NUTRITION AND CANCER, *54*(1), 69–78

Molecular Mechanisms Involved in Chemoprevention of Black Raspberry Extracts: From Transcription Factors to Their Target Genes

Haitian Lu, Jingxia Li, Dongyun Zhang, Gary D. Stoner, and Chuanshu Huang

Abstract: *Berries have attracted attention for their chemopreventive activities in last a few years. Dietary freeze-dried blackberries have been shown to reduce esophagus and colon cancer development induced by chemical carcinogen in rodents. To elucidate molecular mechanisms involved in chemoprevention by berry extracts, we employed mouse epidermal Cl 41 cell line, a well-characterized in vitro model in tumor promotion studies. Pretreatment of Cl 41 cells with methanol-extracted blackberry fraction RO-ME resulted in a dramatical inhibition of B(a)PDE-induced activation of AP-1 and NFκB, and expression of VEGF and COX-2. The inhibitory effects of RO-ME on B(a)PDE-induced activation of AP-1 and NFκB appear to be mediated via inhibition of MAPKs and IκBα phosphorylation, respectively. In view of the important roles of AP-1, NFκB, VEGF and COX-2 in tumor promotion/progression, and VEGF and COX-2 are target of AP-1 and NFκB, we anticipate that the ability of black raspberries to inhibit tumor development may be mediated by impairing signal transduction pathways leading to activation of AP-1 and NFκB, subsequently resulting in down-regulation of VEGF and COX-2 expression. The RO-ME fraction appears to be the major fraction responsible for the inhibitory activity of black raspberries.*

Introduction

Epidemiological studies have demonstrated a correlation between high intake of vegetables and fruit and a decreased incidence of cancer in multiple organ sites such as the lung, larynx, oral pharynx, gastrointestinal tract, and pancreas in humans (1, 2). Dietary supplements with various vegetables and fruit have also been protective against tumor development in animal model systems (3-6). It is thought that the chemopreventive effects of vegetables and fruit are due to their contents of phytochemicals, vitamins, vitamin precursors, minerals and compounds that have not, as yet, been identified (7-10).

Berries of various types, including blackberries, strawberries, raspberries and blueberries, have received recent attention for their potential health benefits, including cancer preventive activity (1, 3). Berries contain a number of known chemopreventive agents, such as vitamin A, C, E, and folic acid; calcium and selenium; phytosterols including beta-sitosterol; and polyphenols including anthocyanins, gallic acid, ellagic acid, ferulic acid, coumaric acid, and quercetin (11). The anthocyanins are quite abundant in certain berry types, such as black raspberries and blackberries, and are regarded as natural antioxidants (12-14) which improve the overall antioxidant defense in human plasma (14). Other berry phenolics such as ellagitannins and proanthocyanidins were found to be protective against both lipid and protein oxidation (15). The chemopreventive activities of berry derivatives have been demonstrated in several recent studies (3, 11, 16-18). *In vitro* studies using Syrian hamster embryo (SHE) cells showed that methanol extracts from black raspberries and strawberries reduced benzo[a]pyrene (B[a]P)-induced cell transformation (1). In addition, the proliferation of colon and breast cancer cells was inhibited by berry extracts in a concentration-dependent manner (16). Previous *in vivo* studies also demonstrated that freeze-dried black raspberries and strawberries inhibited chemical-induced tumors in colon, esophagus, and oral cavity of rodents (11, 17, 18). There have been relatively few studies on the mechanisms of chemoprevention by berries. In the *N*-nitrosomethylbenzylamine (NMBA)-induced rat esophageal cancer model, pretreatment of rats with dietary berries including black raspberries and strawbeirres resulted in the reduction of O^6-methylguanine adduct formation from NMBA in esophageal DNA (11,17,18), indicating that berries may influence the metabolism of NMBA. In this same model, both berry types were effective in inhibiting tumor formation when added to the diet following treatment of the rats with NMBA (i.e., post-initiation). In this protoocol, berries were found to reduce the proliferation rate of premalignant cells (11). Recently, in an *in vivo* hemangioma

H. Lu, J. Li, D. Zhang, and C. Huang are affiliated with the Nelson Institute of Environmental Medicine, New York University School of Medicine,Tuxedo, NY 10987. G. D. Stoner is affiliated with the Division of Hematology and Oncology, Department of Internal Medicine and Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210.

model, edible wild blueberry extracts and berry mixtures were found to impair angiogenesis, an indispensable process in cancer development (19). A few studies have been conducted to identify the chemical contents of berries that are responsible for their chemopreventive activities (16, 20-22). For instance, correlation studies indicated that antioxidants, such as Vitamin C and other substances, might play a synergistic role in the inhibition of cancer cell proliferation by berry extracts (16). Moreover, ellagic acid, a widely-distributed component in various berries, was found to inhibit chemical–induced tumors in rodents (20). Potential mechanisms for chemopreventive activities of ellagic acid include inhibition of metabolic activation of carcinogens, interference in the binding of the reactive metabolites of carcinogens to DNA, as well as the induction of phase II enzymes involved in carcinogen detoxification (21–23). Although ellagic acid may be a promising target for future mechanistic studies addressing berry chemoprevention, it is also clear that ellagic acid is not the major agent responsible for tumor-inhibitory activities of berries (17).

A number of critical oncogenic signaling pathways including activator protein 1 (AP-1), nuclear factor kappa B (NFκB), and nuclear factor in activated T cells (NFAT) have been found in the past several years (24-28). We are attempting to elucidate the molecular mechanisms involved in the chempreventive activities of berries by evaluating their effects on components of these signaling pathways. We found that extracts from several berry derivatives had inhibitory effects on the carcinogen-induced activation of transcription factor AP-1, NFκB and NFAT, as well as the expression of their target genes, cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF). These results contribute to an in-depth understanding of molecular mechanisms for chemoprevention by berries.

Methods

Berry Extracts and Cell Culture Conditions

Black raspberry extracts were obtained as described previously (3). After picking, ripe black raspberries (*Rubus occidentalis,* RO) were washed, frozen, freeze-dried, and then extracted with methanol. The initial methanol extract was filtered, dried and identified as F001. The F001 extract was partitioned between dichloromethane and water and separated into a water-soluble fraction (F003), an organic soluble fraction (F004), and an insoluble fraction (F005). The F001 extract was also eluted through a silica gel chromatography column using dicholoromethane:methanol, producing a non-polar fraction DM, and a polar fraction ME.

Mouse JB-6 Cl 41 epidermal cells have been used extensively for *in vitro* studies of the mechanisms of tumor promotion (29). Transformation-sensitive (P+) JB6 cells are unique in defining neoplastic transformation, as well as transformation related events such as AP-1 activation induced by tumor promoters including phorbol esters and growth factors (30, 31). These cells have contributed to our understanding of molecular events involved in the regulation of the extracellular signal-regulated protein kinases (ERKs)-mitogen-activated protein kinase (MAPKs)/AP-1, nuclear factor κ B (NFκB), and phosphatidylinositol 3-kinase (PI-3K) pathways that lead to tumor promotion (29-31). To date, most of the observations in *in vitro* studies using Cl41 cells have been confirmed *in vivo* animal models (31). Thus, the results using Cl41 cells can usually be extrapolated to *in vivo* studies of tumor promotion and for the identification of molecular targets for cancer prevention (31). To evaluate transcription factor activation and the expression of their downstream target genes, we used Cl 41 cells stably transfected with certain luciferase reporter constructs (i.e., Cl 41 AP-1, NFκB, NFAT, and COX-2 mass1 cells) as described in previous studies (27, 32). All these cell lines were routinely cultured in Eagle's Minimal Essential Medium (MEM, Calbiochem San Diego, CA) supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine (Life Technologies, Inc. Rockville, MD). The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in air.

To investigate the inhibitory effects of berry extracts on carcinogenesis, we routinely expose cells to carcinogens that produce alterations in signaling transduction pathway. B[a]P, a widely distributed environmental carcinogen, induces tumors in various organ sites in animal models (33). Benzo[a]pyrene-7,8-diol-9,10-epoxide (B[a]PDE), the ultimate carcinogenic metabolite of B[a]P, acts as a complete carcinogen by binding to cellular DNA and protein at neutrophilic sites (34). The carcinogenic activity of B[a]PDE is strongly associated with the development of smoking-related cancers (35, 36). Since our previous studies demonstrated that black raspberry extracts inhibit B[a]P-induced cell transformation, we examine the effects of berry fractions on B[a]P/B[a]PDE exposed Cl 41 cells to develop an understanding of the chemopreventive effects of berries.

Assays for AP-1, NF B, and NFAT Transcription Activation, as Well as COX-2 Promotor Activity

Confluent monolayer cells of the transfectants were trypsinized, and 8×10^3 viable cells in 100 μl of 5% FBS MEM were added to each well of 96-well plates. Plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. After the cell density reached 80–90%, the cells were pretreated with black raspberry fraction RO-ME, or anthocyanins including cyanidin chloride (CyCl) and kuromanin chloride (KurCl, chemical name: cyanidin-3-O-glucoside chloride) at indicated concentrations for 30min, then exposed to 2 μM of B[a]PDE. The luciferase activity was determined by the luciferase assay at indicated different time points using a luminometer (Wallac 1420 Victor 2 multiplate counter system, Perkin Elmer Life Science, Inc.) after the addition of 50 μl of lysis buffer for 30 min at 4°C. The results were expressed as AP-1, NFκB, NFAT, and COX-2 transcriptional induction relative to medium control**.** The

Student's *t* test was used to determine the significance of the differences of AP-1, NFκB, NFAT, and COX-2 induction among various transfectants. The differences were considered significant at a $P \leq 0.05$.

Western Blot Analysis

8×10^4 cells of Cl 41 cells were seeded into each well of 6-well plates. After the cell density reached 70-80%, cells were pretreated with 10 μg/ml (for Akt and p70 S6 kinase (p70S6K)) or 25μg/ml (for COX-2) RO-F003, RO-F004, RO-DM, and RO-ME for 30 min, and then exposed to B[a]PDE (2μM) for 60min or 120min. The cells were collected by adding 100 μl Tris-Glycine SDS sample buffer (Invitrogen, Carlsbad, CA) until they reach 95-100% confluence. Western blots were performed with either phosphospecific antibodies or nonphosphorylated antibodies against Akt, p70S6K, and rabbit anti-mouse COX-2 polyclonal antibody. p38 kinase was immuodetected as a protein loading control. The protein band specifically bound to the primary antibody was detected using an anti-rabbit IgG-AP-linked and an ECF western blotting system (Amersham Biosciences, Piscataway, NJ).

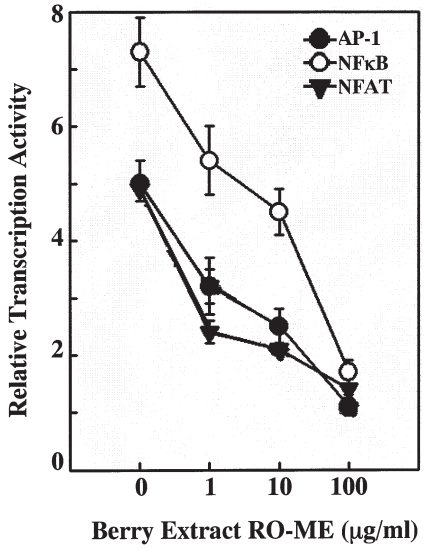

Figure 1. Inhibition of B[a]PDE-induced activation of AP-1, NFκB, and NFAT by black raspberry extracts. Eight $\times 10^3$ of Cl 41 AP-1, NFκB, and NFAT mass1 cells were seeded into each well of 96-well plates, and cultured in 5% FBS MEM at 37 °C. After the cell density reached 80-90%, the cells were first pretreated with black raspberry fraction RO-ME at various concentrations as indicated for 30 min, and then exposed B[a]PDE (2μM) for AP-1, NFκB, and NFAT induction. The results are expressed as AP-1, NFκB, and NFAT induction relative to control medium containing DMSO. Each bar indicates the mean and standard deviation of triplicate assay wells.

Results

The Effects of Berry Extracts on AP-1 Induction

The transcription factor, AP-1, is a collective form of various homodimers or heterodimers consisted of members from several protein families including Jun and Fos (two major families for mammalian AP-1 proteins), as well as ATF and MAF (37). After stimulation with growth factors, cytokines, carcinogens and/or oncoproteins, the AP-1 complex rapidly binds to specific DNA sequences that are implicated at different stages of tumorigenesis (38). Multiple AP-1 components are found to be highly expressed, and the signaling pathways leading to increased AP-1 DNA binding activity are often activated in human tumor cells (39). Moreover, AP-1 activation is required for the Cl 41 cell transformation induced by tumor promoters (29). By regulating the transactivation of certain target genes, AP-1 plays an essential role in carcinogenesis, including effects on tumor cell proliferation, survival, and invasive growth and metastasis (24). AP-1 proteins with different components may also contribute to carcinogenesis by down-regulating certain tumor suppressor genes (24) and by cooperating with oncogenes such as Ras (40).

Previously, we have shown that B[a]PDE induces AP-1 activation in Cl41 cells (35,41). Based upon this observation, we determined whether AP-1 is a target for chemoprevention of berry extracts (32). After pretreatment of Cl 41 cells with various black raspberry fractions, including RO-F003, RO-F004, RO-DM, and RO-ME, AP-1 luciferase reporter transfected cells were exposed to B[a]PDE. Of the fractions tested, the RO-ME fraction was found to be the most potent inhibitor of AP-1 induction by B[a]PDE, consistent with its inhibition of B[a]P-induced cell transformation (Fig.1) (32). In this study, we also determined the mechanism(s) for the inhibition on B[a]PDE-induced AP-1 activation by berry extracts. Ellagic acid, a well known chemopreventive agent in black raspberries (11), was previously shown to react with B[a]PDE, thus blocking its DNA binding activity (42). Thus, it was logical to ask whether the inhibition of B[a]PDE-induced AP-1 activation was resulted from preventing B[a]PDE from binding to DNA. This possibility was ruled out by the fact that pretreatment of RO-ME failed to cause any significant reduction in B[a]PDE-DNA adduct formation in Cl 41 cells (32). Further studies showed that RO-ME inhibited B[a]PDE-induced AP-1 activation by targeting MAPK pathways, including ERKs, c-Jun-NH_2-terminal kinases (JNKs), and p38K (32), which is consistent with our previous findings that B[a]PDE induced the activation of AP-1 through activation of ERKs, JNKs, and p38K (35). Moreover, published results from our group suggested that the PI-3K/Akt pathway is required for the B[a]PDE-induced AP-1 activation in Cl 41 cells (35). Thus, we investigated the involvement of PI-3K/Akt pathway in the inhibition of B[a]PDE-induced AP-1 activity. We found that pretreatment of Cl 41 cells with RO-ME markedly inhibits B[a]PDE-induced PI-3K activation (28) and phosphorylation of Akt at Thr308/Ser473 and p70S6K at Thr421/Ser424 (Fig.2). Collectively, our data indicate that black raspberry fractions inhibit B[a]PDE-induced AP-1 activation through inhibiting activation of the PI-3K/Akt pathway.

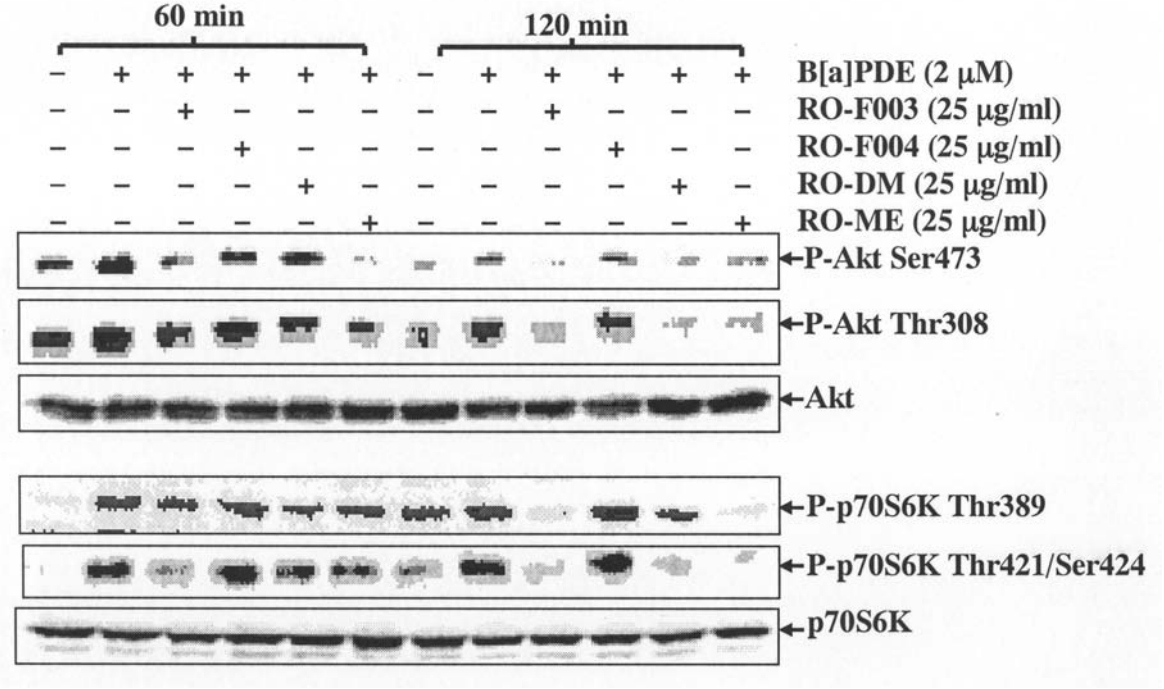

Figure 2. Inhibition of B[a]PDE-induced activation of Akt and p70S6K by black raspberry extracts. Eight $\times 10^4$ of Cl 41 VEGF mass1 cells were seeded into each well of six-well plates, and cultured in 5% FBS MEM at 37°C. After the cell density reached 70-80%, cells were first pretreated with black raspberry extract fractions (25μg/ml) for 30 min, then exposed to B[a]PDE (2 μM) for 60 min or 120 min. Western blots were performed with either phospho-specific antibodies or non-phosphorylated antibodies against Akt and p70S6K. The protein band specifically bound with primary antibodies was detected by using anti-rabbit IgG-AP-linked and ECF western blotting system (32).

NF B, Another Target of Blackberry Extracts

NFκB is a dimeric transcription factor consisting of Rel family members (44). It is an omnipotent transcription factor with extensive regulatory roles for both immune response and carcinogenesis (45, 46). NFκB may act as a tumor promoter by suppressing apoptosis, stimulating cell proliferation, as well as targeting genes that facilitate tumor invasion and metastasis (45, 46). We demonstrated that exposure to B[a]PDE induces NFκB activation in Cl 41 cells (40). Thus, we were interested in determining if the chemopreventive effects of black raspberry extracts could be attributed, at least in part, to their effects on B[a]PDE-induced NFκB activation (32). Our results indicated that black berry fraction RO-ME dramatically inhibited B[a]PDE-induced NFκB activation (Fig. 1) (32). Similar to inhibition of AP-1 activity, pretreatment or co-incubation of cells with RO-ME is required for inhibition of B[a]PDE-induced NFκB activation (32).

NFκB is inhibited in its expression by the cytoplasmic inhibitor, inhibitory subunit kappa B (IκB) α (44). The activation of NFκB is mediated by initiating the phosphorylation, ubiquitination, and subsequent degradation of IκBα (44). Upon degradation of IκBα, NFκB is released from IκBα inhibition and translocates into nucleus where it functions as a transcription factor for its target genes (44,47). The effects of berry extracts on B[a]PDE-caused IκBα phosphorylation and degradation were investigated. Our studies indicated that 90 minutes after the treatment of Cl41 cells with B[a]PDE, the phosphorylation of IκBα is up-regulated when compared to non-treatment controls (32). Pretreatment of cells with RO-ME inhibited IκBα phosphorylation (32). 270 minutes later, the inhibitory effects of RO-ME on the degradation of IκBα were observed (32). These results suggests that the inhibitory effects of RO-ME on B[a]PDE-induced NFκB activation occur indirectly through effects on IκBα. Previous studies have shown that Akt is implicated in tumor necrosis factor (TNF)- and platet-derived growth factor (PDGF)-induced NFκB activation (48,49). Since the PI3K/Akt pathway was found to by us to be inhibited by RO-ME, the impairment of Akt by RO-ME may also contribute to its inhibition of NFκB activation.

NFAT: A Novel Target for Chemoprevention by Berry Extracts

NFAT is a calcium-regulated transcription factor expressed in both T cells and non-lymphoid tissue cells (50-55). After its activation and translocation into the nucleus, NFAT forms a heterodmeric transcriptional complex with AP-1 as co-activators to initiate NFAT-dependent transactivation (54). NFAT is implicated in carcinogenesis since it was found to be induced by various carcinogens (55-57), and involved in the expression of Interleukin-8, COX-2, TNFα, and other cytokines that are associated with cancer development (26,58-59). NFAT is also known to promote the invasive capabilities of human carcinomas (26). With the application of NFAT3 siRNA, we demonstrated the requirement of NFAT in B[a]PDE-induced TNFα expression

in Cl 41 cells, which in turn leads to COX-2 expression and cell transformation in Cl 41 cells (Huang et al., unpublished data). Thus, the effects of black rapberry fractions on B[a]PDE-induced NFAT activation were also assessed. Cl 41 stably transfected with a NFAT luciferase reporter were pretreated with different extract fractions from black raspberries, and then exposed to B[a]PDE. The RO-ME fraction was found to be the major fraction exhibiting inhibitory effects on B[a]PDE-induced NFAT activation among the fractions tested (Fig. 1).

Down-Regulation of Transcription Factor Target Genes by Berry Extracts

As indicated above, AP-1, NFκB, and NFAT are involved in cancer development (24-28). The roles of these transcription factors in carcinogenesis are attributed to their transcriptional regulation of target genes that regulate cellular proliferation, apoptosis, angiogenesis, and tumor invasion and metastasis (24-28). Thus, with the knowledge that berry fractions inhibit B[a]PDE-induced activation of the transcription activators, AP-1, NFκB and NFAT, we conducted studies to determine the effects of these fractions on the expression of the target genes of these activators, VEGF and COX-2.

Inhibition of B[a]PDE-induced expression of vascular endothelial growth factor (VEGF): VEGF is a group of related growth factors; i.e., VEGFs B, C, D and E, placenta growth factor and the *orf* virus-encoded factor (60). VEGF binds to its receptor kinases VEGFRs to activate intracellular signaling pathways, eliciting angiogenesis by inducing the migration and proliferation of endothelial cells (61, 62). Overexpression of VEGF has been found in virtually all tumor types, including tumors that have an activated *ras* oncogene and/or inactivated *p53* gene (64). VEGF stimulates tumor angiogenesis, induces tumor cell proliferation and migration (65-67), mediates tumor growth, invasion, and metastasis (68-71). With regard to the regulation of VEGF expression by transcription factors, the gene encoding VEGF D was identified as a target gene of c-Fos, a major AP-1 component (72). AP-1, associated with hypoxia inducible factor 1 (HIF-1), promotes VEGF expression under hypoxic conditions (73). A few studies have shown that NFκB activation is also associated with the up-regulation of VEGF expression (74, 75). Considering the critical role of VEGF in cancer development and its regulation by AP-1 and NFκB, we were interested in the effects of berry fractions on B[a]PDE-induced VEGF expression. Using Cl 41 cells stably transfected with a VEGF luciferase reporter, we investigated the effects of berry fractions on expression of this gene. RO-ME, the most potent inhibitory fraction for B[a]PDE-induced activation of AP-1 and NFκB, was also found to inhibit VEGF expression induced by B[a]PDE (28). These observations suggest that the inhibition, by berry fractions, of AP-1 and NFκB in Cl 41 cells may be associated with their inhibitory effects on VEGF induction by B[a]PDE.

Several signaling pathways appear to mediate the induction of VEGF expression (63, 76-78). In human glioblastoma cells, VEGF is induced via the activation of ERK1/2 signaling pathway (63), whereas in human prostate cancer cells, arsenite induced VEGF through PI-3K/Akt pathway (76). Inhibition of VEGF expression by the potential anti-tumor agents, apigenin and SU5416, was shown recently to occur through the inhibition of the PI3K/Akt/p70S6K1 signaling pathway (77, 78). In our studies, B[a]PDE-induced VEGF expression in Cl 41 cells was inhibited by black raspberry fraction RO-ME, which also inhibited the activation of PI3K/Akt/p70S6K and the MAPK pathways. To provide direct evidence for the role of PI-3K in B[a]PDE-induced VEGF expression, a VEGF luciferase reporter and dominant-negative PI-3K p85 mutant, Δp85, were co-transfected into Cl 41 cells. The blockage of PI-3K activation by Δp85 overexpression inhibited B[a]PDE-induced VEGF expression (28), indicating a requirement for PI-3K in B[a]PDE-induced VEGF expression in Cl 41 cells. Since our group previously demonstrated that B[a]PDE-induced AP-1 activation was PI3K/Akt/JNKs pathway-dependent (35), and RO-ME inhibited B[a]PDE-induced activation of AP-1, we suspect that the PI3K/Akt/AP-1 pathway is a target for RO-ME inhibition of B[a]PDE-induced VEGF expression.

COX-2, an important target of black raspberry extracts: COX-2 is involved in multiple physiological and pathological processes, and its expression has been found to be regulated by cytokines, growth factors, tumor promoters, and oncogenes under tight control by several transcription factors (45, 46, 59, 79, 90-93). Prostaglandins, the major products of COX-2 enzyme activity, are important mediators of inflammation (80). COX-2 expression is also enhanced in many cancer types, promoting cell proliferation, neoangiogenesis, tumor cell migration and metastasis, and inhibiting apoptosis (81-89). AP-1 is one eukaryotic transcription factor implicated in the regulation of COX-2 expression (90-92). COX-2 is also a target gene of the transcription factor, NFκB (45, 46), and NFκB inhibitors may cause regression of colorectal cancer development via the inhibition of COX-2 (46). In addition, the NFAT regulatory element is found in the 5'-flanking promoter region of human COX-2 (59, 93), and our most recent studies show that NFAT-mediated COX-2 induction plays an essential role in TNFα-induced cell transformation (27). NFAT has also been shown to induce COX-2 both in Jurkat human leukemic T cells and in glomerular mesangial cells (93,94).

The extensive roles of COX-2 in cancer development led us to investigate whether berry fractions influence the expression of this enzyme. Our results indicate that B[a]PDE markedly induced COX-2 transcription and protein expression (Figs. 3a and 3b), and both RO-F003 and RO-ME berry fractions strongly inhibited COX-2 expression. The RO-DM fraction produced only a marginal effect, and the RO-F004 fraction had no effect, on COX-2 expression (Fig. 3a). Different dose and time point studies demonstrated the inhibition of B[a]PDE-induced COX-2 expression by RO-ME in a wide

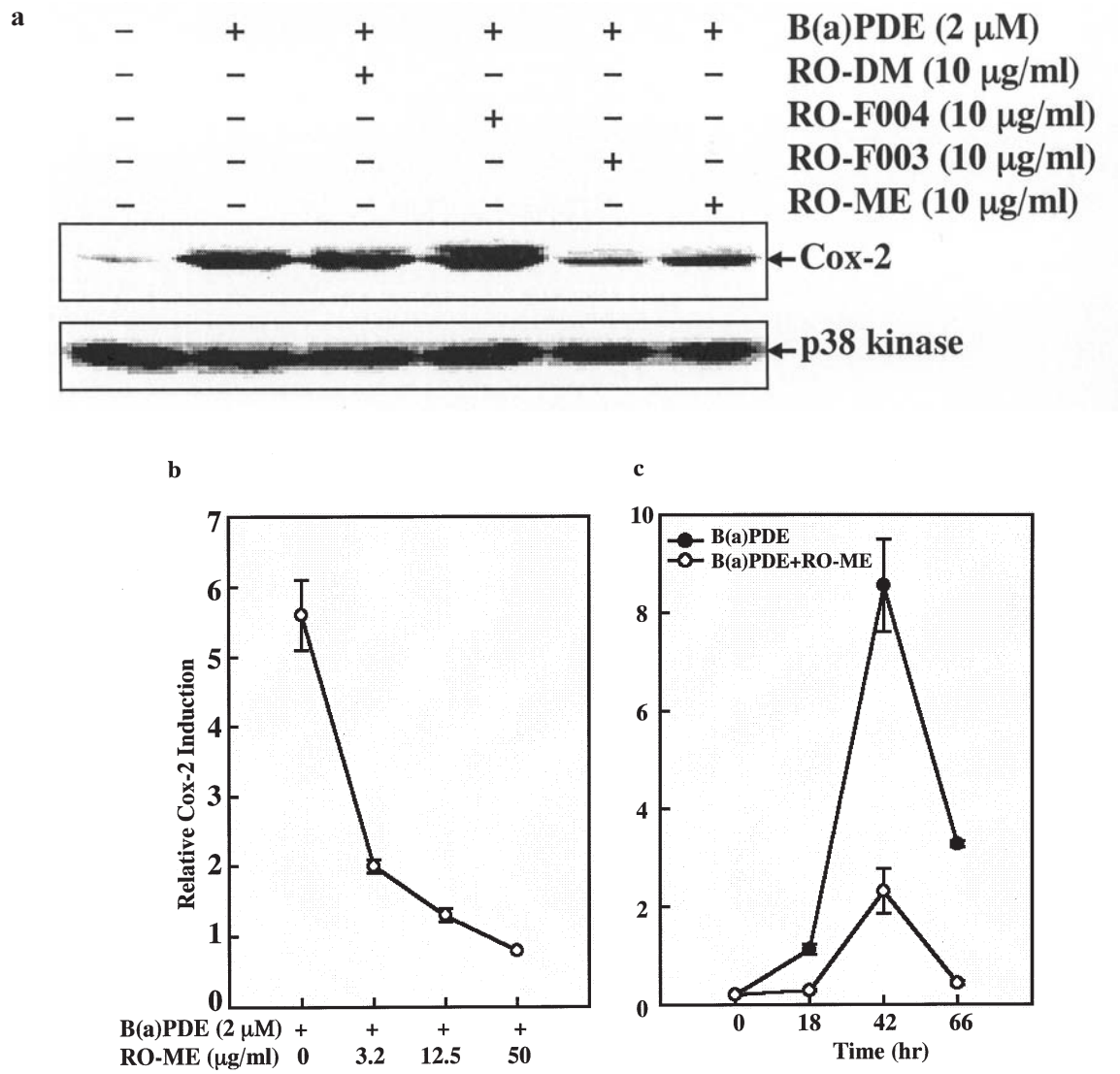

Figure 3. Effects of black raspberry extract fractions on COX-2 induction by B[a]PDE. A: Eight $\times 10^4$ of Cl 41 COX-2 mass1 cells were seeded into each well of six-well plates, and cultured in 5% FBS MEM at 37°C. After the cell density reached 70-80%, cells were first pretreated with black raspberry fractions (10 μg/ml) for 30 min, then exposed to B[a]PDE (2 μM) for 36 hr. Western blots were performed with antibodies against COX-2, or p38 kinase. p38K was used as a protein loading control. The protein band specifically bound with primary antibodies was detected by using anti-rabbit IgG-AP-linked and ECF western blotting system (32). B and C: Eight $\times 10^3$ of Cl 41 COX-2 mass1 cells were seeded into each well of 96-well plates, and cultured in 5% FBS MEM at 37°C. After the cell density reached 80-90%, the cells were first pretreated with black raspberry fraction RO-ME at various concentrations as indicated in the figure for 30 min, and then exposed B[a]PDE for COX-2 induction for 42 hr (B) or at different time points as indicated in the figure (C), the cells were extracted with lysis buffer, and luciferase activity was measured using Promega Luciferase assay reagent with a luminometer (Wallac 1420 Victor2 multiplate counter system) after addition of 50 μl of lysis buffer for 30 min at 4°C. The results are expressed as COX-2 induction relative to control medium containing DMSO. Each bar indicates the mean and standard deviation of triplicate assay wells.

dose range from 3.2-50 μg/ml, as well as at all the time points observed (Figs. 3b and 3c).

Since COX-2 is a major target gene of AP-1, NFκB and NFAT, and black raspberry fraction RO-ME dramatically inhibits B[a]PDE-induced activation of all three transcription factors, it is reasonable to link RO-ME inhibition to reduction of COX-2 expression. Recent studies demonstrate that COX-2 inhibitor, celecoxib, inhibited the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced AP-1 activation by inhibiting p38 MAP kinase (95). AP-1 is regulated by MAPKs pathways (43), and inhibition of B[a]PDE-induced AP-1 activation by black raspberry fraction RO-ME is accompanied by the repression of MAPKs (32). Thus, with our most recent finding of the involvement of NFAT for the induction of COX-2, we anticipate that RO-ME inhibits B[a]PDE-induced COX-2 expression by impairing NFAT and MAPKs/AP-1 pathways.

Discussion

Epidemiological studies suggested that the consumption of fruits and vegetables reduces the incidence of many human cancers including lung, larynx, oral pharynx, gastroin-

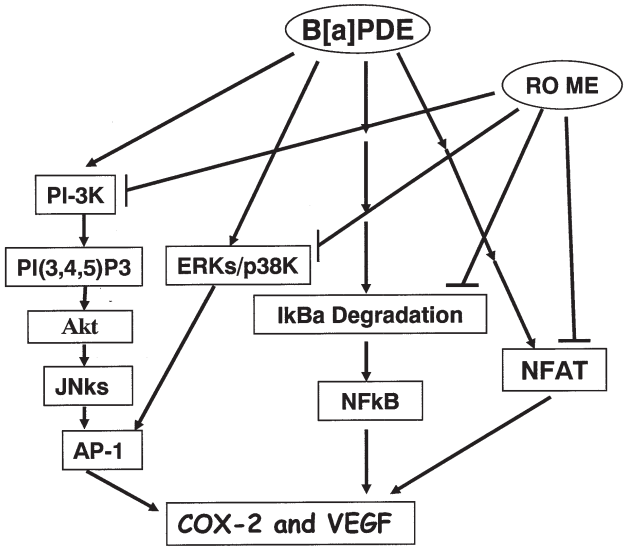

Figure 4. Schematic illustration of known molecular mechanisms that may be involved in the chemopreventive activities of black raspberry fraction RO-ME.

testinal tract, and pancreatic cancer (2). Previous studies from our laboratory and others have demonstrated the chemopreventive effects of freeze-dried raspberries on tumor formation *in vivo* (11, 96), and the inhibition of carcinogen-induced cell transformation (3). In the present study, we have summarized current data on molecular mechanisms for the chemopreventive activities of black raspberry fractions identified using the Cl 41 mouse epidermal cell system. Transcription factors AP-1, NFκB, and NFAT are well-known for their roles in carcinogenesis (32). Our results demonstrate that black raspberry fraction RO-ME is the major fraction blocking B[a]PDE-induced activation of AP-1, NFκB, and NFAT. Although we do not know precisely which components in this fraction are responsible for the inhibitory effects, recent studies suggest that anthocyanins play an important role (97). Since activation of the MAPKs pathway and PI3K/Akt/p70S6K pathway are also inhibited by RO-ME, and these pathways are involved in B[a]PDE-induced AP-1 activation (35), we suggest that the inhibitory effects of RO-ME on B[a]PDE-induced AP-1 activation may occur through the blocking of these signaling pathways. Moreover, the inhibition of NFκB activation is attributed to impaired phosphorylation and subsequent degradation of IκBα, the upstream inhibitor for NFκB. PI3K/Akt pathway may also be involved in mediating NFκB activation by activating IκB kinase (IKK). Similar to AP-1 and NFκB activation, the activation of another transcription factor NFAT induced by B[a]PDE was also inhibited by black raspberry fraction RO-ME.

VEGF and COX-2, the downstream target genes of AP-1, NFκB and NFAT, are also inhibited by black raspberry fraction RO-ME. We also examined the effects of black raspberry fractions, including RO-ME, on B[a]PDE-induced nitric oxide synthase (iNOS) expression, however, none of the fractions produced any inhibitory effect (Data not shown), suggesting that inhibition of berry extracts on gene expression is transcription factor and their target-gene specific.

Based on previous studies, our current knowledge on the molecular mechanisms involved in the chemopreventive activity of black raspberry fraction RO-ME can be summarized

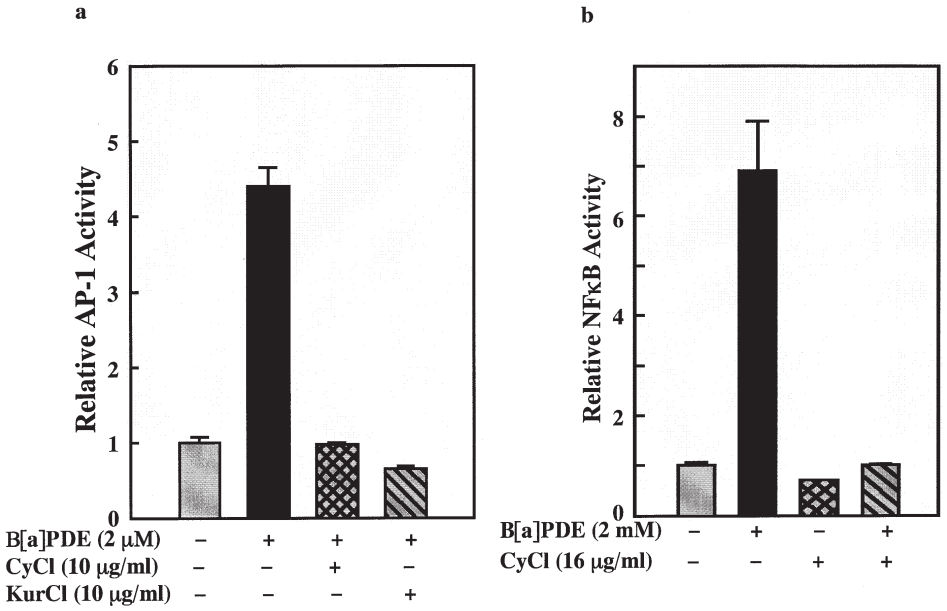

Figure 5. Inhibition of AP-1 and NFκB activation by anthocyanins in mouse Cl 41 cells. Cl 41 AP-1 mass1 (a) or Cl 41 NFκB mass1 (b) cells were seeded into each well of 96-well plates, and cultured in 5% FBS MEM at 37 °C. After the cell density reached 80-90%, the cells were first pretreated with 10 μg/ml of anthocyanins (CyCl or KurCl) for 30 min, and then exposed to B[a]PDE (2 μM) for AP-1 induction. Twenty-four hours after B[a]PDE exposure, the cells were extracted with lysis buffer, and luciferase activity was measured using Promega Luciferase assay reagent with a luminometer (Wallac 1420 Victor2 multiplate counter system) after addition of 50 μl of lysis buffer for 30 min at 4^0C. The results are expressed as relative NFκB activity relative to medium control containing DMSO. Each bar indicates the mean and standard deviation of triplicate assay wells.

into Fig. 4. Although we identified some of the effects of black raspberry fractions on gene expression *in vitro*, it is probable that we are far from a total analysis of the molecular effects of berries leading to cancer prevention. More research is necessary to identify all components of the anti-tumor effects of berry fractions since cancer development is a highly complicated process involving the combined interactions of numerous regulators and effectors. Additional studies are also required to identify the key chemicals that are responsible for the chemopreventive activities of berries. As indicated above, recently we have identified the black raspberry anthocyanins, CyCl and KurCl, as inhibitors of B[a]PDE-induced activation of AP-1 and NFκB in Cl 41 cells (Fig.5). Further investigations of the molecular mechanisms by which athocyanins exhibit chemopreventive effects are ongoing in our laboratory. We believe that the clarification of the molecular mechanisms of cancer chemoprevention by berries, as well as the identification of key inhibitory components in berries, may ultimately be beneficial to the prevention of cancer development in humans.

Acknowledgments and Notes

This work was supported, in part, by grants from NIH/NCI R01 CA103180, R01 CA094964, and R01 CA 112557, and from NIH/NIEHS R01 ES012451. The first two authors have contributed equally to this work.
Address correspondence to Dr. Chuanshu Huang, Nelson Institute of Environmental Medicine, New York University School of Medicine, 57 Old Forge Road, Tuxedo, NY 10987. Phone: 845-731-3519. FAX: 845-351-2320. E-mail: chuanshu@env.med.nyu.edu.

References

1. Block G, Patterson B, and Subar A: Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr Cancer* **18**, 1-29, 1992.
2. Vecchia CL, and Tavani A: Fruit and vegetables, and human cancer. *Eur J Cancer Prev* **7**, 3-8, 1998.
3. Xue H, Aziz RM, Sun N, Cassady JM, Kamendulis LM, et al.: Inhibition of cellular transformation by berry extracts. *Carcinogenesis* **2**, 351-356, 2001.
4. Ahmed S, Rahman A, Mathur M, Athar M, and Sultana S: Anti-tumor promoting activity of Asteracantha longifolia against experimental hepatocarcinogenesis in rats. *Food Chem Toxicol* **39**, 19-28, 2001.
5. Kusamran WR, Ratanavila A, and Tepsuwan A: Thai and Chinese bitter gourd fruits and sweet basil leaves on hepatic monooxygenases and glutathione S-transferase activities, and in vitro metabolic activation of chemical carcinogenesis in rats. *Food Chem Toxicol* **36**, 475-484, 1998.
6. Gotoh T, Yamada K, Yin H, Ito A, Kataoka T, et al.: Chemoprevention of *N*-nitroso-*N*-methylurea-induced rat mammary carcinogenesis by soy foods or biochanin A. *Jpn J Cancer Res* **89**, 137-142, 1998.
7. Mukhtar H, and Ahmad N: Cancer chemoprevention: future holds in multiple agents. *Toxicol Appl Pharmacol* **158**, 207-210, 1999.
8. Krishnan K, Ruffin MT, IV, and Brenner DE: Cancer chemoprevention: a new way to treat cancer before in happens. *Primary Care* **25**, 361-379, 1998.
9. Watternberg LW: An overview of chemoprevention: current status and future prospects. *Proc Soc Exp Biol Med* **216**, 133-141, 1997.
10. Rock CL: Nutritional factors in cancer chemoprevention. *Hematol Oncol Clin North Am* **12**, 975-991, 1998.
11. Kresty LA, Morse MA, Morgan C, Carlton PS, Lu J, et al.: Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilized black raspberries. *Cancer Res* **61**, 6112-6119, 2001.
12. Roy S, Khanna S, Alessio HM, Vider J, Bagchi D, et al.: Anti-angiogenic property of edible berries. *Free Rad Res* **36**, 1023-1031, 2002.
13. McCarty MF: Current prospects for controlling cancer growth with non-cytotoxic agents—nutrients, phytochemicals, herbal extracts, and available drugs. *Med Hypotheses* **56**, 137-154, 2001.
14. Cao G, and Prior RL: Anthocyanins are detected in human plasma after oral administration of an elderberry extract. *Clin Chem* **45**, 574-576, 1999.
15. Viljanen K, Kylli P, Kivikari R, and Heinonen M: Inhibition of protein and lipid oxidation in liposomes by berry phenolics. *J Agric Food Chem* **52**, 7419-7424, 2004.
16. Olsson ME, Gustavsson KE, Andersson S, Nilsson A, and Duan RD: Inhibition of cancer cell proliferation in vitro by fruit and berry extracts and correlations with antioxidant levels. *J Agric Food Chem* **52**, 7264-7271, 2004.
17. Stoner GD, Kresty LA, Carlton PS, Siglin JC, and Morse MA: Isothiocyanates and freeze-dried strawberries as inhibitors of esophageal cancer. *Toxicol Sci* **52** (Suppl.), 95-100, 1999.
18. Carlton PS, Kresty LA, Siglin JC, Morse MA, Lu J, et al.: Inhibition of N-nitrosomethylbenzylamine-induced tumorigenesis in the rat esophagus by dietary freeze-dried strawberries. *Carcinogenesis* **22**, 441-446, 2001.
19. Atalay M, Gordillo G, Roy S, Rovin B, Bagchi D, et al.: Anti-angiogenic property of edible berry in a model of hemangioma. *FEBS Lett* **544**, 252-257, 2003.
20. Mandal S and Stoner GD: Inhibition of *N*-nitrosomethylbenzylamine-induced esophageal tumorigenesis in rats by ellagic acid. *Carcinogenesis* **11**, 55-61, 1990.
21. Teel RW, Dixit R, and Stoner GD: The effects of ellagic acid on the uptake, persistence, metabolism and DNA-binding of benzo[a]pyrene in cultured explants of strain A/J mouse lung. *Carcinogenesis* **6**, 391-395, 1985.
22. Teel RW: Ellagic acid binding to DNA as a possible mechanism for its antimutagenic and anticarcinogenic action. *Cancer Lett* **30**, 329-336, 1986.
23. Ahn D, Putt D, Kresty L, Stoner GD, Fromm D, et al.: The effects of dietary ellagic acid on rat hepatic and esophageal mucosal cytochromes P450 and phase II enzymes. *Carcinogenesis* **17**, 821-828, 1996.
24. Eferl E, and Wagner EF: AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer* **3**, 859-867, 2003.
25. Lin A, and Karin M: NFκB in cancer: a market target. *Semin Cancer Biol* **13**, 107-114, 2003.
26. Jauliac S, Lopez-Rodriguez C, Shaw LM, Brown LF, Rao A, et al.: The role of NFAT transcription factors in integrin-mediated carcinoma invasion. *Nat Cell Biol* **4**, 540-544, 2002.
27. Yan Y, Ouyang W, Li J, Ma Q, Hu Y et al.: Nuclear factor of activated T cells 3 (NFAT3) is specifically required for tumor necrosis factor-α (TNF-α)-induced cyclooxygenase-2 (COX-2) expression and cell transformation in C141 cells. *J Cell Science,* in press.
28. Huang C, Li J, Song L, Zhang D, Tong OS, et al.: Black rasberry extracts inhibit benzo[a]pyrene diol-epoxide-induced AP-1 activation and VEGF transcription by targeting the PI-3K/Akt pathway. *Cancer Res* **66,** 581–587, 2006.
29. Li J, Chen H, Tang MS, Shi X, Amin S, et al.: PI-3K and Akt are mediators of AP-1 induction by 5-MCDE in mouse epidermal Cl41 cells. *J Cell Biol* **165**, 77-86, 2004.
30. Bernstein LR, and Colburn NH: AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. *Science* **244**, 566-569, 1989.

31. Dhar A, Young MR, and Colburn NH: The role of AP-1, NFκB, and ROS/NOS in carcinogenesis: the JB6 model is predictive. *Mol Cell Biochem* **234/235**, 185-193, 2002.
32. Huang C, Huang Y, Li J, Hu W, Aziz R, et al.: Inhibition of benzo[a]pyrene diol-epoxide-induced transactivation of activated protein 1 and nuclear factor κB by black raspberry extracts. *Cancer Res* **62**, 6857-6863, 2002.
33. Phillips DH: Fifty years of benzo[a]pyrene. *Nature* **303**, 468-472, 1983.
34. Hanelt S, Helbig R, Hartmann A, Lang M, Seidel A, et al.: A comprehensive investigation of DNA adducts, DNA strand breaks and gene mutations induced by benzo[a]pyrene and (+/-)-anti-benzo[a]pyrene-7,8-diol 9,10-oxide in cultured human cells. *Mutat Res* **390**, 179-188, 1997.
35. Smith LE, Denissenko MF, Bennett WP, Li H, Amin S, et al.: Targeting of lung cancer mutation hotspots by polycyclic aromatic hydrocarbons. *J Natl Cancer Inst* **92**, 803-811, 2000.
36. Li J, Tang MS, Liu B, Shi X, and Huang C: A critical role of PI-3K/Akt/JNKs pathway in benzo[a]pyrene diol-epoxide (B[a]PDE)-induced AP-1 transactivation in mouse epidermal Cl41 cells. *Oncogene* **23**, 3932-3944, 2004.
37. Shaulian E, and Karin M: AP-1 as a regulator of cell life and death. *Nat Cell Biol* **4**, E131-E136, 2002.
38. Angel P, and Karin M: The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* **1072**, 465-469, 2002.
39. Mathas S, Hinz M, Anagnostopoulos I, Krappmann D, Lietz A, et al.: Aberrantly expressed c-Jun and JunB are a hallmark of Hodgkin lymphoma cells, stimulate proliferation and synergize with NFκB. *EMBO J* **21**, 4104-4113, 2002.
40. Johnson R, Spiegelman B, Hanahan D, and Wisdom R: Cellular transformation and malignancy induced by ras require c-jun. *Mol Cell Biol* **16**, 4504-4511, 1996.
41. Li J, Chen H, Ke Q, Feng Z, Tang MS, et al.: Differential effects of polycyclic aromatic hydrocarbons on transactivation of AP-1 and NFκB in mouse epidermal Cl41 cells. *Mol Carcinog* **40**, 104-115, 2004.
42. Sayer JM, Yagi H, Wood A, Conney AH, and Jerina DM: Extremely facile reaction between the ultimate carcinogen benzo(a)pyrene-7,8-diol 9,10-epoxide and ellagic acid. *J Amer Chem Soc* **104**, 5562-5564, 1982.
43. Karin M, Liu Z, and Zandi E: AP-1 function and regulation. *Curr Opin Cell Biol* **9**, 240-246, 1997.
44. Karin M, and Ben-Neriah Y: Phosphorylation meets ubiquitination: the control of NFκB activity. *Annu Rev Immunol* **18**, 621-663, 2000.
45. Li Q, and Verma M: NFκB regulation in the immune system. *Nat Rev Immunol* **2**, 725-734, 2002.
46. Karin M, Cao Y, Greten FR, and Li ZW: NFκB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* **2**, 301-310, 2002.
47. Viatour P, Merville MP, Bours V, and Chariot A: Phosphorylation of NFκB and IκB proteins: implications in cancer and inflammation. *Trends Biochem Sci* **30**, 43-52, 2005.
48. Romashkova JA, and Makarov SS: NFκB is a target of Akt in anti-apoptotic PDGF signaling. *Nature* **401**, 86-90, 1999.
49. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, et al.: NFκB activation by tumor necrosis factor requires the Akt serine-threonine kinase. *Nature* **401**, 82-85, 1999.
50. Rincon M, and Flavell RA: Transcription mediated by NFAT is highly inducible in effector CD4+ T helper 2 (Th2) cells but not in Th1 cells. Mol Cell Biol **17**,1522-1534, 1997.
51. Durand DB, Shaw JP, Bush MR, Replogle RE, Belagaje R, et al.: Characterization of antigen receptor response elements within the interleukin-2 enhancer. Mol Cell Biol **8**, 1715-1724, 1988.
52. Serfling E, Barthelmas R, Pfeuffer I, Schenk B, Zarius S, et al.: Ubiquitous and lymphocyte-specific factors are involved in the induction of the mouse interleukin 2 gene in T lymphocytes. EMBO J **8**, 465-473, 1989.
53. Shaw JP, Utz PJ, Durand DB, Toole JJ, Emmel EA, et al.: Identification of a putative regulator of early T cell activation genes. Science **241**, 202-205, 1988.
54. Rao A, Luo C, and Hogan PG: Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol **15**, 707-747, 1997.
55. Huang C, Mattjus P, Ma WY, Rincon M, Chen NY, et al.: Involvement of nuclear factor of activated T cells activation in UV response. Evidence from cell culture and transgenic mice. *J Biol Chem* **275**, 9143-9149, 2000.
56. Huang C, Li J, Costa M, Zhang Z, Leonard SS, et al.: Hydrogen Peroxide Mediates Activation of Nuclear Factor of Activated T Cells (NFAT) by Nickel Subsulfide. *Cancer Res* **61**, 8051-8057, 2001.
57. Huang C, Ding M, Li J, Leonard SS, Rojanasakul Y, et al.: Vanadium-induced nuclear factor of activated T cells through hydrogen peroxide. *J Biol Chem* **276**, 22397-22403, 2001.
58. Chow CW, Rincon M, Cavanagh J, Dickens M, and Davis RJ: Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway. *Science* **278**, 1638-1641, 1997.
59. Iniguez MA, Martinez-Martinez S, Punzon C, Redondo JM, and Fresno M: An essential role of the nuclear factor of activated T cells in the regulation of the expression of the cyclooxygenase-2 gene in human T lymphocytes. *J Biol Chem* **275**, 23627-23635, 2000.
60. Miyoshi C, and Ohshima N: Vascular endothelial growth factor (VEGF) expression regulates angiogenesis accompanying tumor growth in a peritoneal disseminated tumor model. *In Vivo* **15**, 233-238, 2001.
61. Ferrara N: Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney Int* **56**, 794-814, 1999.
62. Carmeliet P, and Jain RK: Angiogenesis in cancer and other diseases. *Nature* **407**, 249-257, 2000.
63. Xu L, Fukumura D, and Jain RK: Acidic extracellular pH induces vascular endothelial growth factor (VEGF) in human glioblastoma cells via ERK1/2 MAPK signaling pathway. *J Biol Chem* **277**, 11368-11374, 2002.
64. Ferrara N, and Davis-Smyth T: The biology of vascular endothelial growth factor. *Endocr Rev* **18**, 4-25, 1997.
65. Ferrara N, and Bunting S: Vascular endothelial growth factor, a specific regulator of angiogenesis. *Curr Opin Nephrol Hypertens* **5**, 35-44, 1996.
66. Folkman J: Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1**, 27-31, 1995.
67. Neufeld G, Cohen T, Gengrinovitch S, and Poltorak Z: Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* **13**, 9-22, 1999.
68. Angelov L, Salhia B, Roncari L, McMahon G, and Guha A: Inhibition of angiogenesis by blocking activation of the vascular endothelial growth factor receptor 2 leads to decreased growth of neurogenic sarcomas. *Cancer Res* **59**, 5536-5541, 1999.
69. Borgstrom P, Hillan KJ, Sriramarao P, and Ferrara N: Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endothelial growth factor neuralizing antibody: novel concepts of angiostatic therapy from intravital videomicroscopy. *Cancer Res* **56**, 4032-4039, 1996.
70. Brekken RA, Overholser JP, Stastny VA, Waltenberger J, Minna JD, et al.: Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. *Cancer Res* **60**, 5117-5124, 2000.
71. Cheng SY, Huang HJ, Nagane M, Ji XD, Wang D, et al.: Suppression of glioblastoma angiogenicity and tumorigenicity by inhibition of endogenous expression of vascular endothelial growth factor. *Proc Natl Acad Sci USA* **93**, 8502-8507, 1996.
72. Marconcini L, Marchio S, Morbidelli L, Cartocci E, Albini A, et al.: c-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis in vivo and in vitro. *Proc Natl Acad Sci USA* **96**, 9671-9676, 1999.
73. Damert A, Ikeda E, and Risau W: Activator-protein-1 binding potentiates the HIF-1-mediated hypoxia-induced transcriptional activation of

vascular-endothelial growth factor expression in C6 glioma cells. *Biochem J* **327**, 419-423, 1997.
74. Shibata A, Nagaja T, Imai T, Funahashi H, Nakao A, et al.: Inhibition of NFkappaB activity decreases the VEGF mRNA expression in MDA-MB-231 breast cancer cells. *Breast Cancer Res Treat* **73**, 237-243, 2002.
75. Okamoto T, Yamagishi S, Inagaki Y, Amano S, Koga K, et al.: Angiogenesis induced by advanced glycation end products and its prevention by cerivastatin. *FASEB J* **16**, 1928-1930, 2002.
76. Gao N, Shen L, Zhang Z, Leonard SS, He H, et al.: Arsenite induces HIF-1α and VEGF through PI3K, Akt and reactive oxygen species in DU145 human prostate carcinoma cells. *Mol Cell Biochem* **255**, 33-45, 2004.
77. Fang J, Xia C, Cao Z, Zheng JZ, Reed E, et al.: Apigenin inhibits VEGF and HIF-1 expression via PI3K/AKT/p70S6K1 and HDM2/p53 pathways. *FASEB J* **19**, 342-353, 2005.
78. Zhong XS, Zheng JZ, Reed E, and Jiang BH: SU5416 inhibited VEGF and HIF-1α expression through the PI3K/AKT/p70S6K1 signaling pathway. *Biochem Biophys Res Commun* **324**, 471-480, 2004.
79. Williams CS, Mann M, and Dubois RN: The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene* **18**, 7908-7916, 1999.
80. Nathan C: Points of control in inflammation. *Nature* **420**, 846-852, 2002.
81. Tsujii M, and Dubois RN: Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* **83**, 493-501, 1995.
82. Tsujii M, Kawano S, and Dubois RN: Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci U S A* **94**, 3336-3340, 1997.
83. Liu XH, Kirschenbaum A, Yao S, Lee R, Holland JF, et al.: Inhibition of cyclooxygenase-2 suppresses angiogenesis and the growth of prostate cancer in vivo. *J Urol* **164**, 820-825, 2000.
84. Prescott SM, and Fitzpatrick FA: Cyclooxygenase-2 and carcinogenesis. *Biochim Biophys Acta* **1470**, M69-78, 2000.
85. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, et al.: Upregulation of cyclooxygenase-2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* **107**, 1183-1188, 1994.
86. Ristimaki A, Honkanen N, Jankala H, Sipponen P, and Harkonen M: Expression of cyclooxygenase 2 in human gastric cancer. *Cancer Res* **57**, 1276-1280, 1997.
87. Hwang D, Scollard D, Byrne J, and Levine E: Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. *J Natl Cancer Inst* **90**, 455-460, 1998.
88. Okami J, Yamamoto H, Fujiwara Y, Tsujie M, Kondo M, et al.: Overexpression of cyclooxygenase-2 in carcinoma of the pancreas. *Clinical Cancer Res* **5**, 2018-2024, 1999.
89. Hida T, Yatabe Y, Achiwa H, Muramatsu H, Kozaki K, et al.: Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Res* **58**, 3761-3764, 1998.
90. Guo YS, Hellmich MR, Wen XD, and Townsend CM, Jr: Activator protein-1 transcription factor mediates bombesin-stimulated cyclooxygenase-2 expression in intestinal epithelial cells. *J Biol Chem* **276**, 22941-22947, 2001.
91. Subbaramaiah K, Lin DT, Hart JC, and Dannenberg AJ: Peroxisome proliferator-activated receptor gamma ligands suppress the transcriptional activation of cyclooxygenase-2. Evidence for involvement of activator protein-1 and CREB-binding protein/p300. *J Biol Chem* **276**, 12440-12448, 2002.
92. Subbaramaiah K, Norton L, Gerald W, and Dannenberg AJ: Cyclooxygenase-2 is overexpressed in HER-2/neu-positive breast cancer: evidence for involvement of AP-1 and PEA3. *J Biol Chem* **277**, 18649-18857, 2002.
93. de Gregorio R, Iniguez MA, Fresno M, and Alemany S: Cot kinase induces cyclooxygenase-2 expression in T cells through activation of nuclear factor of activated T cells. *J Biol Chem* **276**, 27003-27009, 2001.
94. Sugimoto T, Haneda M, Sawano H, Isshiki K, Maeda S, et al.: Endothelin-1 induces cyclooxygenase-2 expression via nuclear factor of activated T-cell transcription factor in glomerular mesangial cells. *J Am Soc Nephro*l **12**, 1359-1368, 2001.
95. Chun KS, Kim SH, Song YS, and Surh YJ: Celecoxib inhibits phorbol ester-induced expression of COX-2 and activation of AP-1 and p38 MAP kinase in mouse skin. *Carcinogenesis* **25**, 713-722, 2004.
96. Stoner GD, and Gupta A: Etiology and chemoprevention of esophageal squamous cell carcinoma. *Carcinogenesis* **22**, 1737-1746, 2001.
97. Hecht SS, Huang C, Stoner GD, Li J, Kenney PMJ, et al.:Identification of cyanidin glycosides as constituents of freeze-dried black raspberries which inhibit anti-benzo[a]pyrene-7,8-diol-9,10-epoxide induced NFκB and AP-1 activity. *Carcimogenesis,* in press.