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

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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

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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