

## Perillyl Alcohol Is an Angiogenesis Inhibitor

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### ABSTRACT

Aberrant angiogenesis is essential for the progression of solid tumors and hematological malignancies. Thus, antiangiogenic therapy is one of the most promising approaches to control cancer. In the present work, we examined the ability of perillyl alcohol (POH), a dietary monoterpene with well-established tumor chemopreventive and chemotherapeutic activity, to interfere with the process of angiogenesis. POH remarkably prevented new blood vessel growth in the *in vivo* chicken embryo chorioallantoic membrane assay and proved to be effective in inhibiting the morphogenic differentiation of cultured endothelial cells into capillary-like networks both in collagen gel and Matrigel models. In addition, POH reduced the cell number in a proliferation assay and induced apoptosis of endothelial cells as indicated by the POH-mediated increase of caspase-3 ac-

tivity and DNA fragmentation. Consistent with the observed antisurvival effect, POH treatment resulted in a significant inhibition of Akt phosphorylation in endothelial cells. Finally, POH was able to differentially modulate the release of two important angiogenic regulators: vascular endothelial growth factor (VEGF) and angiopoietin 2 (Ang2). POH decreased the release of VEGF from cancer cells but stimulated the expression of Ang2 by endothelial cells, indicating that it might suppress neovascularization and induce vessel regression. Overall, these data underscore the antiangiogenic potential of POH and suggest that POH, in addition to its anticancer activity, may be an effective agent in the treatment of angiogenesis-dependent diseases.

Perillyl alcohol (POH) (*p*-metha,1,7-diene-6-ol or 4-isopropenyl-cyclohexenecarbinol) is a naturally occurring non-nutritive dietary monoterpene, found in the essential oils of several plants (lavandin, mints, cherries, etc.) and synthesized by the mevalonate pathway. It has well established chemopreventive activity in rodent mammary, skin, liver, lung, colon, and forestomach cancers and also chemotherapeutic activity in pancreatic, mammary, and prostatic animal tumor models, leading to regression of existing malignant tumors (Gould, 1997; Belanger, 1998; Crowell, 1999). In animals fed a standard chew containing 2% POH (estimated daily intake of POH approximately 1.76 g/kg), regression of the tumor mass has been observed (Belanger, 1998). In the body, POH is converted to perillaldehyde, perillic acid, and dihydroperillic acid, which exhibit biological activity. Data

from rodents have shown that tumor regression occurs when plasma levels of perillic acid and dihydroperillic acid reach 390 to 480  $\mu\text{M}$  and 110 to 230  $\mu\text{M}$ , respectively (Belanger, 1998; Crowell, 1999). Additionally, POH has a cytostatic and cytotoxic effect against a variety of cancer cell lines (Crowell, 1999; Burke et al., 2002; Clark et al., 2002). Treatment of pancreatic tumor cells with POH for 2 days results in a concentration-dependent decrease in cell proliferation, with  $\text{IC}_{50}$  values of 290 and 480  $\mu\text{M}$  for the human and hamster cell lines, respectively. A similar treatment of murine B16 ( $\text{F}_{10}$ ) melanoma cells with POH inhibits cell proliferation with an  $\text{IC}_{50}$  of 250  $\mu\text{M}$ . Incubation of malignant hamster pancreatic ductal epithelial cells with 100 to 500  $\mu\text{M}$  POH caused a 2.6- to 18-fold higher rate of apoptosis and a 2-fold higher expression of the pro-apoptotic protein Bak compared with untreated malignant cells (Belanger, 1998, and refs. therein). Recent evidence suggests that POH can also inhibit cell migration *in vitro* (Wagner et al., 2002) and *in vivo* (Teruszkin Balassiano et al., 2002), and it is capable of sensitizing glioma cells to ionizing radiation and conventional

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**ABBREVIATIONS:** POH, perillyl alcohol; VEGF, vascular endothelial growth factor; Ang, angiopoietin; CAM, chorioallantoic membrane; BLMVEC, bovine lung microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; PMA, phorbol 12-myristate 13-acetate; ELISA, enzyme-linked immunosorbent assay.

chemotherapy (Rajesh et al., 2003). Given its proved anticancer activity and its favorable toxicity profile, POH is currently being tested in phase I and phase II clinical trials in patients with refractory solid malignancies. Pharmacokinetic studies in humans revealed that levels of 390 to 480  $\mu\text{M}$  of the POH metabolite perillic acid are achieved in patients receiving nontoxic doses of POH orally (Belanger, 1998; Hudes et al., 2000; Azzoli et al., 2003).

Several mechanisms have been proposed to mediate the antitumor effects of POH. It has been shown that POH affects the expression of several regulators of cell cycle and apoptosis (Ariazi et al., 1999; Bardon et al., 2002; Shi and Gould, 2002). There is also evidence that POH inhibits the post-translational isoprenylation of the Ras small GTPase superfamily of proteins (Hohl and Lewis, 1995; Stayrook et al., 1998; Holstein and Hohl, 2003), which are known to play a key role in many signal transduction pathways, including those that stimulate tumor-associated angiogenesis (Kranenburg et al., 2004).

The development of an angiogenic supply from the existing vasculature is critical for the growth of solid tumors, as well as for the progression of hematological malignancies (Saaristo et al., 2000; Bergers and Benjamin, 2003; Moehler et al., 2003). Angiogenesis, under both physiological and disease-associated conditions, is a complex multistep process involving the orchestrated interaction of endothelial cells with the extracellular matrix and soluble angiogenic factors. Among the known angiogenic factors, the families of vascular endothelial growth factors (VEGFs) and angiopoietins (Ang1 to Ang4) have emerged as essential coordinators of angiogenesis, selectively targeting the endothelium through their endothelial cell-specific tyrosine kinase receptors (Yancopoulos et al., 2000; Jones et al., 2001; Ferrara et al., 2003). To date, much effort has been directed toward discovering antiangiogenic agents and evaluating them as cancer therapeutics (Eskens, 2004). Among them, several dietary and nondietary phytochemicals have been investigated for their antiangiogenic activity in both animal models and cell culture systems (Singh and Agarwal, 2003). In the present work, we have investigated the potential usefulness of POH as an effective antiangiogenic agent. We have studied the activities of POH with regard to growth of new blood vessels in the chicken embryo chorioallantoic membrane (CAM) in vivo, morphological differentiation of endothelial cells in three-dimensional collagen gel and Matrigel, endothelial cell proliferation and apoptosis, and production of the angiogenic growth factors VEGF and Ang2. The effect of the enzymatically synthesized ester of POH with decanoic acid was also examined in parallel in most of the above-mentioned studies.

## Materials and Methods

**Materials.** Bovine lung microvascular endothelial cells (BLMVECs) were obtained from Vec Technologies (Rensselaer, NY), and K562 cells were originally obtained from American Type Culture Collection (Manassas, VA). Tissue culture plastic ware was obtained from Corning-Costar (Corning, NY). Cell culture media and supplements, fetal calf serum, trypsin, and antibiotics were obtained from Invitrogen (Carlsbad, CA). POH was obtained from Fluka (Buchs, Switzerland). Lipase B from *Candida antarctica* was kindly offered by Novo Nordisk (Bagsvaerd, Denmark), and silica gel plates were purchased from Merck (Darmstadt, Germany). Rat tail collagen, type I was from BD Biosciences (San Jose, CA). The Supersignal Chemi-

luminescent Substrate was obtained from Pierce Chemical (Rockford, IL). The anti-Akt and anti-phospho-Akt were from Cell Signaling Technology Inc. (Beverly, MA). X-ray film was obtained from Eastman Kodak (Rochester, NY). The anti-rabbit HRP-labeled secondary antibody was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). VEGF and Ang2 ELISA Duo set kits were obtained from R&D Systems (Minneapolis, MN). Reagents for SDS-polyacrylamide gel electrophoresis and western blotting were obtained from Bio-Rad (Hercules, CA). The EnzChek and CyQUANT kits for determining caspase-3 activity and cell number, respectively, were purchased from Molecular Probes (Eugene, OR). All other reagents included were obtained from Sigma-Aldrich (St. Louis, MO).

**Cell Culture.** BLMVECs were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics (10 U/ml penicillin and 100 mg/ml streptomycin) and used up to passage 12. Human umbilical vein endothelial cells (HUVECs) were isolated as previously described (Hatzia Apostolou et al., 2003) and cultured in M199 supplemented with 15% fetal calf serum, 200  $\mu\text{g}/\text{ml}$  endothelial cell growth supplement, 4 U/ml heparin sodium, L-glutamine, and antibiotics and were used at passages 1 to 5. The human lymphoblastoma K562, B16 mouse skin melanoma, and MDA-MB-231 human mammary gland cancer cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, and antibiotics. Cells were incubated in a humidified 37°C incubator containing 5% CO<sub>2</sub>.

**Synthesis of POH Esters.** The enzymatic synthesis and purification of POH decanoic ester was performed as previously described (Skouridou et al., 2003). Briefly, 5 mmol POH and 5 mmol decanoic acid were added in 10 ml of hexane, and the esterification was catalyzed by immobilized lipase B from *C. antarctica* at 50°C for 48 h under continuous shaking. POH ester was isolated by preparative thin-layer chromatography: the reaction mixture was concentrated by vacuum evaporation, applied to 60 F264 silica gel plates, and eluted with hexane/diethyl ether 8/2 (v/v). The ester was extracted from the plates with acetone and after solvent removal was used with no further purification. Stock solutions (1 M) of POH and POH ester were prepared in absolute ethanol and further dilutions were made in culture media.

**CAM Assay.** The in vivo chicken embryo CAM angiogenesis model was used, as previously described (Papadimitriou et al., 2001). In brief, Leghorn fertilized eggs were incubated for 4 days at 37°C, when a window was opened on the egg's shell, exposing the CAM. The window was covered with tape, and the eggs were returned to the incubator. Different amounts of POH (2, 10, and 20 nmol) in a solution containing 0.1% ethanol or vehicle (0.1% ethanol) were applied onto an area of 1 cm<sup>2</sup> (restricted by a plastic ring) of the CAM on day 9 of embryo development. Forty-eight hours after treatment and subsequent incubation at 37°C, CAMs were fixed in situ, excised from the eggs, placed on slides, and left to air dry. Pictures were taken through a stereoscope equipped with a digital camera, and the total length of the vessels was measured using image analysis software (Scion Image; Scion Corporation, Frederick, MD). Assays for each test sample were carried out three times, and each experiment included 8 to 10 eggs per data point.

**In Vitro Angiogenesis Assays.** Three-dimensional cultures of BLMVECs in collagen gels were established as previously described (Papapetropoulos et al., 1997). Briefly, rat tail collagen type I at a final concentration of 2 mg/ml was mixed with 10 $\times$  M199, neutralized with sterile 1 M NaOH, and the solution held at 4°C. Cells (2  $\times$  10<sup>6</sup> cells/ml) were added immediately to the collagen solution, and five drops (0.1 ml each) of the mixture was put in 60-mm Petri dishes and placed in a 37°C humidified 5% CO<sub>2</sub> incubator to permit gel formation. Five milliliters of culture medium containing POH (0.25, 0.5, or 1.0 mM) or ethanol vehicle (0.1%) was then added over the gel and the dishes returned to the incubator. After 2 h, phorbol 12-myristate 13-acetate (PMA) was pipetted into the medium at a final concentration of 100 nM, and cells were allowed to differentiate over a 5-day period. Gels were photographed, and their diameters were

monitored macroscopically. To evaluate the formation of structures resembling tubes with lumens, cell cultures were washed with PBS examined by phase-contrast microscopy and microphotographed.

The Matrigel tube formation assay was performed as previously described (Pipili-Synetos et al., 1998). Briefly, Matrigel was used to coat the wells of 96-well tissue culture plates (0.04 ml/well) and left to solidify for 1 h at 37°C; 15,000 HUVECs were then suspended in 0.15 ml of M199 supplemented with 5% fetal calf serum and added to each well. Different concentrations of POH (0.1, 0.5, and 1 mM) were added to the corresponding wells simultaneously with the cells. After 6 h of incubation at 37°C, the medium was removed, the cells were fixed, and the length of structures that resembles capillary cords was measured in the total area of the wells using image analysis software (Scion Image), as previously described (Papadimitriou et al., 2001; Hatzia Apostolou et al., 2003).

**Cell Proliferation Assay.** BLMVECs or K562 cells were plated at  $2 \times 10^3$  or  $15 \times 10^3$  cells/well, respectively, in a 96-well plate and 24 h later were treated with fresh media containing POH, POH ester (0.1, 0.5, and 1 mM), or ethanol vehicle (0.1%) and further cultured for 48 to 72 h. Thereafter, the cell number was measured by using the CyQUANT cell proliferation assay kit according to the manufacturer's instructions. This assay has a linear detection range extending from 50 to 50,000 cells in 200- $\mu$ l volumes using the dye provided by the kit at  $1 \times$  concentration. Briefly, medium was removed from the wells, cells were carefully washed once with PBS, and then 200  $\mu$ l of CyQUANT GR dye/cell lysis buffer was added to each well and incubated for 5 min at room temperature, protected from light. The sample fluorescence was measured in a fluorescence microplate reader using excitation and emission filters at 480 and 520 nm, respectively. A reference standard curve using BLMVECs or K562 cells (from 50–50,000) was created for converting sample fluorescence to cell numbers.

**Apoptosis Assays.** Cell apoptosis was monitored by measuring caspase-3 activity and by determining DNA fragmentation. For the caspase-3 activity assay, K562 cells ( $1 \times 10^6$ ) or BLMVECs grown to confluence in 12-well plates were incubated with fresh media containing various concentrations of POH (0.1, 0.5, and 1 mM), POH ester (1 mM), ethanol vehicle (0.1%), or 5 to 10  $\mu$ M cyclohexamide as a positive apoptosis control. After 16, 24, 48, or 72 h, cells floating in the supernatant were combined with the adherent cells that were collected by trypsinization, and cell pellets were washed once with PBS. Samples were then processed according to the EnzCheck Caspase-3 Assay kit instructions. Briefly, cells were lysed with 60  $\mu$ l of lysis buffer, lysates were collected by centrifugation at 13,000g for 20 min at 4°C, and protein concentrations were measured. Fifty microliters of lysates were transferred to 96-well microplates and incubated for 30 min with a caspase-3 substrate. The release of the fluorescent product was measured using a fluorescence microplate reader (excitation at 350 nm and emission detection at 450 nm).

For the DNA fragmentation assay, confluent cultures of cells grown in 100-mm dishes were incubated with fresh media containing 1 mM POH, 0.1% ethanol vehicle, or 5  $\mu$ M cyclohexamide for 48 h. Floating and adherent cells were then harvested, washed once with PBS, and cytosolic DNA was prepared from cell pellets as described by (Leist et al., 1997) with some modifications. Briefly, cell pellets were lysed in 100  $\mu$ l of lysis buffer (20 mM Tris-HCl, 10 mM EDTA, and 1% Triton X-100, pH 8.0), the cytosolic fraction was collected by centrifugation at 13,000g for 20 min at 4°C, and protein concentration was determined. Cytosol aliquots containing equal amounts of protein were extracted with phenol/chloroform. One-tenth volume of 3 M sodium acetate was added to the solution, and DNA was precipitated by adding an equal volume of isopropanol. After storing at -20°C overnight, a DNA pellet was obtained by centrifugation at 13,000g for 15 min at 4°C and washed once with 75% ethanol. The pellet was dried and resuspended in 20  $\mu$ l of 20 mM Tris-HCl, pH 8.0. After digesting RNA with RNase (0.1 mg/ml) at 37°C for 30 min, samples (10  $\mu$ l) were electrophoresed through a 1% agarose gel, and DNA was photographed under visualization with UV light.

**Western Blotting.** BLMVECs grown to confluence in six-well plates were treated with 0.5 mM POH or 0.1% ethanol for the indicated time periods (15, 30, 60, and 120 min) and then subjected to cell lysis and western blotting. Cells were collected and solubilized with ice-cold cell lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 50 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 0.5% sodium deoxycholate, 1 mM EDTA, 0.1 mM EGTA, 1% Triton-X, 1% SDS, 1 mM PMSF, 10  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml leupeptin, and 10 ng/ml pepstatin). After centrifugation at 13,000g for 20 min at 4°C, protein concentration of supernatants was determined. Lysates containing equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis on 10% gels, transferred to nitrocellulose membranes, and probed with primary antibodies that specifically recognize the Ser473 phosphorylated form of Akt or total Akt. Immunoreactive bands were visualized by enhanced chemiluminescence detection. A similar protocol was followed for K562 cells; primary antibodies recognizing Erk1/2 when phosphorylated at Thr202/Tyr204 or total levels of endogenous Erk1/2 were used.

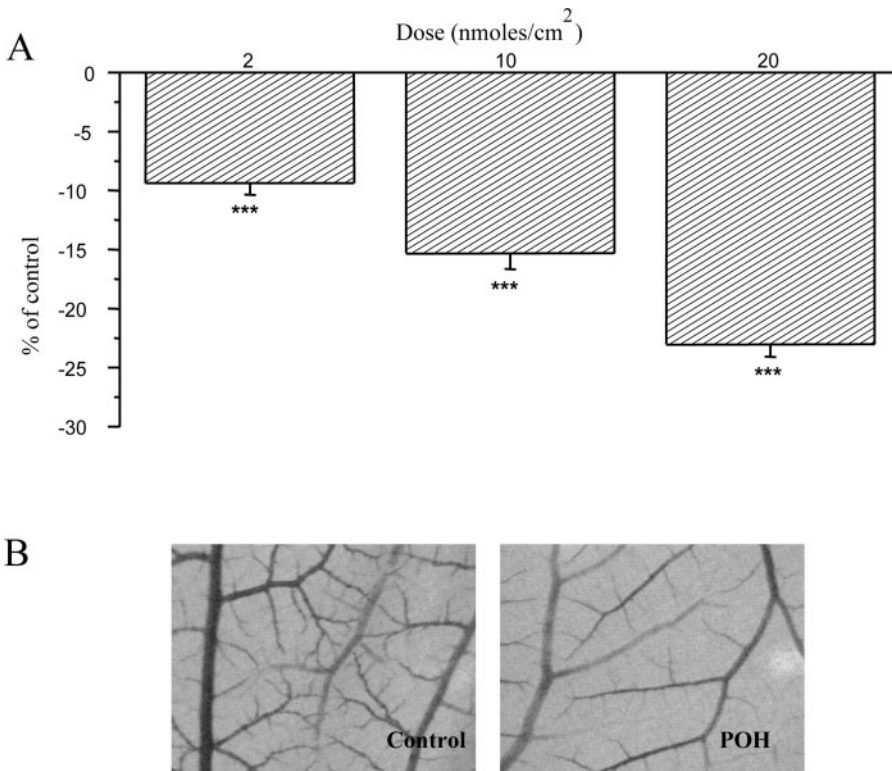
**ELISA for VEGF and Ang2 Detection.** Cells grown to confluence in 24-well plates were treated with POH (0.1, 0.2, 0.5, and 1 mM), POH ester (1 mM), or vehicle (0.1% ethanol) over various time periods (24–72 h). Conditioned media from these cultures were analyzed by a VEGF or Ang2 ELISA according to the manufacturer's specifications. In brief, 100  $\mu$ l of sample dilution in assay diluent (1% bovine serum albumin in PBS) were added to a 96-well microplate pre-coated with polyclonal antibody specific for human VEGF or human Ang2. Recombinant human VEGF or Ang2 was used for the standard curve. After incubation for 2 h at room temperature, the wells were washed, and polyclonal biotinylated anti-human VEGF or Ang2 antibodies were added. Incubation was continued as above, plates were washed, and streptavidin conjugated to horseradish peroxidase was added and further incubated for 20 min. Plates were washed again, developed using the substrate solution (equal volumes of  $\text{H}_2\text{O}_2$  and tetramethylbenzidine), and the reaction was stopped by adding 2 N  $\text{H}_2\text{SO}_4$ . The optical density was measured at 450 nm with correction wavelength set at 630 nm. The standard curve was generated using the GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA) with a four-parameter logistic curve fit.

**Data Analysis and Statistics.** Data are presented as means of the percentage of control  $\pm$  S.E.M. of the indicated number of observations. Statistical comparisons between groups were performed using the one-way ANOVA followed by a post hoc test (Neuman-Keuls, Dunnett's, or least significant difference), as appropriate. Differences among means were considered significant when  $p < 0.05$ .

## Results

**POH Suppresses Angiogenesis in the CAM.** To evaluate the effects of POH on neovascularization in vivo, we used the CAM model of angiogenesis. The results from the morphometric analysis (Fig. 1A) showed that POH decreased the number of CAM vessels in a dose-dependent manner up to  $23 \pm 1\%$  for the 20 nmol dose. For comparison, it is noted that dexamethasone, a steroid known to inhibit angiogenesis, inhibited vessel length in the same assay by 10.5 and 12% at 8 and 80 nmol, respectively. Figure 1B shows representative pictures of the CAM vascular network following vehicle or POH treatment. The decrease in the number of CAM vessels was not due to toxicity, as verified on CAM paraffin sections stained with eosin-hematoxylin (H. Loutrari and A. Papatropoulos, data not shown).

**POH Attenuates Capillary-Like Organization of Endothelial Cells in Vitro.** The effect of POH on the differentiation of endothelial cells into tube-like structures was studied in two different in vitro angiogenesis models, namely the collagen gel and the Matrigel assay. In the first model, three-



**Fig. 1.** Effect of POH on the CAM. POH or vehicle was applied onto 1 cm<sup>2</sup> of the CAM on day 9 and incubated for 48 h at 37°C. CAMs were fixed and excised from the eggs. A, total length of the vessel network was measured using image analysis software. Results are expressed as means ± S.E.M.; *n* ≥ 24; \*\*\*, *p* < 0.001 from the control. B, representative photographs showing the CAM vascular network following treatment with vehicle (control) or with 20 nmol POH.

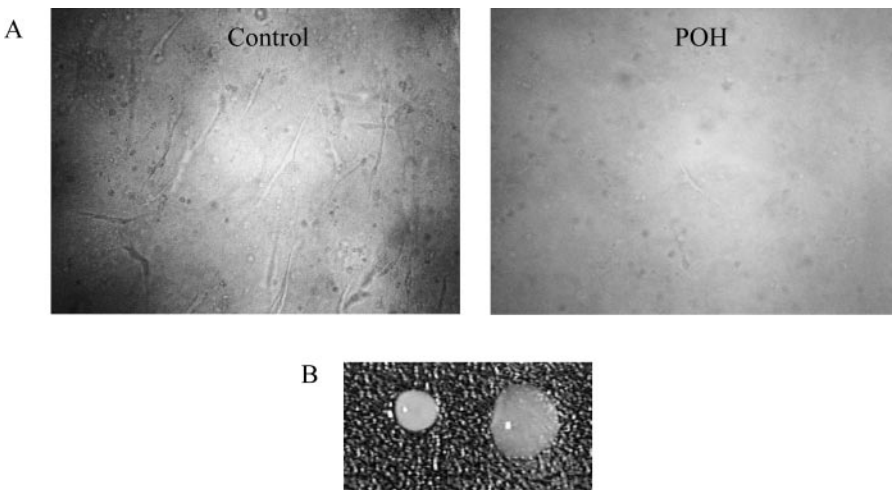
dimensional cultures of BLMVECs were preincubated with POH or vehicle, and vessel-like structure formation was stimulated by addition of PMA. As early as 24 h after PMA treatment, control cells formed networks that became more complex with prolonged incubation (Fig. 2A) and caused a macroscopically visible contraction of the collagen gel droplet (Fig. 2B, left). In contrast, pretreatment with POH induced a concentration-dependent inhibition of cell organization into networks, the cells remained interspersed throughout the gel for the entire incubation period (Fig. 2A), and collagen gel contraction was prevented (Fig. 2B, right).

Similar results were obtained with the Matrigel *in vitro* angiogenesis assay. Culturing untreated HUVECs on Matrigel triggered their morphological differentiation in structures imitating geometric tubule-like networks (Fig. 3B). In contrast, as revealed by microscopic observation (Fig. 3B) and morphometric analysis (Fig. 3A), treatment with POH

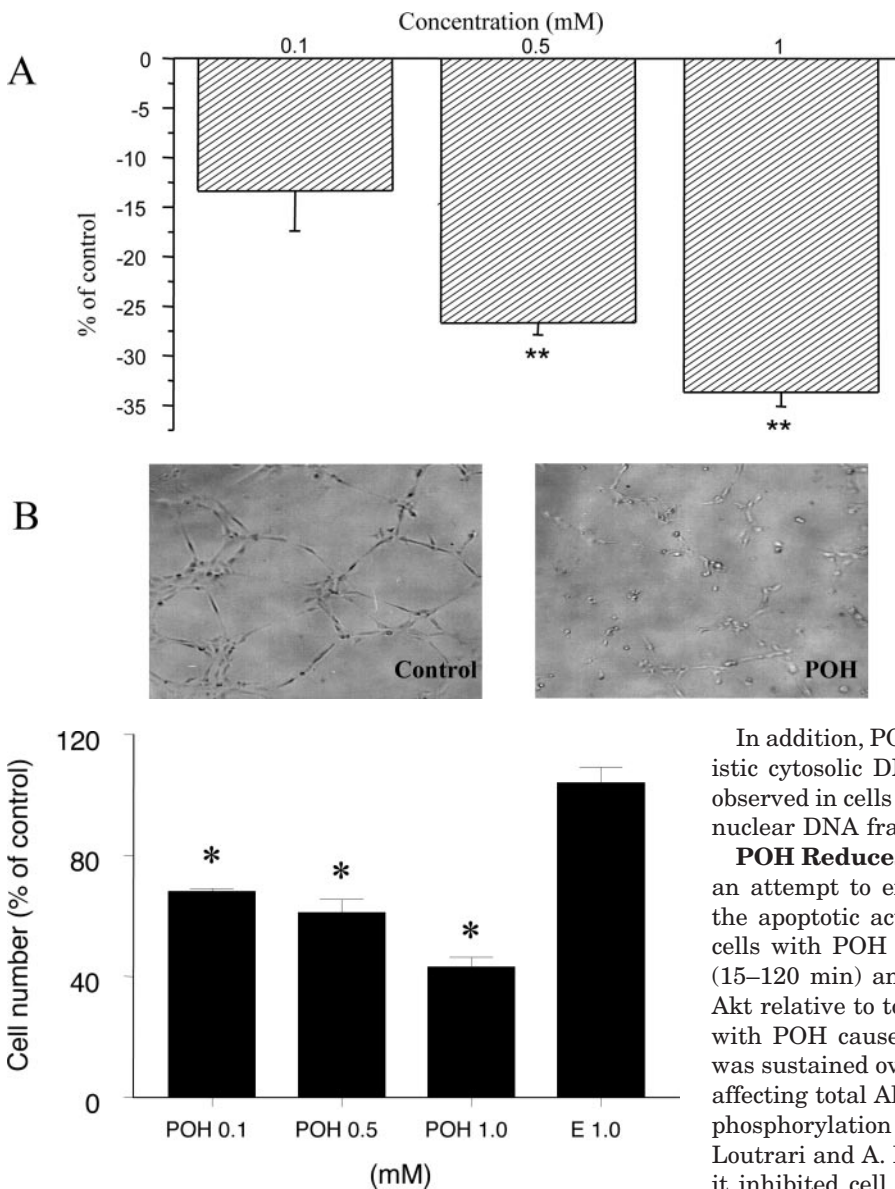
caused a concentration-dependent reduction of the total length of the above-mentioned structures.

**POH Reduces Endothelial Cell Number and Inhibits Cell Survival.** Signal transduction pathways promoting endothelial cell proliferation and survival are essential for the angiogenic process. We observed (Fig. 4) that treatment of exponentially growing endothelial cells with POH caused a concentration-dependent decrease in cell number in a cell proliferation assay. In contrast, addition of the decanoic acid ester of POH was without effect. Furthermore, in line with earlier studies on a variety of mammalian cancer cells, we found that POH induced a concentration-dependent reduction in cell number of K562 cells. Incubation with 0.1, 0.5, and 1.0 mM POH for 72 h reduced K562 cell number to 72.1 ± 5.0, 51.5 ± 4.1, and 32.4 ± 2.0% of control.

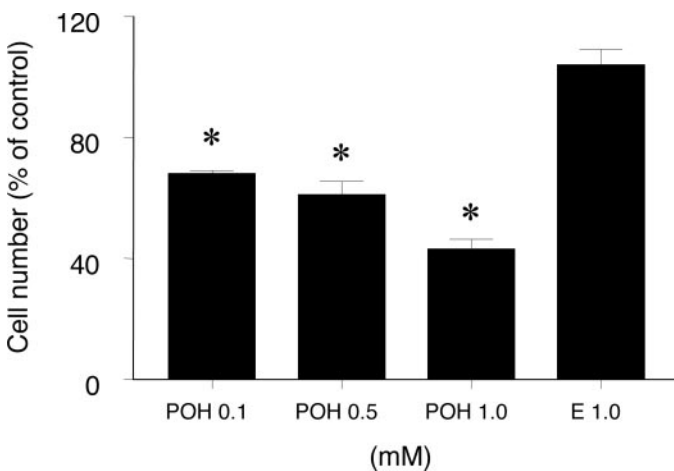
We also examined the potential effect of POH on apoptosis induction in BLMVECs by evaluating two different apoptosis



**Fig. 2.** POH inhibits differentiation of endothelial cells in the collagen gel assay. BLMVECs cultured in collagen gels (2 × 10<sup>6</sup> cells/ml) were treated with 0.5 mM POH or 0.1% ethanol (control). Two hours later, PMA (100 nM) was added, and cells were further maintained in culture for 5 days. A, phase-contrast photomicrographs of control or POH-treated cells. B, macroscopic photomicrograph of collagen gels from control (left) and POH-treated (right) cultures.



**Fig. 3.** POH inhibits differentiation of endothelial cells in the Matrigel assay. HUVECs ( $10^5$  cells/ml) were added onto Matrigel-coated wells in 96-well plates in the presence of POH or vehicle (control) and incubated for 6 h. A, length of the tube network was measured in the total well area. Results are expressed as means  $\pm$  S.E.M.;  $n = 9$ ; \*\*,  $p < 0.01$  from control. B, representative photomicrographs showing the formation of tube-like structures on Matrigel after control or POH (1 mM) treatment.



**Fig. 4.** Effect of POH on endothelial cell proliferation. BLMVECs were plated in a 96-well plate at 2000 cells/well. The next day, POH, POH ester (E), or vehicle were added, cultures were incubated for 48 h, and cell numbers were measured using the CyQUANT cell proliferation assay kit. Results are expressed as means  $\pm$  S.E.M.;  $n = 9$ ; \*,  $p < 0.05$  from control.

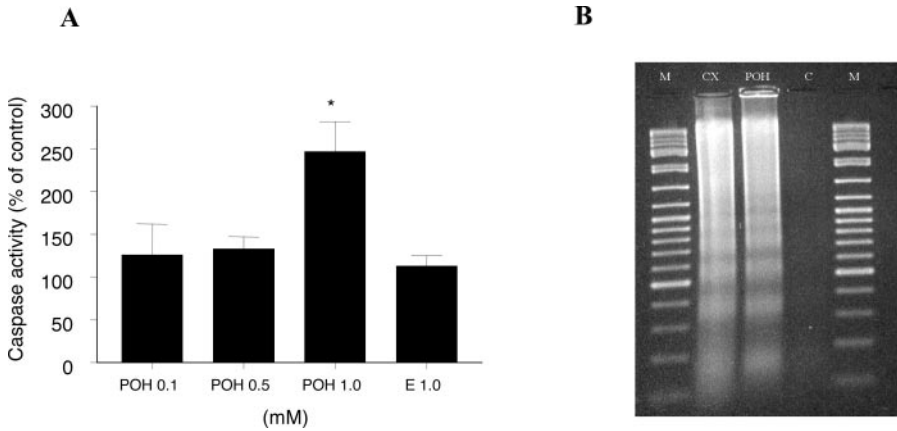
indicators: caspase-3 activation and DNA fragmentation. As shown in Fig. 5A, treatment of cells with 1 mM POH for 48 h caused a statistically significant increase in caspase-3 activity ( $246.5 \pm 34.7\%$  of control); when cells were treated with 5  $\mu$ M cycloheximide, a much greater increase in caspase activity was observed ( $1118.3 \pm 46.1\%$  of control). However, incubation of endothelial cells with lower POH concentrations or with the decanoic ester of POH had no effect. It has been previously reported that POH induces apoptosis in several cancer cell lines. In agreement with these observations, POH caused a concentration-dependent stimulation of caspase-3 activity in K562 cells: after incubation with 0.1, 0.2, and 0.5 mM POH for 72 h, caspase-3 activity was  $162.9 \pm 7.0$ ,  $336.5 \pm 30.0$ , and  $423.7 \pm 49.0\%$  of control, respectively, whereas cycloheximide (10  $\mu$ M) caused a  $264.8 \pm 6\%$  increase.

In addition, POH-treated BLMVECs exhibited a characteristic cytosolic DNA laddering pattern, very similar to that observed in cells incubated with cycloheximide, as a result of nuclear DNA fragmentation of apoptotic cells (Fig. 5B).

**POH Reduces Akt Activation in Endothelial Cells.** In an attempt to explore the signaling mechanism mediating the apoptotic activity of POH in BLMVEC, we treated the cells with POH (0.5–1.0 mM) for various incubation times (15–120 min) and determined the levels of phosphorylated Akt relative to total Akt. As illustrated in Fig. 6, incubation with POH caused a reduction of Akt phosphorylation that was sustained over the examined incubation periods without affecting total Akt levels. In contrast, POH did not alter Akt phosphorylation levels in K562 lymphoblastoma cells (H. Loutrari and A. Papapetropoulos, data not shown), although it inhibited cell proliferation and induced apoptosis in this cell line. Interestingly, we observed that POH inhibited ERK1/2 phosphorylation in K562 cells (pERK1/2/totalERK1/2 ratios were 0.46 and 0.2 for vehicle- and POH-treated cultures after 15 min of incubation).

**Differential Modulation of VEGF and Ang2 Expression by POH.** The effect of POH or POH ester on VEGF and Ang2 release from cancer and endothelial cells, respectively, was examined by ELISA. As illustrated in Fig. 7, treatment of K562 human lymphoblastoma cells with POH caused a concentration- and time-dependent decrease in the basal production of VEGF. A similar response to POH treatment, although to a lesser degree, was noted with the B16 mouse skin melanoma and the MDA-MB-231 human mammary gland cancer cell lines. Following incubation with 0.2, 0.5, and 1.0 mM POH for 24 h, VEGF release was  $95.4 \pm 0.4$ ,  $88.1 \pm 1.5$ , and  $65.9 \pm 1.3\%$  of control, respectively for B16 cells and  $89.6 \pm 2.7$ ,  $81.5 \pm 2.6$ , and  $69.3 \pm 3.1\%$  of control, respectively for MDA-MB-231 cells.

In contrast, POH treatment induced a concentration-dependent increase in Ang2 release from BLMVECs (Fig. 8). It should be noted that the decanoic ester of POH had only



**Fig. 5.** POH induces apoptosis in endothelial cells. A, BLMVECs grown to confluence were treated with POH, POH ester (E), or vehicle (control) for 48 h and then analyzed for caspase-3 activity. Results are expressed as means  $\pm$  S.E.M.;  $n = 9$ ; \*,  $p < 0.05$  from control. Caspase-3 activity in cycloheximide-treated cells was  $1118.3 \pm 46.1\%$  of control. B, confluent cultures of BLMVECs were incubated with  $5 \mu\text{M}$  cycloheximide (CX), 1 mM POH, or vehicle (C) for 48 h. Cells were then lysed, and DNA was extracted from cytosolic aliquots containing equal amounts of protein. DNA samples and markers (M) were electrophoresed through a 1% agarose gel and photographed under UV light. Control samples contain no cytosolic DNA since vehicle-treated cultures do not undergo programmed cell death. Similar results were obtained in two experiments.

minimal effects on the secretion of both angiogenic factors (Figs. 7 and 8).

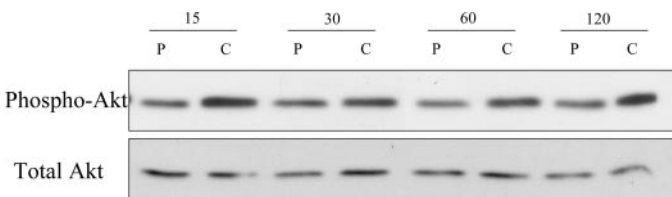
**Discussion**

The monoterpene POH has been previously shown to exert a significant antitumor activity in animal models, which has been attributed to the cytostatic and cytotoxic actions of POH on cancer cells (Gould, 1997; Belanger, 1998; Crowell, 1999). However, in vivo antitumor activity could also result from inhibition of neovascularization. To evaluate the ability of POH to interfere with the angiogenic process in vivo, we used a well-established model of neo-vessel formation and observed that POH dose-dependently reduced the number of vessels in the CAM. Since neovascularization is a multistep process that requires coordinated activation of many signaling pathways triggered by a number of angiogenic inducers (Yancopoulos et al., 2000; Jones et al., 2001), we speculated that POH might affect different stages of angiogenesis. To test this hypothesis, we designed a series of in vitro experiments evaluating the potentially pleiotropic activities of POH. Based on preliminary experiments and on information in the literature regarding the action of POH on tumor cell lines, 0.1 to 1 mM was used in all our assays (Belanger, 1998, and refs. therein).

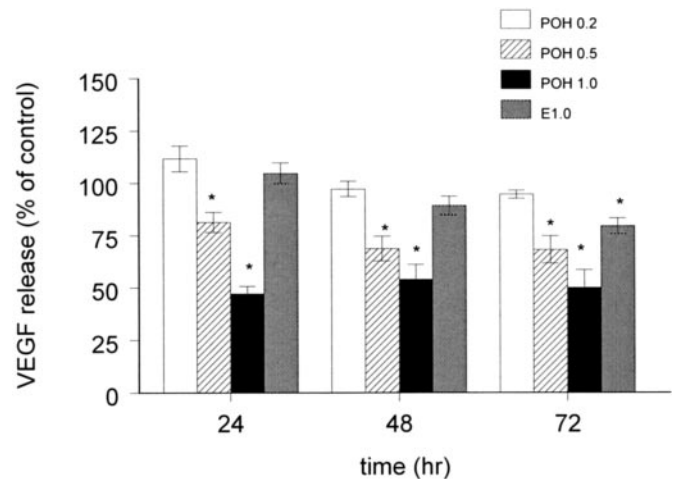
The effect of POH on the differentiation of endothelial cells was examined in two different in vitro angiogenesis models. It is well established that culture of endothelial cells in a three-dimensional scaffold of extracellular matrix protein accelerates their morphological differentiation into tube-like structures. This process involves dramatic changes in endothelial cell cytoskeletal dynamics, resulting in enhanced mo-

tility and alterations in cell shape (Connolly et al., 2002; Davis et al., 2002). We observed that POH inhibited in a concentration-dependent manner the organization of endothelial cells into structures that resemble capillaries in both collagen gel and Matrigel assays. Furthermore, POH prevented the contraction of collagen gels, suggesting that it might reduce endothelial cell actin stress fiber formation (Hoang et al., 2004). It has been widely demonstrated that Rho GTPases interact with the cytoskeleton and are, thus, major regulators of cytokinesis (Bishop and Hall, 2000). More recent evidence suggests that Rho GTPases participate in signaling pathways that control endothelial cell phenotype during neovascularization (Connolly et al., 2002; Cascone et al., 2003; Hoang et al., 2004). Thus, it is possible that POH attenuates the formation of tube-like networks by inhibiting the post-translational isoprenylation and association with the membrane and subsequent activation of Rho GTPases in endothelial cells through a mechanism that might be similar to the one observed in tumor cells (Hohl and Lewis, 1995; Stayrook et al., 1998; Holstein and Hohl, 2003).

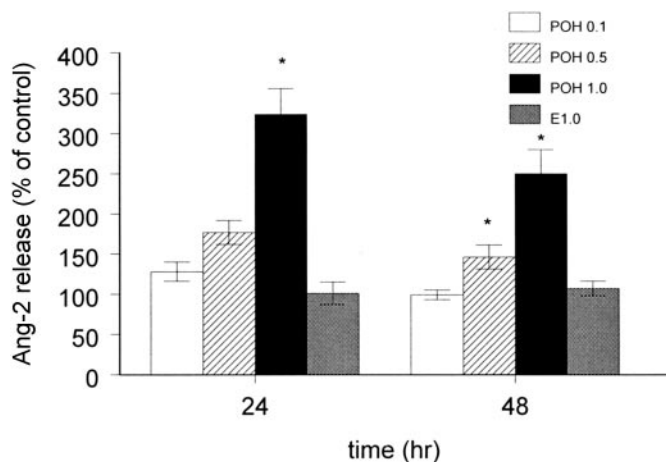
Endothelial cell proliferation and survival are important steps in the angiogenic process. Most pro-angiogenic growth factors stimulate endothelial cell proliferation (Yancopoulos et al., 2000; Jones et al., 2001; Ferrara et al., 2003), whereas



**Fig. 6.** POH inhibits Akt phosphorylation in endothelial cells. BLMVECs grown to confluence were incubated with 0.5 mM POH (P) or vehicle (C) for the indicated times (minutes) and then solubilized with lysis buffer. Equal amounts of protein lysates were run on a 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with phospho-specific anti-Akt or anti-total Akt antibodies. Immunoreactive bands were visualized with ECL. The pAkt/Akt ratios for POH-treated cells were 39, 85, 57, and 37% of vehicle-treated cells (control) after 15, 30, 60, and 120 min.



**Fig. 7.** POH reduces VEGF release from cancer cells. K562 cells ( $3 \times 10^6$  cells/ml) were treated with POH, POH ester (E), or vehicle (control). At the indicated times, culture supernatants were collected and measured by ELISA for the presence of VEGF. Results are expressed as means  $\pm$  S.E.M.;  $n = 9$ ; \*,  $p < 0.05$  from control. Concentrations of POH and E are in millimolar.



**Fig. 8.** POH stimulates Ang2 release from endothelial cells. BLMVECs grown to confluence were incubated with POH, POH ester (E), or vehicle (control). At the indicated times, culture supernatants were collected and measured by ELISA for the presence of Ang2. Results are expressed as means  $\pm$  S.E.M.;  $n = 9$ ; \*,  $p < 0.05$  from control. Concentrations of POH and E are in millimolar.

other important molecules involved in neovessel formation, like Ang1, do not directly stimulate endothelial cell growth but rather stabilize vascular networks by promoting endothelial cell survival (Papapetropoulos et al., 1999, 2000). POH has well-established cytostatic and apoptotic activities in a variety of mammalian cancer cells (Crowell, 1999; Burke et al., 2002; Clark et al., 2002). We also observed the induction of programmed cell death in the cancer cell lines used in our study. Interestingly, POH also reduced endothelial cell number and stimulated apoptosis of endothelial cells, as indicated by the increase in caspase-3 activity and DNA fragmentation. The reduction in EC number brought about by low POH concentrations might be due to inhibition of cell proliferation, whereas the lower cell number seen with increased POH concentrations is likely due to stimulation of apoptosis. To determine pathways potentially involved in POH's effect on apoptosis, we tested the ability of POH to affect activation of Akt, a key kinase in survival signaling pathways (Franke et al., 2003). We found that POH decreased the levels of phosphorylated (active) form of Akt, indicating that POH-induced suppression of endothelial cell survival could be mediated through inhibition of Akt signaling. An interesting observation was that although POH very efficiently attenuated cell proliferation and induced apoptosis in K562 cancer cells, it had no effect on Akt phosphorylation in these cells (H. Loutrari and A. Papapetropoulos, unpublished data). In line with the notion that POH blocks small GTPase signaling, POH-mediated apoptosis in K562 cells correlated with an inhibition of ERK1/2 phosphorylation, a downstream target of the Ras/Raf pathway. These results suggest that the pro-apoptotic action of POH may involve different molecular targets in endothelial and cancer cells.

We finally determined the effects of POH on angiogenic growth factor production. Due to their proven importance in neovascularization, we focused on two such factors, namely VEGF and Ang2. VEGF is mainly produced by cancer cells, in response to multiple stimuli (activation of oncogenic Ras proteins, hypoxia, and ultraviolet radiation) and activates its cognate receptors on endothelial cells leading to prolifera-

tion, migration, survival, and vascular permeability (Ferrara et al., 2003). Ang2, on the other hand, is primarily produced by endothelial cells and exerts its biological actions through the antagonism of Ang1-stimulated phosphorylation of Tie2 on vascular endothelium (Yancopoulos et al., 2000; Jones et al., 2001). In the absence of VEGF, Ang2 stimulates vessel regression, whereas in the presence of VEGF, neovascularization is favored (Holash et al., 1999; Yancopoulos et al., 2000; Vajkoczy et al., 2002). In our experiments, we observed that POH down-regulated the basal production of VEGF in three different cancer cell lines and up-regulated the release of Ang2 from endothelial cells, favoring the balance of the two growth factors toward a state that leads to vessel regression.

In most of our experiments, we examined the effect of POH ester with decanoic acid in parallel with POH. In a previous study using the flavonoid rutin, we noted that addition of a fatty acid chain on this compound improved its biological activity that was probably due to an increase of its lipophilicity (Kodelia et al., 1994). However, in the present work, we observed that esterification of POH on its unique  $-OH$  group with decanoic acid essentially abolished its biological activity suggesting that a free  $-OH$  group is indispensable for maximal POH biological activity. Further experiments using alternative POH esters with a smaller carbon chain length are currently in progress.

In conclusion, we have provided evidence that POH possesses antiangiogenic properties (inhibition of new vessel growth in the CAM assay, inhibition of endothelial cell proliferation and organization into tube-like structures, alteration in the production of angiogenic growth factors, and induction of apoptosis) that complement its toxic effects on tumor cells. Thus, the combined effects of POH on tumor and endothelial cells might explain its strong anticancer activity. The observed inhibition of VEGF release coupled to the increase in Ang2 production provides an additional mechanism through which POH treatment might cause tumor regression by destroying the vascular networks supporting the tumor mass.

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