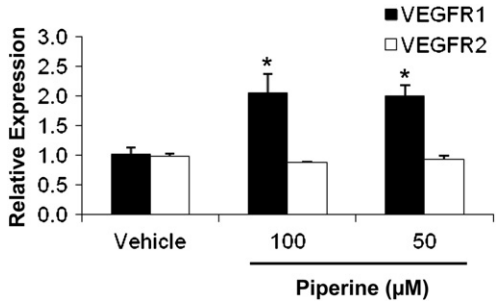

Fig. 8. Piperine increases HUVEC expression of VEGFR1. HUVECs were cultured for 24 h in the presence of medium, vehicle (DMSO) or the indicated concentrations of piperine. Cells were then stained with anti-VEGFR1 Ab, anti-VEGFR2 Ab or an isotype control, fixed and analyzed by flow cytometry. The MCF of VEGFR1 and VEGFR2 expression (less the MCF of the isotype control) was normalized relative to HUVECs treated with medium alone. Results shown are the mean±S.E.M. of three independent experiments; *$P<.05$ when compared to the respective vehicle control, as determined by ANOVA with the Tukey–Kramer multiple-comparisons posttest.

4. Discussion

Angiogenesis is an important component of tumor progression [2] and, as such, is considered to be a pivotal target for cancer treatment [3]. The dietary phytochemical piperine has previously been reported to kill several different types of cancer cells [13–15]; however, to our knowledge, we are the first to show antiangiogenic activity by piperine. Importantly, LDH release and Annexin-V staining established that piperine was not simply cytotoxic for HUVECs but potently inhibited *in vitro* proliferation and migration of endothelial cells, as well as tubule formation *in vitro* and *ex vivo* and breast cancer cell-induced angiogenesis *in vivo*. However, piperine did not inhibit HUVEC expression of MMP-2 and MMP-9, which are involved in endothelial cell migration [22].

Surprisingly, the antiangiogenic activity of piperine was not mediated through TRPV1, which functions as a receptor for piperine [16]. Since different TRPV1 antagonists exhibit different potencies for inhibiting piperine-induced oral aversiveness in rats [25], we used three different TRPV1 antagonists to rule out differences in antagonist potency and receptor affinity as possible confounding factors. All

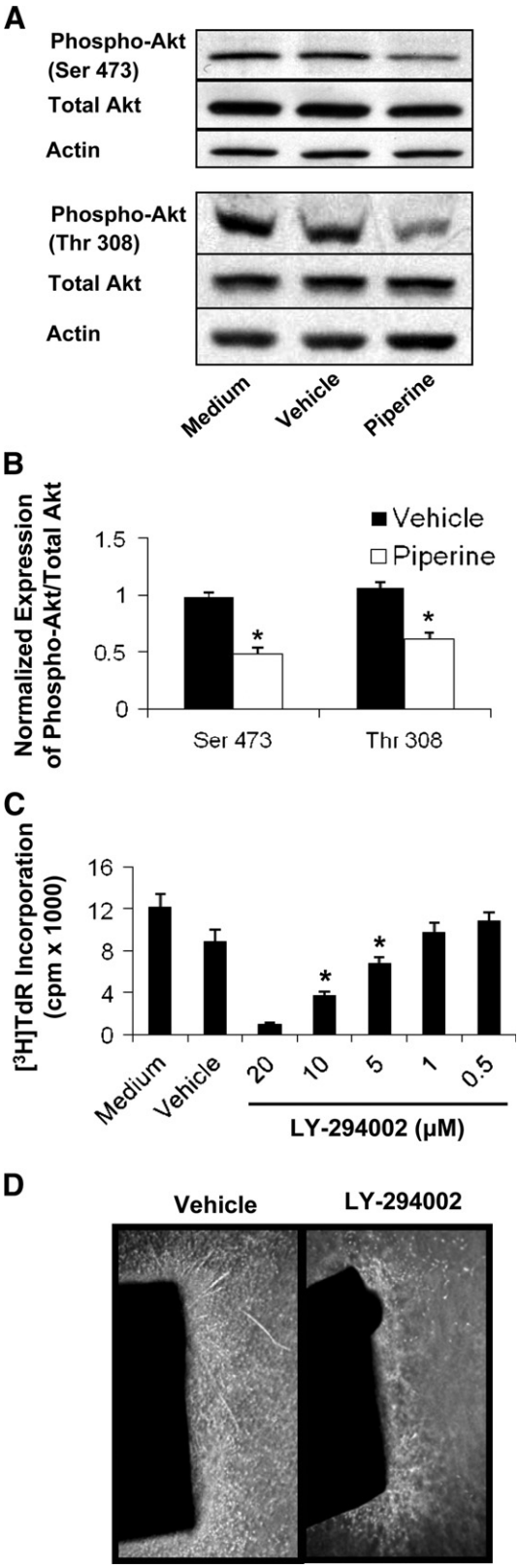

Fig. 7. Piperine inhibits Akt phosphorylation. (A) HUVECs cultured for 24 h in the presence of medium, vehicle (DMSO) or the indicated concentrations of piperine were lysed, and total protein was collected for Western blot analysis. Membranes were probed with Abs against phospho-Akt (Ser 473), phospho-Akt (Thr 308) or total Akt and then washed and probed with anti-actin Ab to confirm equal protein loading. Results from a representative experiment ($n=4$) are shown. (B) Relative expression of phospho-Akt was calculated as the ratio of phospho-Akt to total Akt expression after each band was normalized to actin, as determined by densitometric analysis. Expression was then normalized to the medium control. Results shown are the mean±S.E.M. of three independent experiments; *$P<.05$ when compared to the vehicle control, as determined by ANOVA with the Tukey–Kramer multiple-comparisons posttest. (C) HUVECs were cultured for 24 h in the presence of medium, vehicle (DMSO) or the indicated concentrations of LY-294002. Cells were pulsed with [^{3}H]TdR for the last 6 h of culture and harvested, and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data shown are the mean of three independent experiments±S.E.M.; *$P<.05$ when compared to the vehicle control, as determined by ANOVA with the Tukey–Kramer multiple-comparisons posttest. (D) Rat aortas were excised, inverted, cut into segments and incubated in a supplemented collagen matrix with vehicle (DMSO) or 10 μM LY-294002. Blood vessel development was monitored and photographed (4× magnification) after 7 days. Images of representative aortas ($n=2$) are shown.

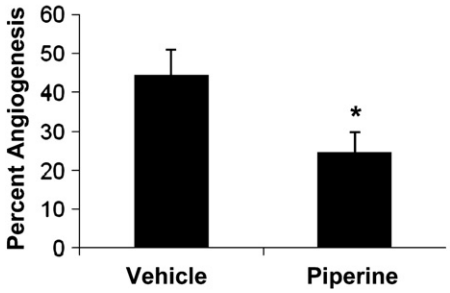

Fig. 9. Piperine inhibits breast cancer cell-induced angiogenesis *in vivo*. Mesh onplants containing MDA-MB-231 breast cancer cells and either vehicle (DMSO) or 50 μM piperine were embedded in collagen, placed on 10-day-old chick embryos and incubated for 3 days. Onplants were then photographed, and the percent angiogenesis was calculated; $^{*}P<.05$ when compared to the vehicle control, as determined by Student's *t* test.

three TRPV1 antagonists failed to interfere with the inhibitory effect of piperine on HUVEC proliferation. Moreover, TRPV1 mRNA was not detected in HUVECs, although TRPV1 expression has been reported on cerebromicrovascular [26] and pulmonary arterial endothelial cells [27], as well as on immortalized HUVEC-12 cells [28]. It is possible that TRPV1 is differentially expressed on various types of endothelial cells, as is the case for other TRP channels such as TRPP2, which is expressed on human renal artery endothelial cells but not on HUVECs [29]. In addition, we cannot rule out possible differential expression of TRPV1 depending on the culture conditions. Nevertheless, the absence of TRPV1 mRNA in our HUVECs combined with the failure of TRPV1 antagonists to relieve piperine-mediated inhibition of HUVEC proliferation leads us to conclude that piperine does not mediate its antiangiogenic activities through TRPV1.

The molecular basis for the myriad physiological effects of piperine is poorly understood. Piperine has been reported to inhibit the translocation of nuclear transcription factors nuclear factor κ-light-chain-enhancer of activated B cells, c-Fos, activating transcription factor 2, and cAMP response element-binding in B16-F10 mouse melanoma cells [30], as well as Wnt signaling in MCF7 breast cancer cells [31], and signal transducers and activators of transcription 1 phosphorylation in interferon (IFN)-α-, IFN-β- or lipopolysaccharide-treated macrophages [32]. In endothelial cells, piperine inhibits tumor necrosis factor (TNF)-induced inhibitor of κB (IκB)α phosphorylation but not baseline phosphorylation of IκBα [8]. Our findings indicate that piperine also interferes with Akt activation in response to angiogenesis-promoting growth factors such as VEGF that are contained in EGM-2 medium, although further investigation is required to determine whether this effect is direct or occurs upstream of Akt phosphorylation. Since Akt is an important signaling molecule during angiogenesis [17], it is reasonable to conclude that piperine-mediated inhibition of Akt phosphorylation at Ser 473 and Thr 308 residues is likely responsible for the inhibitory effect of piperine on angiogenesis. This conclusion is in line with evidence that Akt activation is required for cell cycle progression [33], as well as endothelial cell migration and tubule formation [34]. Moreover, we confirmed the importance of Akt to HUVEC proliferation and tubule formation by rat endothelial cells by demonstrating inhibition of these angiogenesis-related processes in the presence of LY-294002, which is a selective inhibitor of PI3K that is known to function upstream of Akt in endothelial cells [35]. Inhibition of Akt activation could also be responsible for the cell cycle block and decrease in cyclin D3 expression observed in piperine-treated HUVECs since Akt signaling prevents G_1 arrest by inhibiting the nuclear import of $p27^{Kip1}$ [33], a cell cycle inhibitor protein that inhibits the activity of cyclin-dependent kinase-cyclin complexes [36]. Akt signaling also inhibits the degradation of D-type cyclins, including cyclin D3 [33,37]. In addition, the PI3K/Akt pathway is involved in VEGF expression by different types of cancer cells [38,39]. However, we cannot rule out the possible contribution of piperine-mediated effects on other signaling pathways to the suppression of angiogenesis. In this regard, increased HUVEC expression of VEGFR1 in the presence of piperine may lead to reduced signaling through VEGFR2 since VEGFR1 binds VEGF with higher affinity than does VEGFR2 [40] but is not required for the *in vitro* proliferation and migration of endothelial cells [41]. Endothelial cell-associated VEGFR1 and soluble VEGFR1 are therefore suggested to function as decoy receptors for VEGF [23]. Nevertheless, taken together, our data suggest that inhibition of Akt activation is an important component of the antiangiogenic action of piperine.

Interestingly, despite Akt activation being important for endothelial cell survival [17], HUVECs did not show decreased viability following piperine treatment, which suggests that residual Akt function in piperine-treated cells was sufficient to maintain cell survival even though other cellular functions involved in angiogenesis were inhibited. Alternatively, other prosurvival signaling pathways in endothelial cells may be refractory to piperine, therefore allowing sufficient prosurvival signaling to prevent the death of piperine-treated HUVECs.

In addition to its antiangiogenic properties, piperine shows *in vitro* cytotoxic activity against a range of cancer cell types, including B16-F10 melanoma cells [13], Dalton's lymphoma ascites and Ehrlich ascites carcinoma [14]. Nevertheless, inhibition of angiogenesis is likely to be an important component of piperine-mediated reduction of tumor progression *in vivo* [13]. Piperine may also be useful for cancer chemoprevention since antiangiogenic agents have the capacity to inhibit the development of tumor vasculature, thereby limiting the growth of hyperplastic foci and possibly preventing the development of clinically significant disease [42]. Chronic inflammation of tissues, which has been linked to carcinogenesis, is characterized by a sustained infiltrate of inflammatory cells that secrete both inflammation- and angiogenesis-promoting factors [43]. Interestingly, piperine inhibits endothelial cell expression of adhesion molecules and the subsequent adhesion of neutrophils to activated endothelial cells [8,44], suggesting that piperine might reduce the migration of inflammatory cells into inflamed tissues. In addition, piperine blocks macrophage activation and production of the proinflammatory factors TNF and nitric oxide both *in vitro* and *in vivo* [45]. Piperine also exhibits chemopreventive antioxidant activity *in vivo* in carcinogen-induced intestinal damage [46] and chemically induced lung carcinogenesis models [47], as well as reducing lipid peroxidation and antioxidant loss in a high-fat-diet-induced oxidative stress model [48]. The combination of antiangiogenic, anti-inflammatory and antioxidant activities argues that piperine may function as an effective chemopreventive agent.

In conclusion, our data show that piperine inhibits multiple aspects of the angiogenic process *in vitro* and *ex vivo*, as well as suppressing breast cancer cell-induced angiogenesis *in vivo*. These effects are independent of TRPV1 but likely involve piperine-mediated inhibition of Akt phosphorylation. Piperine is therefore identified as a novel inhibitor of angiogenesis that warrants further study as a nutraceutical with possible application in the prevention and/or treatment of cancer, as well as other angiogenesis-dependent diseases.

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