Black Raspberry Extracts Inhibit Benzo(*a*)Pyrene Diol-Epoxide–Induced Activator Protein 1 Activation and VEGF Transcription by Targeting the Phosphotidylinositol 3-Kinase/Akt Pathway

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Abstract

Previous studies have shown that freeze-dried black raspberry extract fractions inhibit benzo(*a*)pyrene [B(*a*)P]–induced transformation of Syrian hamster embryo cells and benzo(*a*)pyrene diol-epoxide [B(*a*)PDE]–induced activator protein-1 (AP-1) activity in mouse epidermal Cl 41 cells. The phosphotidylinositol 3-kinase (PI-3K)/Akt pathway is critical for B(*a*)PDE-induced AP-1 activation in mouse epidermal Cl 41 cells. In the present study, we determined the potential involvement of PI-3K and its downstream kinases on the inhibition of AP-1 activation by black raspberry fractions, RO-FOO3, RO-FOO4, RO-ME, and RO-DM. In addition, we investigated the effects of these fractions on the expression of the AP-1 target genes, vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS). Pretreatment of Cl 41 cells with fractions RO-F003 and RO-ME reduced activation of AP-1 and the expression of VEGF, but not iNOS. In contrast, fractions RO-F004 and RO-DM had no effect on AP-1 activation or the expression of either VEGF or iNOS. Consistent with inhibition of AP-1 activation, the RO-ME fraction markedly inhibited activation of PI-3K, Akt, and p70 S6 kinase ($p70^{S6k}$). In addition, overexpression of the dominant negative PI-3K mutant Δp85 reduced the induction of VEGF by B(*a*)PDE. It is likely that the inhibitory effects of fractions RO-FOO3 and RO-ME on B(*a*)PDE-induced AP-1 activation and VEGF expression are mediated by inhibition of the PI-3K/Akt pathway. In view of the important roles of AP-1 and VEGF in tumor development, one mechanism for the chemopreventive activity of black raspberries may be inhibition of the PI-3K/Akt/AP-1/VEGF pathway. (Cancer Res 2006; 66(1): 581-7)

Introduction

Epidemiologic studies indicate that the majority of human cancer is caused by exposure to environmental chemical carcinogens (1). Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants found in cigarette smoke, charred foods, and the atmosphere from incomplete combustion of fossil fuels (2–4). Exposure to PAH-containing substances increases the risk of lung, skin, and, possibly, other cancers (3). The carcinogenicity of PAHs is associated with metabolic activation of reactive diol epoxide intermediates that bind covalently to critical targets in DNA (4, 5). Benzo(*a*)pyrene [B(*a*)P] is one of the most ubiquitous environmental PAHs and it induces tumors in multiple organ sites in animals (3). Benzo(*a*)pyrene-7,8-diol-9,10-epoxide [B(*a*)PDE], the ultimate metabolite of B(*a*)P, contributes to carcinogenesis through interaction with nucleophilic sites in cellular DNA, RNA, and protein (6). Although it is possible to minimize exposure to PAH carcinogens, it is unlikely that human exposure will ever be eliminated.

Population-based studies suggest that consuming relatively large amounts of vegetables and fruits can prevent the incidence of cancer in several organs, including the lungs, larynx, oral pharynx, gastrointestinal tract, and pancreas (7, 8). Among fruits, berries contain numerous known chemopreventive agents including vitamins A, C, E, and folic acid; calcium and selenium; polyphenols such as gallic acid, ellagic acid, ferulic acid, coumaric acid, quercitin, and multiple anthocyanins; and various phytosterols such as β-sitosterol (9). The anthocyanins are responsible for the red, violet, purple, and blue colors of fruit. The dietary consumption of anthocyanins in elderberries has been shown to improve the overall antioxidant defense status of human plasma (10). Our recent studies have shown chemopreventive effects of strawberries and black raspberries for the rodent esophagus and colon (9, 11–13). Both berry types exhibit high antioxidant activity and strawberry consumption has been shown to increase the antioxidant capacity of human plasma (14).

A previous investigation with our collaborators showed that a methanol fraction of black raspberries inhibited B(*a*)P-induced transformation of Syrian hamster embryo cells in a dose-dependent manner (15). In that study, we were in error by designating the methanol fraction as RU-ME rather than RO-ME. The correct genus and species for black raspberries is *Rubus occidentalis* (RO) rather than *Rubus urinus* (RU). We later reported that the RO-ME fraction inhibits the activation of activator protein 1 (AP-1) and nuclear factor κB (NF-κB) induced by B(*a*)PDE in mouse epidermal Cl 41 cells (16). The precise molecular mechanism(s) by which RO-ME inhibits AP-1 activation, however, remains unclear. Because our previous studies have shown that the phosphotidylinositol 3-kinase (PI-3K)/Akt pathway is required for B(*a*)PDE-induced AP-1 activation (17) and that the PI-3K/Akt pathway is a mediator for the induction of vascular endothelial growth factor (VEGF; ref. 18), in the present study, we investigate the effect of black raspberry extract fractions on VEGF and inducible nitric oxide synthase (iNOS) expression and the possible involvement of the PI-3K/Akt pathway in these effects.

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Materials and Methods

Cells and culture conditions. Mouse epidermal cell line, JB6 clone 41 (Cl 41), and three cell lines derived from Cl 41 cells were transfected stably with either an AP-1-luciferase reporter (P^+1-1), a VEGF-luciferase reporter (Cl 41 VEGF mass1), or a VEGF-luciferase reporter and dominant-negative PI-3K mutant (Δp85) (Cl 41 VEGF Δp85 mass1). All four of the cell lines (i.e., Cl 41, P^+1-1, Cl 41 VEGF mass1, and Cl 41 VEGF Δp85 mass1) were cultured in Eagle's MEM supplemented with 5% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 25 μg gentamicin/mL (16–18). Eagle's MEM was purchased from Calbiochem (San Diego, CA); L-glutamine, gentamicin, and FBS were from Life Technologies, Inc. (Rockville, MD). The cultures were dissociated with trypsin and transferred to new 75-cm^2 culture flasks (Fisher, Pittsburgh, PA) every 4 to 5 days.

Black raspberries and fractions. Ripe black raspberries (*R. occidentalis*) obtained from the Stokes Raspberry Farm in Wilmington, Ohio, were washed immediately after picking, frozen at −20°C, then freeze-dried as previously described (11). The black raspberry fractions used in this study were prepared as previously described (16). Briefly, ∼450 g of freeze-dried berries was extracted in 3 volumes of methanol overnight for 3 nights. The extract was filtered and then dried under vacuum at 60°C to produce fraction RO-F001. The residue from fraction RO-F001 (RO-F002) represented solid residue that was not additionally processed. A portion of RO-F001 was partitioned with water/dichloromethane (1:1). The aqueous layer was concentrated under vacuum and dried (RO-F003). The organic (dichloromethane) layer was vacuumed dried at 60°C, resulting in a water-insoluble fraction (RO-F004). A small amount of insoluble fraction, RO-F005, was obtained from the interface between the aqueous and organic layer. Additional RO-F001 was dissolved in methanol and allowed to evaporate. The resulting precipitate was chromatographed on a silica gel column and eluted by dichloromethane/methanol (1:1). The resulting nonpolar eluate (RO-DM) and polar fraction (RO-ME) were obtained. All of the fractions were stored at −20°C in the dark until use in the assays. For cell treatment, each fraction was dissolved in DMSO to give a final concentration of 50 mg/mL of fraction and frozen at −70°C for use in the assays.

Other reagents. The substrate for the luciferase assay was purchased from Promega (Madison, WI); B(*a*)P was purchased from Sigma (St. Louis, MO); and the B(*a*)PDE was kindly provided by Dr. Shantu Amin (Institute for Cancer Prevention, Valhalla, NY). Both B(*a*)P and B(*a*)PDE were dissolved in DMSO to achieve a stock concentration of 2 mmol/L. The phosphospecific antibodies against phosphorylated sites of Akt and p70 S6 kinase ($p70^{S6k}$) were from Cell Signaling Technology (Beverly, MA).

AP-1 activity assay. Confluent monolayers of P^+1-1 cells were trypsinized and 8×10^3 viable cells, suspended in 100 μL of MEM supplemented with 5% FBS, were added to each well of 96-well plates. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After the cell density reached 80% to 90%, the culture medium was replaced with an equal volume of MEM supplemented with 0.1% FBS and 2 mmol/L L-glutamine. Twelve hours later, the cells were treated with the different berry fractions dissolved in DMSO for 30 minutes at concentrations ranging from 1 to 100 μg/mL. Cells were then exposed to either B(*a*)P or B(*a*)PDE at a final concentration of 2 μmol/L for AP-1 induction. After 24 hours of incubation, the cells were extracted with lysis buffer (Promega) and the luciferase activity was determined by the luciferase assay using a luminometer (Wallac 1420 Victor 2 multilable counter system) after the addition of 50 μL of lysis buffer for 30 minutes at 4°C. The results are expressed as AP-1 activity relative to control medium containing DMSO (0.1% v/v).

VEGF induction. The same procedure as described above for measuring the effects of berry fractions on B(*a*)P- and B(*a*)PDE-induced AP-1 activity in P^+ 1-1 cells was used for determining the effects of the berry fractions on B(*a*)PDE- and B(*a*)P-induced VEGF activity in Cl 41 VEGF mass1 cells and Cl 41 VEGF Δp85 mass1 cells. The results were expressed as VEGF activity relative to control medium containing DMSO (0.1% v/v).

PI-3K assay. PI-3K activities were determined as previously described (17, 18). In brief, Cl 41 cells were cultured in 100-mm tissue culture dishes using 5% FBS MEM. After the cell density reached 70% to 80%, the medium was replaced with MEM supplemented with 0.1% FBS, 2 mmol/L L-glutamine, and 25 μg gentamicin/mL. After 45 hours, the cells were incubated with fresh serum-free MEM for 3 to 4 hours at 37°C. After preincubation with berry extracts for 30 minutes, B(*a*)PDE was added to the cultures for PI-3K induction at a final concentration of 2 μmol/L. Cells were washed once with ice-cold PBS and lysed in 400 μL of lysis buffer [20 mmol/L Tris (pH 8), 137 mmol/L NaCl, 1 mmol/L $MgCl_2$, 10% glycerol, 1% NP40, 1 nmol/L DTT, 0.4 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonylfluoride] per dish. The lysates were centrifuged and the supernatants were incubated at 4°C with 40 μL of agarose beads (previously conjugated overnight with the monoclonal antiphosphotyrosine antibody Py20). The beads were washed twice with each of the following buffers: (buffer A) PBS with 1% NP40, 1 mmol/L DTT; (buffer B) 0.1 mol/L Tris (pH 7.6), 0.5 mol/L LiCl, 1 mmol/L DTT; and (buffer C) 10 mmol/L Tris (pH 7.6), 0.1 mol/L NaCl, 1 mmol/L DTT. The beads were then incubated for 5 minutes on ice in 20 μL of buffer C. Phosphatidylinositol was sonicated in 50 mmol/L HEPES, 1 mmol/L EGTA, 1 mmol/L NaH_2PO_4, and then 20 μL of 0.5 mg/mL phosphatidylinositol were added into each reaction. After 5 minutes at room temperature, 10 μL of the reaction buffer (50 mmol/L $MgCl_2$, 100 mmol/L HEPES (pH 7.6), 250 mmol/L ATP containing 5 mCi of [γ-^{32}P]ATP) were added and the beads were incubated for an additional 15 minutes. Reactions were stopped by the addition of 15 μL of 4 N HCl and 130 μL of chloroform/methanol (1:1). After vortexing for 30 seconds, 30 μL of the phospholipid-containing chloroform phase were spotted onto TLC plates coated with silica gel H containing 1.3% potassium oxalate and 2 mmol/L EDTA in H_2O/methanol (3:2). Plates were heated at 110°C for a minimum of 3 hours before use, and then were placed in tanks containing chloroform/methanol/NH_4OH/H_2O (600:470:20:113) for 40 to 50 minutes until the solvent reached the top of the plates. Plates were dried at room temperature and autoradiographed (17, 18).

Phosphorylation assay for Akt and $p70^{S6k}$. Cl 41 cells (3×10^5) were cultured in each well of six-well plates to 70% to 80% confluence with 5% FBS MEM. The medium was replaced with MEM supplemented with 0.1% FBS, 2 mmol/L L-glutamine, and 25 μg gentamicin/mL, and the cells were cultured for an additional 45 hours. Cells were then incubated in serum-free MEM for 3 to 4 hours at 37°C and were treated with berry extracts for 30 minutes and exposed to B(*a*)PDE for different time periods. Cells were washed once with ice-cold PBS and extracted with an SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with specific antibodies to rabbit phosphospecific Akt (Thr^{308}), phosphospecific Akt (Ser^{473}), nonphosphorylated Akt, phosphospecific $p70^{S6k}$ (Thr^{389}), phosphospecific $p70^{S6k}$ (Ser^{421}/Ser^{424}), and nonphosphorylated $p70^{S6k}$. The Akt and $p70^{S6k}$ protein bands specifically bound to primary antibodies were detected using an antirabbit immunoglobulin G (IgG)-AP–linked antibody and enhanced chemifluorescence Western blotting system (19, 20).

Cell proliferation assay. Cell proliferation was determined by Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega). Briefly, Cl 41 cells (1×10^3) were cultured in 96-well plates to 70% to 80% confluence with 5% FBS MEM and then exposed to different doses of B(*a*)PDE in 0.1% FBS MEM. Twelve hours later, the medium was replaced with 20 μL of PBS. After equilibration for 30 minutes at room temperature, 20 μL of substrate were added into each well of the plates and the incubation was then prolonged for another 10 minutes at room temperature. The luminescence signal was measured with a luminometer (Wallac 1420 Victor 2 multilable counter system). The results were expressed as relative proliferation index, which is normalized to the control cells cultured with 0.1% DMEM containing 0.1% DMSO.

Statistical analysis. The Student's *t* test was used to determine the significance of differences of AP-1, iNOS, and VEGF induction in cells treated with berry fraction plus B(*a*)PDE, B(*a*)PDE alone, berry fraction plus B(*a*)P, B(*a*)P alone, or DMSO alone. The results are expressed as mean ± SE. The differences of AP-1, iNOS, and VEGF induction were considered significant at $P < 0.05$.

Results

Berry fractions inhibit B(*a*)PDE-mediated induction of VEGF, but not iNOS, in Cl 41 cells. There is growing evidence showing that VEGF and iNOS are involved in cancer development

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(21, 22). To assess whether the anticarcinogenic effects of berry extracts might be due, in part, to modulation of the expression of iNOS and VEGF, we first determined whether treatment of Cl 41 cells with B(*a*)PDE or B(*a*)P leads to the induction of VEGF and iNOS. As shown in Fig. 1*A* and *B*, treatment of Cl 41 cells with B(*a*)PDE resulted in a marked induction of VEGF expression, but only a marginal induction of iNOS, whereas B(*a*)P exposure led to iNOS induction, but not of VEGF. The induction of VEGF by B(*a*)PDE was confirmed by dose-response studies. The B(*a*)PDE dose for maximal VEGF induction was within the range of 2.25 to 2.75 μmol/L (Fig. 1*C*) whereas B(*a*)P did not induce VEGF at any of the doses tested (Fig. 1*C*). In contrast, B(*a*)P significantly induced iNOS expression between the concentrations of 3 and 5 μmol/L (Fig. 1*D*) whereas B(*a*)PDE did not show any significant effect on iNOS induction at all tested doses (0.5-4 μmol/L). These results suggest that although B(*a*)P may be metabolized into B(*a*)PDE *in vivo*, these two compounds seem to elicit different cellular responses *in vitro*. To address whether the B(*a*)PDE-induced VEGF induction is caused by either a nonspecific proliferative response or a toxic effect of B(*a*)PDE, we analyzed the effects of various doses of B(*a*)PDE on the proliferation of Cl 41 cells. The results showed that treatment of cells with lower concentrations of B(*a*)PDE (<1.5 μmol/L) resulted in a slight increase in cell proliferation (Fig. 1*E*) but had no effect on VEGF induction. However, treatment of cells with B(*a*)PDE at a dose of 2.5 μmol/L, which showed maximal inductive effect on VEGF expression, did not significantly affect cell proliferation (Fig. 1*E*), suggesting that VEGF induction by B(*a*)PDE is not due to either the induction of cell proliferation or the toxic effects of B(*a*)PDE.

To directly assess the effects of berry fractions on B(*a*)PDE-mediated VEGF induction and B(*a*)P-mediated iNOS induction, Cl 41 VEGF mass1 or Cl 41 iNOS mass1 cells were pretreated with berry fractions for 30 minutes and then exposed to B(*a*)PDE or B(*a*)P, respectively. The results showed that preincubation of Cl 41 cells with either RO-F003 or RO-ME led to a marked inhibition of B(*a*)PDE-mediated VEGF induction whereas fractions RO-F004 and RO-DM had no effect on VEGF induction (Fig. 2*A*). The RO-ME fraction was the most potent inhibitor of B(*a*)PDE-mediated VEGF induction among the fractions tested (Fig. 2*A*) and its inhibitory effect was dose dependent (Fig. 2*B*). In contrast, B(*a*)P-mediated induction of iNOS in Cl 41 cells was not inhibited by any of the berry fractions (data not shown). These results indicate that black raspberry extracts, especially the RO-ME extract, are capable of specifically inhibiting the induction of VEGF.

The PI-3K/AP-1 pathway is involved in inhibition of B(*a*)PDE-induced VEGF induction by RO-ME in C1 41 cells. Previous studies have shown that AP-1 is a major transcription factor responsible for VEGF induction in several cell culture systems (23). We reported that B(*a*)PDE induces AP-1 transactivation in Cl 41 cells and that this induction is inhibited by berry fraction RO-ME (16). It is interesting to test whether RO-ME inhibits the B(*a*)PDE-mediated VEGF induction via a mechanism that inhibits the B(*a*)PDE-induced AP-1 transactivation. Therefore, we first examined the effect of this extract on AP-1 activation in Cl 41 cells stably transfected with the AP-1 luciferase reporter (P^+1-1). Treatment of P^+1-1 cells with B(*a*)PDE markedly stimulated AP-1 activation whereas B(*a*)P exerted a lower effect (Fig. 3*A*), and the

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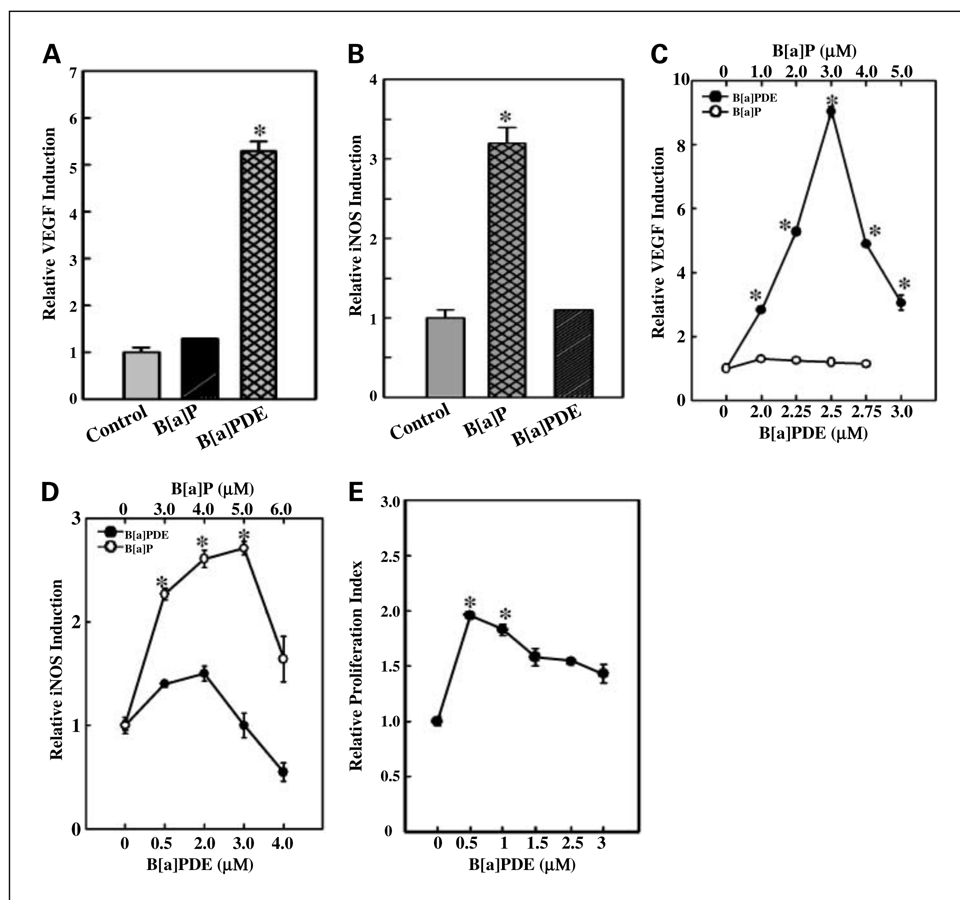

Figure 1. B(*a*)PDE- and B(*a*)P-induced VEGF and iNOS expression in mouse Cl 41 cells, respectively. Cl 41 VEGF mass1 cells (*A* and *C*) or Cl 41 iNOS mass1 cells (*B* and *D*; 8×10^3) were seeded into each well of 96-well plates and cultured at 37°C in MEM supplemented with 5% FBS. After reaching a density of 80% to 90% in the plates, both cell types were exposed to either B(*a*)P or B(*a*)PDE and cultured in MEM with 0.1% FBS for 18 hours. The cells were extracted with lysis buffer and luciferase activity for either VEGF or iNOS was measured as described in Materials and Methods. The results are expressed as VEGF or iNOS induction in carcinogen-treated cells relative to cells grown in control medium containing 0.1% DMSO. *Columns,* mean from triplicate assays; *bars,* SD. *E,* Cl 41 VEGF mass1 cells (1×10^3) were seeded into 96-well plates and cultured to 70% to 80% confluence with 5% FBS MEM and then exposed to different doses of B(*a*)PDE in 0.1% FBS MEM. Twelve hours later, cells were harvested and the luminescence signal was measured as described in Materials and Methods. Results are expressed as relative proliferation index, which is normalized to the control cells cultured with 0.1% DMEM containing 0.1% DMSO. *, $P < 0.05$, significant increase from medium control.

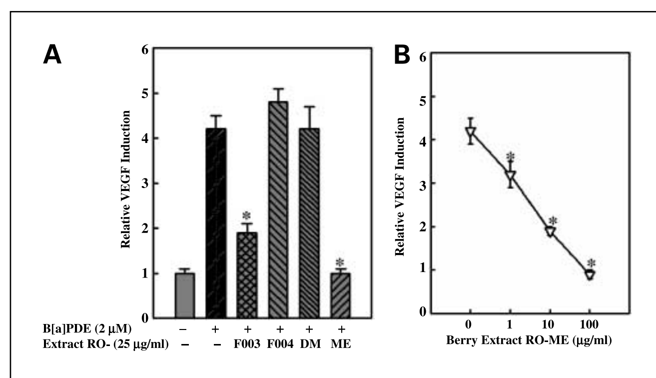

Figure 2. Effects of black raspberry extract fractions on VEGF and iNOS induction by B(a)PDE and B(a)P, respectively. Cl 41 VEGF mass1 cells (8×10^3) were seeded into each well of 96-well plates and cultured at 37°C in MEM supplemented with 5% FBS. After reaching a density of 80% to 90% in the plates, both cell types were pretreated with black raspberry extract fractions for 30 minutes and then exposed to B(a)PDE for VEGF induction. After 24 hours of incubation, the cells were extracted with lysis buffer and luciferase activity for VEGF was measured as described in Materials and Methods. The results are expressed as VEGF induction in carcinogen-treated cells relative to cells grown in control medium. *Columns,* mean from triplicate assays; *bars,* SD. *, $P < 0.05$, significant decrease in VEGF induction in cells treated with B(a)PDE plus berry extracts versus those treated with B(a)PDE only.

activation of AP-1 by B(a)PDE was inhibited by pretreatment of the cells with the RO-ME extract (Fig. 3*B*). These results are consistent with VEGF induction by B(a)PDE and VEGF inhibition by RO-ME (Figs. 1 and 2). These data, together with the previous finding that AP-1 is a major transcription factor for VEGF induction, suggest that inhibition of AP-1 by RO-ME may be a mechanism involved in its inhibition of VEGF induction by B(a)PDE. Because our previous studies had shown that PI-3K is required for AP-1 activation in Cl 41 cells following B(a)PDE treatment (17), it was of interest to determine the role of PI-3K in B(a)PDE-stimulated VEGF induction in Cl 41 cells. We examined the effect of B(a)PDE treatment of Cl 41 VEGF Δp85 mass1 cells, which are cotransfected with a VEGF-luciferase reporter and the Δp85, a dominant-negative PI-3K p85 mutant (18). We showed that overexpression of Δp85 completely blocked B(a)PDE-mediated VEGF induction (Fig. 4*A*). These observations were further confirmed by time-course and dose-response studies (Fig. 4*B* and *C*) showing that activation of the PI-3K/AP-1 pathway is essential for VEGF induction by B(a)PDE.

Effect of berry extracts on B(a)PDE-induced PI-3K activation in Cl 41 cells. PI-3K is a dimeric enzyme composed of a catalytic subunit (p110) and a regulatory subunit (p85) and phosphorylates the lipid phosphatidylinositol to generate phosphatidylinositol trisphosphate. The results above showed that the PI-3K/AP-1 pathway plays a role in B(a)PDE-stimulated VEGF induction in Cl 41 cells. In addition, the RO-ME extract inhibits B(a)PDE-mediated VEGF induction in these cells. This led us to investigate whether the inhibition of VEGF expression by RO-ME is mediated by inhibition of PI-3K activation. We examined the effect of RO-ME on PI-3K activation induced by B(a)PDE by determination of its effect on phosphatidylinositol trisphosphate generation. As shown in Fig. 5*A*, treatment of Cl 41 cells with B(a)PDE led to a marked activation of PI-3K, which was significantly inhibited by pretreatment with RO-ME. These data indicate that berry components are capable of inhibiting B(a)PDE-induced PI-3K activity, which could be the mechanism through which they inhibit B(a)PDE-mediated AP-1 and VEGF expression.

Effect of berry extracts on B(a)PDE-induced Akt and $p70^{S6k}$ activation in mouse Cl 41 cells. Because the above results showed that the RO-ME extract could block B(a)PDE-induced PI-3K activation in Cl 41 cells and our previous studies indicated that Akt and $p70^{S6k}$ are two separate PI-3K downstream kinases (17, 18, 20), it was important to determine whether berry extracts exhibit an effect on the activation of Akt and $p70^{S6k}$. Preincubation of Cl 41 cells with RO-ME extracts led to dramatic decreases in B(a)PDE-induced phosphorylation of Akt at both Thr^{308} and Ser^{473} and of $p70^{S6k}$ at both Thr^{389} and Thr^{421}/Ser^{424} (Fig. 5*B*). The inhibitory effects of the RO-ME fraction on phosphorylation of both Akt and $p70^{S6k}$ were observed at a concentration as low as 3 μg/mL (Fig. 5*B*). These data indicate that berry extract RO-ME can block Akt and $p70^{S6k}$ activation induced by B(a)PDE. In view of our previous observations that the activation of AP-1 by B(a)PDE is totally dependent on PI-3K in the same cell line, it is reasonable to speculate that the inhibition of B(a)PDE-mediated VEGF induction by the RO-ME extract is mediated by the PI-3K/Akt/AP-1 pathway.

Discussion

Numerous studies indicate that the dietary consumption of fruits and vegetables is associated with a reduced risk of cancer in humans (7, 8). Black raspberries have been shown to be one of the fruits that contain many known chemopreventive agents. Our previous studies have shown the protective effects of dietary freeze-dried black raspberries on the occurrence of chemically induced tumors in the esophagus and colon of rodents (9, 13). The protective effect of the berries seems to be due to localized absorption of berry compounds by the epithelial lining cells of the aerodigestive tract. Primary mechanistic studies indicate that the berries inhibit the initiation stage of tumor development in animals by reduction of DNA adduct formation (13). Berries also inhibit the promotion/progression stages of tumor development, in part, by reducing the growth rate of premalignant cells and by

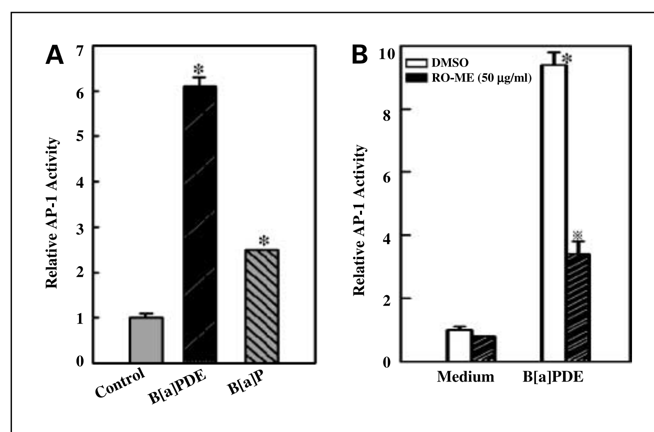

Figure 3. Inhibition of AP-1 transactivation by black raspberry extract fraction RO-ME. P+1-1 cells (8×10^3) were seeded into each well of 96-well plates and cultured in 5% FBS MEM at 37°C. After the cell density reached 80% to 90%, the cells were exposed to either B(a)PDE (2 μmol/L) or B(a)P (2 μmol/L) for AP-1 induction (*A*) or were pretreated with RO-ME (50 μg/mL) for 30 minutes and then exposed to B(a)PDE (2 μmol/L) for AP-1 induction (*B*). After 24 hours of incubation, cells were extracted with lysis buffer and luciferase activity measured. Results presented as AP-1 induction in either carcinogen- or RO-ME-carcinogen–treated cells relative to control medium. *Columns,* mean of triplicate assays; *bars,* SD. *, $P < 0.05$, significant increase from medium control; ⋇, $P < 0.05$, significant decrease from B(a)PDE.

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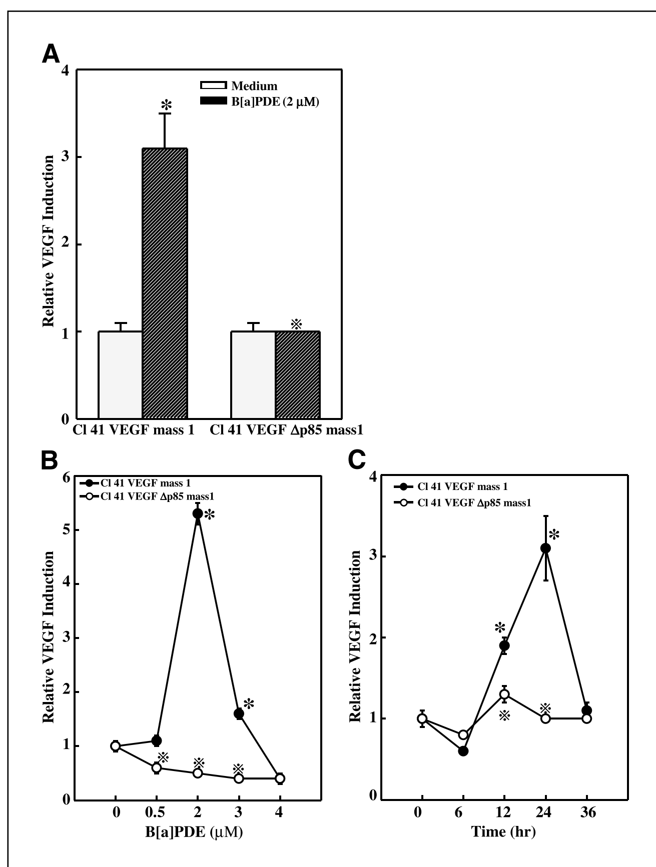

Figure 4. PI-3K activation is involved in B(*a*)PDE-induced VEGF expression. Cl 41 VEGF mass1 cells and Cl 41 VEGF Δp85 mass1 cells (8 × 10^3) were seeded into each well of 96-well plates and cultured as described above. After the cell density reached 80% to 90%, the cells were exposed to 2 μmol/L B(*a*)PDE for 24 hours (*A*), to various concentrations of B(*a*)PDE (*B*), or to 2 μmol/L B(*a*)PDE for different time periods (*C*). Results expressed as VEGF induction relative to control medium. *Columns,* mean from triplicate assays; *bars,* SD. *, $P < 0.05$, significant increase from medium control; ⋇, $P < 0.05$, significant decrease from that of Cl 41 VEGF mass1 cells.

stimulating apoptosis (13). However, the molecular events that are involved in berry inhibition of tumor development are far less clarified.

In previous studies, we found that a methanol-soluble (RO-ME) fraction of black raspberries was most effective in inhibiting B(*a*)P-induced transformation of Syrian hamster embryo cells (15) and B(*a*)PDE-induced transactivation of AP-1 and NF-κB in Cl 41 cells (16). The water-soluble (RO-F003) fraction was the second most active one, with the remaining fractions exhibiting lesser or no activity. Data from the present study are in agreement with these reports in that the RO-ME fraction was found to be the most effective in inhibiting both B(*a*)PDE-mediated AP-1 activation and VEGF induction in Cl 41 cells, with the water-soluble fraction showing lesser activity. Consistent with its inhibition of VEGF induction, the RO-ME fraction produced a dramatic inhibition of the activation of PI-3K, Akt, and $p70^{S6k}$ in Cl 41 cells. Because overexpression of the dominant negative PI-3K mutant Δp85 blocked AP-1 activation and VEGF induction (ref. 17; Fig. 4*A*), the inhibitory effects of the RO-ME extract on B(*a*)PDE-induced AP-1 activation and VEGF expression in Cl 41 cells seem to be mediated by inhibition of the PI-3K/Akt pathway. Interestingly, the RO-ME fraction did not block B(*a*)P-induced iNOS expression in Cl 41 cells, indicating that the regulatory effects of the fraction on VEGF expression are specific. This is in contrast to recent *in vivo* data (G. Stoner) which indicate that the dietary administration of whole freeze-dried black raspberries to rats resulted in reduced levels of iNOS expression in carcinogen-exposed esophagus.

As an important mediator of angiogenesis, VEGF can elicit a pronounced angiogenic response (24–26). In the two-stage chemical carcinogenesis mouse skin model induced by 7,12-dimethylbenz(*a,h*)anthracene and 12-*O*-tetradecanoylphorbol-13-acetate, mutation in codon 61 of the Ha-*ras* gene from A to T is believed to be the critical event to promote benign papilloma development (27). Further studies indicated that mutated Ha-*ras* was able to increase the expression of VEGF and the propensity for tumor development in a transgenic mice model (28). Therefore, it is believed that expression of VEGF might be important for tumor progression.

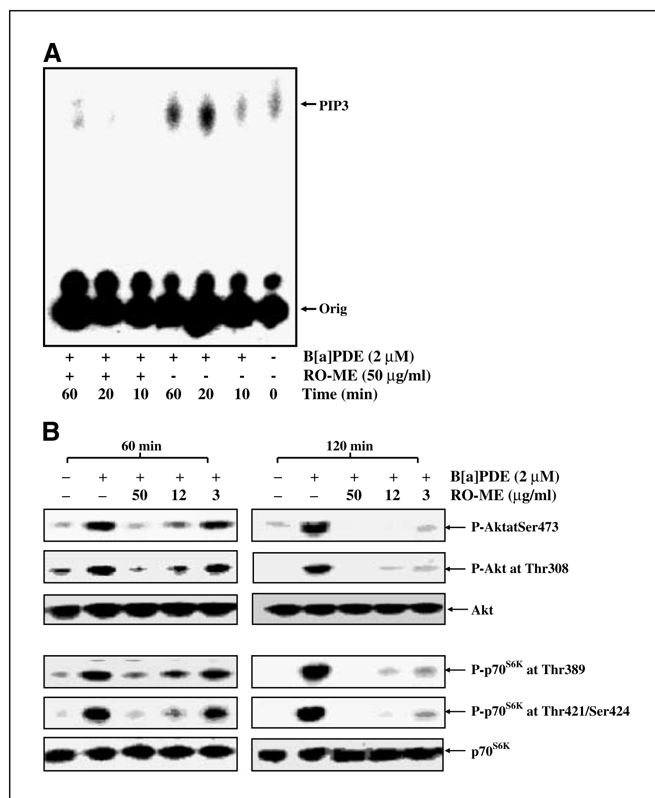

Figure 5. Inhibition of B(*a*)PDE-induced activation of PI-3K (*A*) and Akt and $p70^{S6k}$ (*B*) by black raspberry extract fraction RO-ME. *A,* 3 × 10^5 Cl 41 VEGF mass1 cells were seeded into 100-mm dishes and cultured in MEM supplemented with 5% FBS. After the cells reached a density of 70% to 80% in the dishes, the medium was replaced with MEM containing 0.01% FBS. Forty-five hours later, the cells were incubated in fresh serum-free MEM for 3 to 4 hours. The cells were then pretreated with RO-ME (50 μg/mL) for 30 minutes and exposed to B(*a*)PDE (2 μmol/L) for various time periods for the induction of PI-3K. Cells were washed with ice-cold PBS and lysed in 400 μL of lysis buffer. PI-3K activity was measured as described in Materials and Methods. *B,* 8 × 10^4 Cl 41 VEGF mass1 cells were seeded into each well of six-well plates and cultured in MEM containing 5% FBS. After the cells reached a density of 70% to 80% in the plates, the medium was replaced with 0.1% FBS MEM. Forty-five hours later, the cells were incubated in serum-free MEM for 3 to 4 hours, pretreated with various concentrations of RO-ME for 30 minutes, and then exposed to B(*a*)PDE (2 μmol/L) for either 60 or 120 minutes. Cells were washed once with ice-cold PBS and extracted with SDS sample buffer. Cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with either phosphospecific antibodies or nonphosphorylated antibodies against Akt and $p70^{S6k}$. The protein band specifically bound with the primary antibodies was detected by using an antirabbit IgG-AP–linked antibody and enhanced chemifluorescence Western blotting system.

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In another study, persistent stimulation of human keratinocytes with B(*a*)P for 6 months led to increased secretion of VEGF, and the ability of the cells to grow in soft agar and form tumor mass in nude mice following s.c. injection was also enhanced (29). Thus, it is evident that VEGF also plays a critical role in tumor promotion. Our data indicate that RO-ME fraction of black raspberry strongly inhibits B(*a*)PDE-induced VEGF expression, suggesting that regulation of VEGF expression may be a major mechanism contributing to the anticancer activity of black raspberries.

The precise mechanism(s) underlying VEGF regulation by different stimuli is not fully known. p42/p44 extracellular signal–regulated kinases (ERK) have been proved to be involved in VEGF expression in CCL39 fibroblasts (30). In Ras-transformed fibroblasts, VEGF expression was also inhibited when ERK activity was blocked by treatment with PD98059 whereas the PI-3K inhibitor LY294002 had no effect (31). In Ras-transformed epithelial cells, however, the regulation of VEGF mRNA and protein expression was strongly suppressed by the PI-3K inhibitor (31), suggesting that PI-3K may play a role on VEGF regulation in epithelial cell system. By characterizing the responsive region on VEGF promoter, Milanini et al. (30) found that the stress-activated protein kinases c-jun NH_2-terminal kinase and p38 kinase have no obvious effect on the transcriptional activity of VEGF, either in normoxic or hypoxic conditions. However, PI-3K and its downstream target Akt can exert a regulatory activity (32). Overexpression of the v-P3k protein or cellular PI-3K equipped with a myristylation signal, Myr-P3k, can induce angiogenesis in the chorioallantoic membrane of the chicken embryo (33). Overexpression of the myristylated form of Akt, Myr-Akt, also exerts the same effect. The levels of mRNA for VEGF are elevated in cells expressing activated PI-3K or Myr-Akt. In contrast, VEGF mRNA levels are decreased in cells treated with the PI-3K inhibitor LY294002 and restored by overexpression of v-P3k or Myr-Akt (33). These results indicate that the signaling pathway involved in regulation of VEGF expression is executed in a manner dependent on cell type. To further elucidate the anticancer mechanisms of black raspberries, it is very important to investigate the potential pathway involved in the inhibitory effects of the methanol fraction RO-ME on B(*a*)PDE-induced VEGF expression. In this study, we found that overexpression of Δp85, a dominant-negative mutant of PI-3K, impaired VEGF induction by B(*a*)PDE exposure at all time points and doses tested, showing that B(*a*)PDE-induced VEGF expression is PI-3K dependent. In addition, pretreatment of the same Cl 41 cells with RO-ME resulted in a marked inhibition of both B(*a*)PDE-induced VEGF expression and PI-3K activity, indicating that inhibition of VEGF expression by RO-ME may be through a PI-3K-dependent pathway. In contrast, B(*a*)P-induced iNOS expression was not affected by any of the black raspberry fractions, suggesting that VEGF is the specific mediator of B(*a*)PDE activity. Moreover, we also found that preincubation of cells with RO-ME led to a dramatic decrease in B(*a*)PDE-induced phosphorylation of Akt and $p70^{S6k}$. Because our previous studies showed that B(*a*)PDE induces AP-1 transactivation through a PI-3K/Akt-dependent pathway at a similar dose range (17), and in view of the inhibitory effects of black raspberry fraction on AP-1 transactivation in Cl 41 cells (16), it is reasonable to speculate that the effect of RO-ME on B(*a*)PDE-induced VEGF expression is mediated via inhibition of the PI-3K/Akt/AP-1 pathway.

B(*a*)PDE is the ultimate metabolic of B(*a*)P *in vivo* (2–4, 6). However, an unexpected finding of this study is that B(*a*)PDE exposure is able to induce VEGF expression whereas B(*a*)P treatment leads to iNOS increase, suggesting that B(*a*)P and B(*a*)PDE elicit different cellular responses in Cl 41 cells. One possible explanation may be due to the more dynamic chemical activity of B(*a*)P. In addition to causing cellular toxic effects via conversion into B(*a*)PDE, B(*a*)P may also elicit other effects such as oxidative stress through the induction of cytochrome *P*450 and generation of reactive oxygen species during its *in vivo* metabolism (4, 6). This anticipation is supported by the previous finding that oxidative stress can induce iNOS expression (34). No significant inductive effect of B(*a*)P on VEGF expression seems to be due to relative weak cytochrome *P*450 activities in the cells, which may result in a limited amount of B(*a*)PDE generation and subsequently lead to no effect on VEGF induction. This explanation is also supported by our data that B(*a*)PDE at <2 μmol/L concentration has a very weak VEGF induction.

In summary, the present study shows that RO-ME is the major fraction responsible for inhibition of both AP-1 activation and VEGF induction by B(*a*)PDE. The effect of RO-ME on B(*a*)PDE-induced VEGF expression seems to be mediated by inhibition of the PI-3K/Akt/AP-1 pathway. Although we do not know what specific compounds in the RO-ME fraction might be responsible for its inhibitory effect, the fraction is abundant in the anthocyanins which exhibit a marked ability to down-regulate B(*a*)PDE-induced NF-κB activity in Cl 41 cells (16). Taken together with the previous finding that RO-ME has a potent activity on inhibition of cell transformation (15), it is suggested that inhibition of the PI-3K/Akt/AP-1 signal transduction pathway leading to inhibition of VEGF expression may be one of the mechanisms involved in the anticarcinogenic effects of black raspberries.

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