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Cyanidin-3-glucoside suppresses B[a]PDE-induced cyclooxygenase-2 expression by directly inhibiting Fyn kinase activity

Tae-Gyu Lim [a], Jung Yeon Kwon [a,b], Jiyoung Kim [c,d,e], Nu Ry Song [b], Kyung Mi Lee [a,b], Yong-Seok Heo [f], Hyong Joo Lee [b,c,d,e], Ki Won Lee [a,b,e,*]

[a] Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Republic of Korea
[b] Food Science and Biotechnology Program, Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea
[c] WCU Biomodulation Program, Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea
[d] Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea
[e] Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Republic of Korea
[f] Department of Chemistry, Konkuk University, Seoul 143-701, Republic of Korea

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ABSTRACT

Benzo[a]pyrene-7,8-diol-9,10-epoxide (B[a]PDE) is a well-known carcinogen that is associated with skin cancer. Abnormal expression of cyclooxygenase-2 (COX-2) is an important mediator in inflammation and tumor promotion. We investigated the inhibitory effect of cyanidin-3-glucoside (C3G), an anthocyanin present in fruits, on B[a]PDE-induced COX-2 expression in mouse epidermal JB6 P+ cells. Pretreatment with C3G resulted in the reduction of B[a]PDE-induced expression of COX-2 and COX-2 promoter activity. The activation of activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) induced by B[a]PDE was also attenuated by C3G. C3G attenuated the B[a]PDE-induced phosphorylation of MEK, MKK4, Akt, and mitogen-activated protein kinases (MAPKs), but no effect on the phosphorylation of the upstream MAPK regulator Fyn. However, kinase assays demonstrated that C3G suppressed Fyn kinase activity and C3G directly binds Fyn kinase noncompetitively with ATP. By using PP2, a pharmacological inhibitor for SFKs, we showed that Fyn kinase regulates B[a]PDE-induced COX-2 expression by activating MAPKs, AP-1 and NF-κB. These results suggest that C3G suppresses B[a]PDE-induced COX-2 expression mainly by blocking the activation of the Fyn signaling pathway, which may contribute to its chemopreventive potential.

1. Introduction

Benzo[a]pyrene (B[a]P) is a well-characterized human carcinogen [1–4], and is a ubiquitous environmental contaminant generated from cigarette smoke, charred foods, vehicle exhaust emissions, and industrial processes [1–3]. B[a]P metabolism by cytochrome P450 produces benzo[a]pyrene-7,8-diol-9,10-epoxide (B[a]PDE), which leads to smoking-associated cancers, including skin and lung cancers [4,5]. Although the exact molecular mechanisms are elusive, B[a]PDE might alter cellular events regulating proliferation, apoptosis, and inflammation, and initiate or promote cancers [1,4,6].

Cyclooxygenase (COX) is a rate-limiting enzyme for the conversion of arachidonic acids to prostaglandins. COX has two main isoforms: COX-1 and COX-2. Whereas COX-1 is a constitutively expressed, COX-2 is an inducible isoform [7]. COX-2 is one of the key enzymes which mediating many inflammatory responses, and is induced by tumor promoters, growth factors, and cytokines. In several epithelial cancers, COX-2 is upregulated [8,9]. B[a]PDE leads to cancer by upregulating COX-2 [10]. Numerous lines of evidence indicate that COX-2 plays an important role in development of cancer [11–13]. Overexpression of COX-2 induces cancer cell proliferation [14], inhibits apoptosis [15], promotes angiogenesis [13,16], and increases metastatic potential [12]. Transgenic mice with COX-2 overexpression in basal epidermal cells exhibit a preneoplastic skin phenotype [17,18]. Previous study demonstrated COX-2 is involved in B[a]PDE-induced carcinogenesis through activation of MAPKs/AP-1 and IKKβ/NF-κB, as shown in mouse epidermal C141 cells [10].

The Src family kinases (SFKs) are non receptor tyrosine kinases which consist of nine highly similar tyrosine kinases. SFKs are interacted with transmembrane receptors such as growth factor receptors, hormone receptors, and cytokine receptors. Activated SFKs are related to many cellular events, including differentiation, proliferation, migration, and survival [19–21]. Fyn is a ubiquitously expressed member of the Src family of nonreceptor tyrosine

* Corresponding author at: Food Science and Biotechnology Program, Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea. Tel.: +82 2 880 4861; fax: +82 2 873 5095.
E-mail address: kiwon@snu.ac.kr (K.W. Lee).

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kinases that transmit signals from various cell surface receptors to the cytoplasm [22]. Fyn acts as an oncogene in skin cancer development [23,24]. For example, Fyn mediates epidermal growth factor-induced neoplastic transformation of JB6 P+ mouse skin epidermal cells [25]. COX-2 expression increases as a result of UV or TNF-α activation of Fyn kinase, which regulates MAPKs [23,24]. Thus, Fyn is a good target for chemoprevention of many types of cancer.

Several phytochemicals are reported to act not only as antioxidants but also as small molecules which inhibit the cellular signaling pathways leading to cancer [23,24,26]. One such phytochemical, cyanidin-3-glucoside (C3G) is a naturally occurring polyphenolic anthocyanin widely distributed in fruits, vegetables, and pigmented cereals [27]. C3G scavenge free radicals [28], suppresses inflammation [29], decreases myocardium damage [30] and protect against endothelial dysfunction [31]. C3G might also help prevent cancer as other anthocyanins do [32].

Here we report that C3G inhibits the B[a]PDE-induced COX-2 expression by directly binding Fyn kinase in JB6 P+ mouse skin epidermal cells. We provide evidence that B[a]PDE can stimulate Fyn, which is involved in the B[a]PDE-induced COX-2 expression. In this respect C3G is the Fyn inhibitor that is emerging for the treatment of skin cancer.

2. Materials and methods

2.1. Materials

Chemicals were purchased from the following sources: Cyanidin-3-glucoside (C3G), ChromaDex (Irvine, CA); benzo[a]pyrene-7,8-diol-9,10-epoxide, (B[a]PDE), the Chemical Carcinogen Reference Standards Repository at the National Cancer Institute; 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), Calbiochem (San Diego, CA); Eagle's minimum essential medium (MEM) and G418 sulfate, Mediatech (Herndon, VA); fetal bovine serum (FBS) and penicillin/streptomycin, GIBCO (Grand Island, NY). Primary antibodies recognizing phosphorylated p38 (Tyr^{180}/Tyr^{182}), total p38, phosphorylated MEK ($Ser^{217/221}$), total MEK, phosphorylated SEK1/MKK4 (MKK4, Ser^{257}/Thr^{261}), phosphorylated c-Jun N-terminal kinase (JNK) (Thr^{183}/Tyr^{185}), total JNK, phosphorylated Akt (Ser^{473}) and total Akt were purchased from Cell Signaling Biotechnology (Beverly, MA). Primary antibodies recognizing phosphorylated ERK (Tyr^{204}), total ERK and total MKK4 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for COX-2 and β-actin were purchased from Cayman Chemical (Ann Arbor, MI) and Sigma (St. Louis, MO), respectively. Secondary antibodies recognizing mouse and rabbit IgG and conjugated to horseradish peroxidase were purchased from Zymed (San Francisco, CA).

2.2. Cell culture

JB6 P+ mouse epidermal cells were purchased from American Type Culture Collection (Manassas, VA). The JB6 mouse epidermal cell model is well-established cell system for studying the mechanism of tumor promotion. This cell system includes tumor promotion-sensitive (P^+) and tumor promotion-resistant (P^-) cells [33]. JB6 P+ cells were cultured in MEM supplemented with 5% FBS and 0.1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Western blot analysis

JB6 P+ cells were grown for 48 h and cultured with MEM containing 0.1% FBS for 24 h to eliminate FBS-mediated activation of protein kinases. Cells were treated with various concentrations of C3G or PP2 for 1 h, and then treated with 2 μM of B[a]PDE. Total cell lysates were prepared and equal amounts of protein were separated electrophoretically in 10% SDS–polyacrylamide gels and transferred to Immobilon P membranes (Millipore, Billerica, MA). Membranes were incubated with primary antibodies at 4 °C overnight, then with horseradish peroxidase-conjugated secondary antibodies and developed using enhanced chemiluminescence (ECL) detection kit (GE Healthcare, Pittsburgh, PA).

2.4. Luciferase assay for COX-2, AP-1 and NF-κB transactivation

JB6 P+ cells were stably transfected with COX-2, AP-1, or NF-κB luciferase reporter plasmid containing G418 resistance gene and maintained in 5% FBS-MEM containing 200 μg/ml of G418. Selected cells carrying each reporter plasmid were seeded in MEM containing 0.1% FBS for 24 h, then treated with C3G at 5, 10 or 20 μM or PP2 at 2.5, 5 or 10 μM for 1 h before treatment with 2 μM B[a]PDE for 12 h. Cells were harvested using lysis buffer (0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM DTT and 2 mM EDTA). Luciferase activity was measured using a luminometer (Luminoskan Ascent; Thermo Electron, Helsinki, Finland).

2.5. Fyn kinase assay

Direct Fyn kinase activity assays were conducted using active recombinant Fyn enzyme (Millipore, Bedford, MA) by measuring the ability of Fyn kinase to transfer the radiolabeled phosphate from [γ-^{32}P]ATP to its substrate Src (Millipore, Bedford, MA) in accordance with the manufacturer's instructions. In brief, active Fyn protein and indicated concentrations of C3G were incubated at 30 °C for 10 min in assay buffer (20 mM MOPS (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate and 1 mM DTT). Src substrate peptide was added to the reaction mixture to reach a final concentration of 250 μM, then incubated at 30 °C for 10 min with of diluted [γ-^{32}P]ATP solution in a magnesium acetate–ATP cocktail buffer (Upstate Biotechnology Inc., Lake Placid, NY). Aliquots (15 μl) were transferred onto p81 paper and washed three times with 0.75% phosphoric acid for 5 min and then with acetone for 5 min. The incorporation of radiolabeled phosphate was determined using a scintillation counter.

2.6. Fyn immunoprecipitation using sepharose 4B

To generate a C3G–sepharose 4B complex, sepharose 4B freeze-dried powder (0.3 g) was activated in 30 ml of 1 mM HCl. C3G (1–2 mg) was mixed with activated sepharose 4B in coupling buffer (0.1 M $NaHCO_3$ (pH 8.3) and 0.5 M NaCl), and rotated at 4 °C overnight, transferred to 0.1 M Tris–HCl buffer (pH 8.0) and rotated again at 4 °C overnight, then washed twice with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl. Active Fyn protein (200 ng) was mixed with C3G–sepharose 4B beads or uncomplexed sepharose 4B beads (100 μL, 50% slurry) and various concentrations of ATP in reaction buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 mg/ml bovine serum albumin, 0.02 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg protease inhibitor mixture] and rotated overnight at 4 °C. The beads were then washed five times with washing buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 0.02 mM PMSF]. Immunoprecipitated Fyn bound to the beads was analyzed by Western blot.

2.7. Molecular modeling

The homology model structure of full-length Fyn was kindly provided by Dr. Dubravko Jelic at GlaxoSmithKline [34] and

employed in docking and structure analysis using Insight II software (Accelrys, CA).

2.8. Statistical analysis

All experiments were repeated at least three times. Results are expressed as means ± SD, and the significant differences were determined using one-way ANOVA followed by Duncan's Multiple Range test. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cyanidin-3-glucoside suppresses B[a]PDE-induced upregulation of COX-2

Previous studies have shown that B[a]PDE-induced skin cancer involves abnormal induction of COX-2 [10]. To investigate the chemopreventive activity of C3G, we examined the effect of C3G on B[a]PDE-induced COX-2 expression in mouse epidermal JB6 P+ cells. Treatment with B[a]PDE at 2 μM for 12 h induced the expression of COX-2. Pre-treatment with C3G at 5–20 μM for 1 h significantly reduced the B[a]PDE-induced COX-2 expression in a dose-dependent manner (Fig. 1A). To confirm that C3G regulates B[a]PDE-COX-2 expression, we examined the effect of C3G on COX-2 transcription using a reporter gene assay. Treatment with C3G at 5–20 μM for 1 h significantly inhibited B[a]PDE-induced COX-2 promoter activity by 90–100% (Fig. 1B). These results indicated that C3G suppresses B[a]PDE-induced up-regulation of COX-2 expression in JB6 P+ cells.

3.2. Cyanidin-3-glucoside suppresses B[a]PDE-induced activation of AP-1 and NF-κB

Up-regulation of COX-2 expression by B[a]PDE results from the activation of AP-1 and NF-κB [10]. To determine whether the suppression of COX-2 expression by C3G involves the inhibition of AP-1 and NF-κB transactivation, we measured AP-1 and NF-κB transactivation using JB6 P+ cells stably transfected with a luciferase reporter plasmid bearing AP-1 and NF-κB. Treatment with B[a]PDE at 2 μM for 12 h significantly induced transcriptional activation of AP-1 and NF-κB (normalized to 100%). Pretreatment with C3G at 5–20 μM for 1 h significantly suppressed B[a]PDE-induced transcriptional activation of both AP-1 and NF-κB in a dose-dependent manner (Fig. 2A and B). These results indicated that the C3G suppresses AP-1 and NF-κB transactivation, which might contribute to the anti-inflammatory activity of C3G.

3.3. Cyanidin-3-glucoside suppresses B[a]PDE-induced phosphorylation of ERK, p38 and JNK, and their upstream kinases, MEK, MKK4 and Akt

B[a]PDE-induced AP-1 and NF-κB transcriptional activity involves MAPKs, including ERK, p38, and JNK [35–37]. Previous studies have demonstrated that JNK is regulated by PI3K/Akt signaling in B[a]PDE model [38]. To examine the effect of C3G on B[a]PDE-induced activation of MAPKs and their upstream kinases, we measured the influence of C3G on B[a]PDE-induced phosphorylation of MEK/ERK, MKK4/p38, and Akt/JNK signaling pathways. Pretreatment with C3G attenuated the B[a]PDE-induced phosphorylation of MEK/ERK, MKK4/p38, and Akt/JNK in a dose-dependent manner (Fig. 3A–C). These results indicated the C3G-induced inhibition of MAPKs, which may lead to attenuated B[a]PDE-induced COX-2 expression.

3.4. Cyanidin-3-glucoside inhibits Fyn kinase noncompetitively with ATP

Previous studies demonstrated that phosphorylation of MAPKs in the development of skin cancer results from Fyn activity [23]. Therefore, we examined the effect of C3G on B[a]PDE-induced phosphorylation of Fyn. We found that C3G did not inhibit the

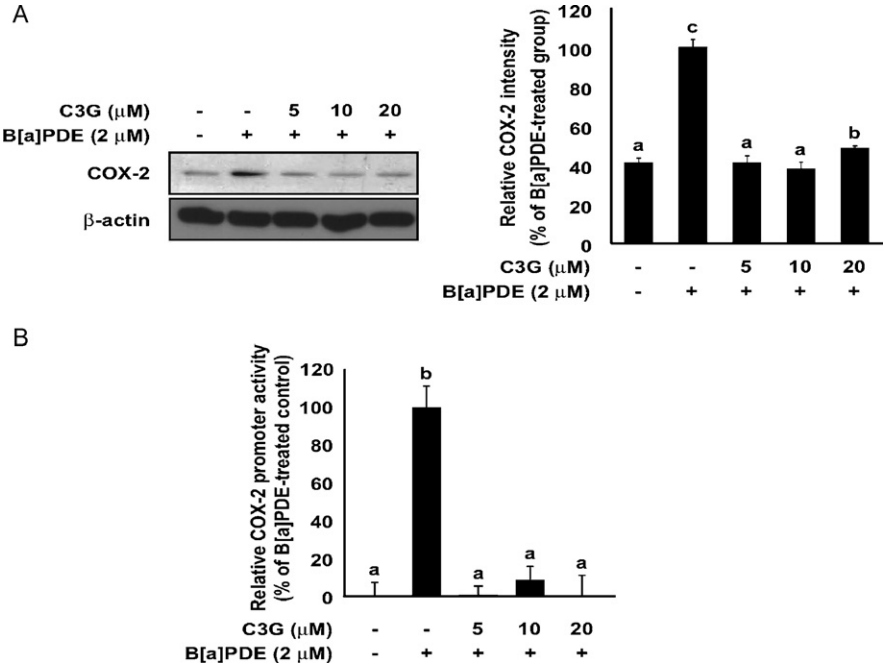

Fig. 1. Cyanidin-3-glucoside (C3G) decreases B[a]PDE-induced COX-2 expression in JB6 P+ cells. After pretreatment of C3G for 1 h, JB6 P+ cells were exposed to B[a]PDE (2 μM) for additional 12 h. (A) The protein levels of COX-2 and β-actin were determined by Western blot. Data are representative of three independent experiments that gave similar results. Each band was quantified by densitometry. (B) COX-2 promoter activity was measured using JB6 P+ cells transfected with COX-2-luciferase reporter plasmid. Data were normalized to promoter activity of B[a]PDE-treated JB6 P+ cells (100%). Data are presented as means ± SE of three independent experiments. Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed using ANOVA followed by Duncan's multiple range test.

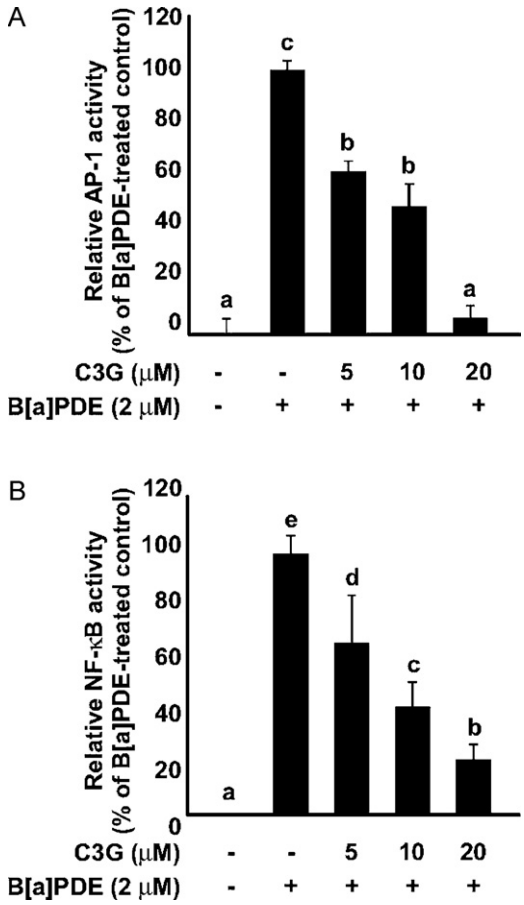

Fig. 2. Cyanidin-3-glucoside (C3G) decreases B[a]PDE-induced transcriptional activation of AP-1 and NF-κB. After pretreatment of C3G for 1 h, JB6 P+ cells were exposed to B[a]PDE (2 μM) for additional 12 h. Transcriptional activity of AP-1 (A) or NF-κB (B) was measured using JB6 P+ cells transfected with AP-1 or NF-κB luciferase reporter plasmid. Data were normalized to the transcriptional activity induced by B[a]PDE in JB6 P+ cells (100%). Data are presented as means ± SE of three independent experiments. Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed using ANOVA followed by Duncan's multiple range test.

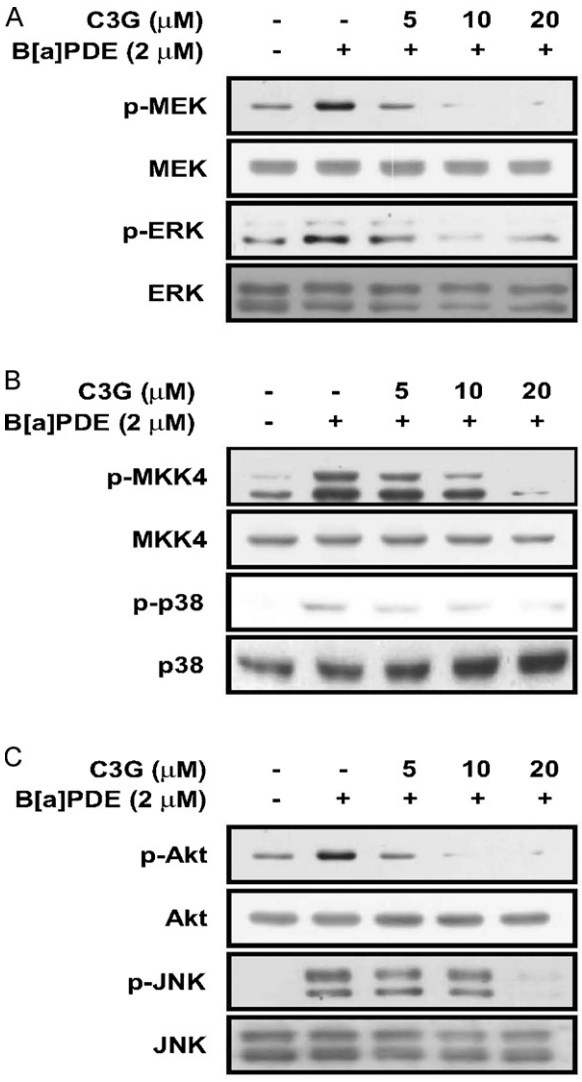

Fig. 3. Cyanidin-3-glucoside (C3G) reduces B[a]PDE-induced phosphorylation of ERK, p38, and JNK, and their upstream kinases, MEK, MKK4, and Akt. After pretreatment of C3G for 1 h, JB6 P+ cells were exposed to B[a]PDE (2 μM) for an additional 30 min (p-MEK, p-MKK4, and p-Akt) or for 3.5 h (p-ERK, p-p38, and p-JNK). The protein levels of phosphorylated- and total-MEK and ERK (A), MKK4 and p38 (B), and Akt and JNK (C) were determined by Western blot. Presented data are representative of three independent experiments that gave similar results.

B[a]PDE-induced phosphorylation of Fyn (Fig. 4A). However, because C3G strongly blocked B[a]PDE-induced MAPKs phosphorylation, which is regulated by Fyn, we hypothesized that C3G directly inhibits Fyn kinase activity. Thus, we measured the effect of C3G on Fyn kinase activity. The activity of Fyn was decreased by C3G treatment in a dose dependent manner (Fig. 4B). To confirm that the inhibition of Fyn activities by C3G is caused by a direct interaction, we performed pull-down assays. Fyn was pulled down by C3G–sepharose 4B (Fig. 4C, lane 3), but not by sepharose 4B alone (Fig. 4C, lane 2). Nevertheless, the addition of ATP at various concentrations did not alter the binding affinity of Fyn to C3G–sepharose 4B (Fig. 4C, lanes 4–6), indicating that C3G inhibits Fyn kinase activity noncompetitively with ATP. These results indicate that C3G can directly bind with the Fyn and subsequently inhibit the activation of MAPKs.

3.5. Fyn mediates B[a]PDE-induced expression of COX-2 and activation of AP-1 and NF-κB

Because C3G directly inhibits Fyn activity, we hypothesized that inhibition of Fyn is responsible for its effect on COX-2 expression. To clarify whether Fyn is involved in the B[a]PDE-induced COX-2 expression, we used a pharmacological SFKs inhibitor, 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine (PP2). Pretreatment of the cells with PP2 at 2.5–10 μM reduced B[a]PDE-induced COX-2 expression (Fig. 5A). Furthermore, PP2 also suppressed B[a]PDE-mediated COX-2 promoter activation (Fig. 5B). Furthermore, PP2 significantly reduced B[a]PDE-induced transactivation of AP-1 and NF-κB in JB6 P+ cells, which correlated with its inhibition of COX-2 expression (Fig. 5C and D).

3.6. Fyn modulates B[a]PDE-induced phosphorylation of ERK, p38 and JNK, and their upstream kinases, MEK, MKK4 and Akt

To determine whether Fyn regulate the B[a]PDE-induced phosphorylation of MAPKs and their upstream kinases, we examined the phosphorylation of MAPKs and their upstream kinases using the PP2. PP2 reduced the B[a]PDE-induced phosphorylation of MEK and ERK (Fig. 6A), MKK4 and p38 (Fig. 6B), and Akt and JNK (Fig. 6C). Overall, these data suggest that Fyn kinase is involved in B[a]PDE-induced activation of ERK, p38 and JNK, through suppression of their upstream kinases, MEK, MKK4 and Akt, respectively.

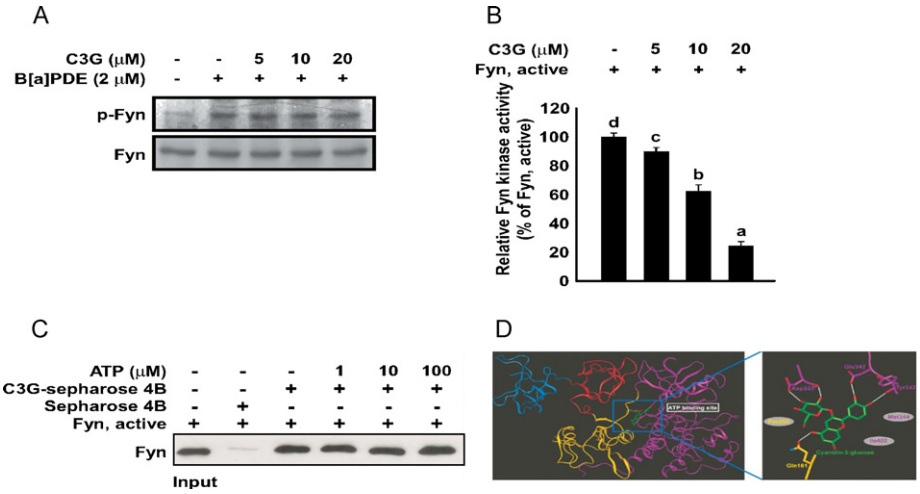

Fig. 4. Cyanidin-3-glucoside (C3G) inhibits the activity of Fyn kinase. (A) After pretreatment of C3G for 1 h, JB6 P+ cells were exposed to B[a]PDE (2 μM) for an additional 5 min. The protein levels of phosphorylated- and total-Fyn were determined by Western blot. (B) Active Fyn was incubated with C3G at the indicated concentrations for 10 min at 30 °C, then with substrate peptide Src and [γ-^{32}P]ATP for an additional 10 min at 30 °C. Incorporated radiolabeled phosphate was normalized to that of Fyn without C3G (100%). Data are presented as means ± SE of three independent experiments. Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed using ANOVA followed by Duncan's multiple range test. (C) Active Fyn (10 ng) and different concentrations (1, 10, or 100 μM) of ATP were incubated with C3G–sepharose 4B or sepharose 4B (as a negative control). Immunoprecipitated Fyn was detected by Western blot. Presented data is representative of three independent experiments that gave similar results. (D) Hypothetical model of Fyn kinase in complex with C3G. Different domains of Fyn kinase are presented in different colors: the N-terminal domain in blue, the kinase domain in violet, and two Src homology domains, SH2 and SH3, in yellow and red, respectively. Cyanidin-3-glucose (atomic color) binds to the putative allosteric site between the SH2 domain and the kinase domain. The ATP binding site is indicated by a white rectangle. In the close-up view of the inhibitor interaction in the allosteric site, the hydrogen bonds are indicated by white lines and the hydrophobic interactions by gray ellipses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

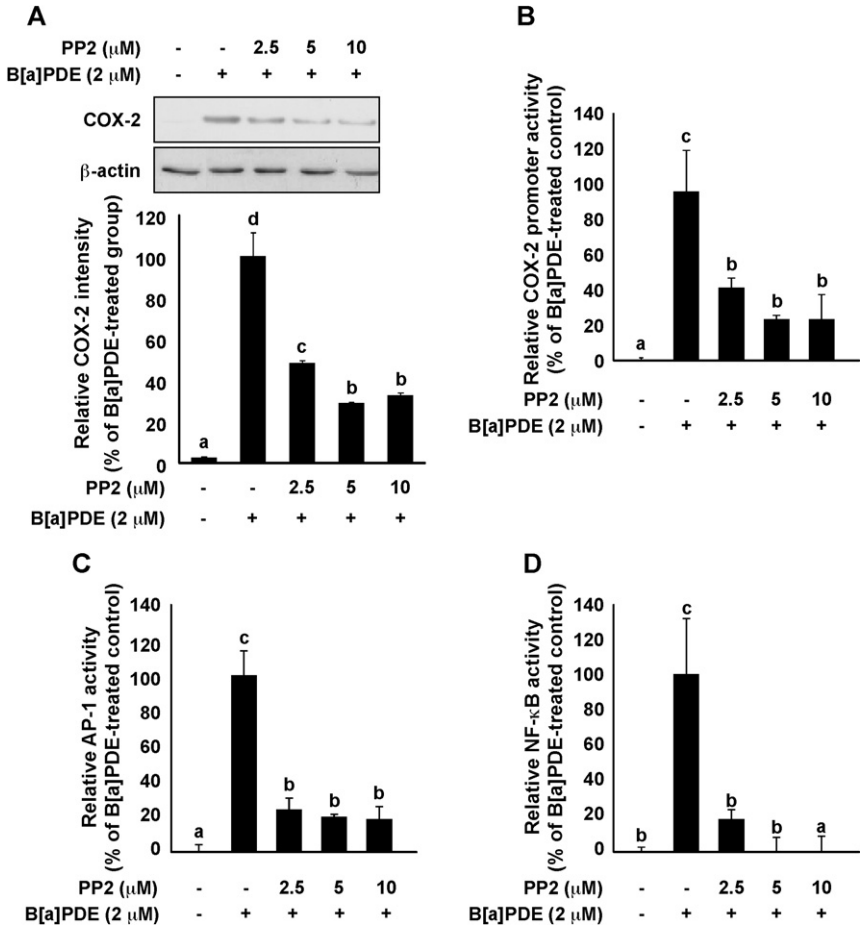

Fig. 5. Pharmacological inhibition of Fyn reduces B[a]PDE-induced transcriptional activation of AP-1 and NF-κB and COX-2. After pretreatment of PP2 for 1 h, JB6 P+ cells were exposed to B[a]PDE (2 μM) for additional 12 h. (A) The protein levels of COX-2 and β-actin were determined by Western blot. Each band was quantified by densitometry. The promoter activity of COX-2 (B) was measured using JB6 P+ cells transfected with COX-2 luciferase reporter plasmid, relatively The promoter activity of transcriptional activity of AP-1 (C) or NF-κB (D) was measured using JB6 P+ cells transfected with AP-1 or NF-κB luciferase reporter plasmid, relatively. Data are representative of three independent experiments that gave similar results. Data were normalized to the luciferase activity induced by B[a]PDE in JB6 P+ cells (100%). Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed using ANOVA followed by Duncan's multiple range test.

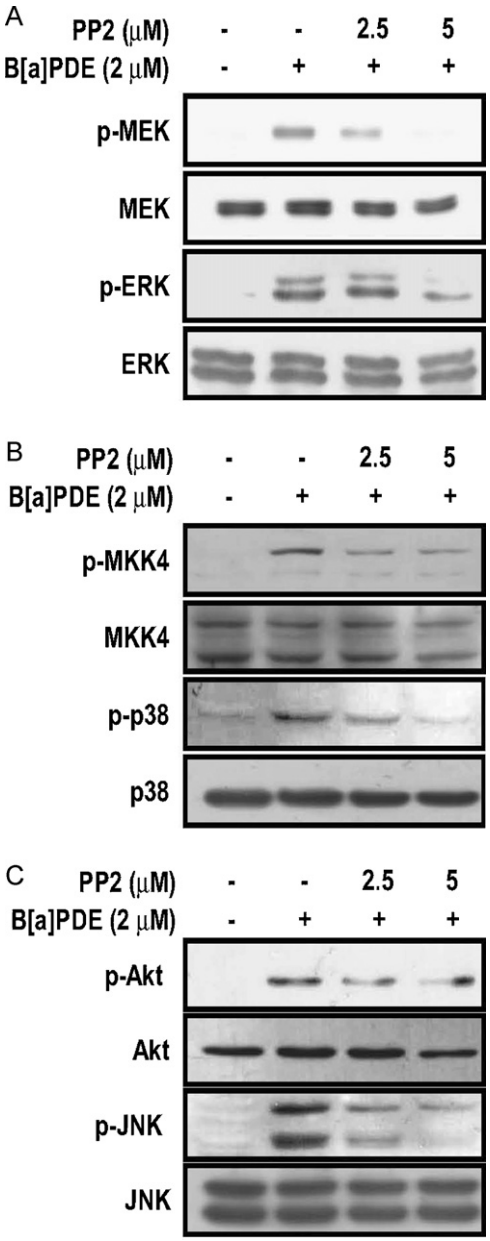

Fig. 6. Pharmacological inhibition of Fyn reduces B[a]PDE-induced phosphorylation of ERK, p38, and JNK and their upstream kinases, MEK, MKK4 and Akt. After pretreatment of PP2 for 1 h, JB6 P+ cells were exposed to B[a]PDE (2 μM) for additional 30 min (MEK, MKK4, and Akt) or 3.5 hr (ERK, p38 and JNK). The protein levels of phosphorylated- and total-MEK and ERK (A), MKK4 and p38 (B), Akt and JNK (C) were determined by Western blot. Presented data are representative of three independent experiments that gave similar results.

4. Discussion

Epidemiological studies have shown that daily intake of fruits and vegetables are associated with a reduced risk of cancer in humans [39,40]. Recently, our group has demonstrated that chemopreventive effect of phytochemicals is caused by not only antioxidant effect but also regulator of cellular signaling [23,24]. It is reported that the C3G, member of the anthocyanidin family, has chemopreventive effect by regulating many cellular signaling [32,41]. Many papers have shown the beneficial effect of cyanidin or its glycosides on many cancer models [32,41,42]. Nevertheless, it has never been reported that the molecular target of C3G in B[a]PDE-induced COX-2 expression model. In this study, we found that C3G inhibits B[a]PDE-induced COX-2 expression by directly binding with Fyn kinase, inhibiting its activity, and subsequently suppressing B[a]PDE-induced MAPKs and transcription factors in JB6 P+ cells. Moreover, we provide the first direct evidence that B[a]PDE leads to the activation of Fyn, which thereby plays a critical role in B[a]PDE-induced COX-2 expression.

Previous research reported that black raspberry shows the inhibitory effect on B[a]PDE-induced COX-2 expression by suppressing inflammatory signaling pathways such as PI3K and ERK [38]. Furthermore, they found that C3G is a major potent constituent of black raspberry in B[a]PDE-induced COX-2 expression model [43]. Abnormal COX-2 expression is closely related to skin carcinogenesis. Topical application of celecoxib, selective COX-2 inhibitor, inhibits UVB-induced skin inflammation and tumor formation [44]. Furthermore, previous study reported that COX-2 has a important role in epidermal cell homeostasis by using COX-2 deficiency mice [45]. The expression of COX-2 was correlated with signal pathway activation. MAPKs are representative COX-2 regulating enzymes. Abnormal activation of MAPK induces transcriptional activation of AP-1 and NF-κB, and subsequently COX-2 expression is upregulated. Thus, targeting COX-2 signaling represents a promising strategy to reduce tumors. In this study, we determined that C3G suppressed B[a]PDE-induced COX-2 expression via inhibition of MAPKs phosphorylation. Accumulating evidences have represented the critical roles of SFKs on many various type of cancer [46–48]. Moreover, it is well known the correlation between Fyn kinase activation and MAPKs phosphorylation. Previously our study demonstrated that Fyn has a critical role in tumor necrosis factor (TNF)-α-induced COX-2 expression as a upstream regulator of MAPKKs and one of the anthocyanines, delphinidin suppresses TNF-α-induced COX-2 expression by directly inhibiting Fyn kinase activity and subsequently suppressing downstream signaling such as MAPK [49]. Additionally, recently our group reported that myricetin, one of the major flavonoids found in various foods directly reduces Fyn kinase activity and subsequently MAPK phosphorylation and COX-2 expression. Therefore, we focused on the critical role of C3G in B[a]PDE-induced COX-2 expression by regulating MAPKs. Our results show that C3G significantly inhibited Fyn activity through direct binding with Fyn.

To investigate the molecular basis of the Fyn kinase inhibition by C3G, we have carried out homology modeling using structure of the full-length Fyn [34]. Fyn includes two Src homology (SH) domains (SH2 and SH3) and a highly conserved catalytic domain including an adenosine triphosphate (ATP)-binding site among other domains [50]. Since we found that C3G does not compete with ATP, we excluded the ATP binding site in the hypothetical model of Fyn kinase in complex with C3G. Interestingly, C3G can fit in the pocket opposite and adjacent to the ATP binding site (Fig. 4D) [34]. Previous study revealed that cyanidin inhibits MKK4 in ATP-competitive manner and the hydroxyl group at 3-position is thought to be important for the interaction by a hydrogen bond [51]. However, the glycosylation at 3-position of C3G would eliminate the interaction and cause steric collisions with the surrounding residues, and thereby reduce the binding affinity of C3G to the ATP binding site of MKK4. Although both C3G and cyanidin are working as ATP noncompetitive inhibitors, C3G binding site of Fyn kinase is opposite to the putative cyanidin binding site of MEK1 and Raf-1. And MEK1 and Raf-1 do not contain SH2 domain, which is thought to be involved in the C3G interaction. In other words, the surface environment of the C3G binding site of Fyn kinase is far from those of MEK1 and Raf-1, providing Fyn selectivity to C3G. The flavonoid moiety of C3G can hydrogen bond with Glu^{342} and Tyr^{343} in the catalytic kinase domain and Gln^{161} in the SH2 domain. C3G can also hydrophobically interact with Ile^{402} and Met^{344} of the kinase domain.

The sugar moiety of C3G can make hydrogen bond with Asp^{323} in the kinase domain and hydrophobically interact with Pro^{250} in the SH2 domain. The interaction between the SH2 domain and C3G could influence protein–protein interactions via SH2 domain. The interaction of C3G with the Fyn kinase domain may distort the ATP or substrate binding site, and thus reduce the catalytic activity of Fyn kinase allosterically. Future investigations using X-ray crystallography would elucidate the exact binding modes of C3G to Fyn kinase.

Among of SFK members, Src, Fyn, and Yes, are highly expressed in various tissues [21]. After activation of Fyn, many inflammatory signaling pathways are subsequently activated. From these processes, the carcinogenesis is progressed. Here, we provide evidence that Fyn activation plays an important role in the B[a]PDE-induced expression of COX-2.

Using pharmacological inhibitor of SFKs, PP2, we found that pretreatment of PP2 suppressed the phosphorylation of MEK/ERK, MKK4/p38, and Akt/JNK signaling pathways as well as COX-2 expression. From these results, we clarified the critical role of Fyn in B[a]PDE-induced COX-2 expression as an upstream MAPK signaling pathways. In our knowledge, this notion is the first suggestion which shows involvement of Fyn in B[a]PDE-induced COX-2 expression. Also, similar with our previous study [49], we suggested that suppressing Fyn kinase activity can be a potential chemopreventive agents for B[a]PDE-induced carcinogenesis model.

In summary, our results reveal a critical role of Fyn in B[a]PDE-induced COX-2 expression, and that C3G suppresses B[a]PDE-induced COX-2 expression by directly suppressing Fyn kinase activation. Based on our findings, C3G shows great potential as a novel chemopreventive agent and may be useful in the treatment of B[a]PDE-associated tumorigenesis.

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