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Fyn kinase is a direct molecular target of delphinidin for the inhibition of cyclooxygenase-2 expression induced by tumor necrosis factor-α

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ABSTRACT

Tumor necrosis factor (TNF)-α-mediated cyclooxygenase (COX)-2 expression plays key roles in inflammation and tumorigenesis, particularly skin carcinogenesis, and hence targeting the TNF-α-mediated signaling pathway might be a promising strategy for developing chemopreventive agents against skin cancer and other skin disorders. Here we report that Fyn kinase – one of the members of the nonreceptor protein tyrosine kinase family – is involved in TNF-α-induced COX-2 expression, and that delphinidin – a major anthocyanidin present in red wine and berries – inhibits these effects by directly inhibiting Fyn kinase activity. Delphinidin strongly inhibited TNF-α-induced COX-2 expression in JB6 P+ mouse epidermal (JB6 P+) cells, whereas two other major phenolic compounds (resveratrol and gallic acid) did not exert significant inhibitory effects. Delphinidin inhibited the TNF-α-induced phosphorylations of JNK, p38 MAP kinase, Akt, p90RSK, MSK1, and ERK, and subsequently blocked the activation of the eukaryotic transcription factors AP-1 and NF-κB. Kinase and pull-down assay data revealed that delphinidin inhibited Fyn kinase activity and directly bound with Fyn kinase noncompetitively with ATP. By using PP2 (a commercial inhibitor of Fyn kinase) and siRNA-Fyn, we confirmed that Fyn kinase is involved in TNF-α-induced COX-2 expression mainly by down-regulating JNK in JB6 P+ cells. Together these findings suggest that the targeted inhibition of Fyn kinase activity and COX-2 expression by delphinidin contributes to the chemopreventive potential of red wine and berries.

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Abbreviations: AP-1, activator protein-1; COX-2, cyclooxygenase-2; CREB, cyclic AMP response element binding protein; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; JB6 P+, JB6 P+ mouse epidermal; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MSK1, mitogen- and stress- activated protein kinase 1; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; p90RSK, p90 ribosomal S6 kinase; SFK, Src family kinase; TNF-α, tumor necrosis factor-α; TNFR, TNF receptor; PMA, phorbol 12-myristate 13-acetate.

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1. Introduction

Tumor necrosis factor (TNF)-α is a key proinflammatory cytokine that acts as a cellular mediator provoking inflammation, proliferation, tumorigenesis, and even apoptosis. TNF-α binds with TNF receptor (TNFR) I (p55 receptor) or II (p75 receptor), and the subsequent association of adaptor proteins with TNFR activates a downstream signaling cascade [1]. The decision between apoptosis and antiapoptosis depends on which downstream signals are recruited, and TNF-α-induced antiapoptotic responses such as proliferation and inflammation are mainly mediated by activation of activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), both of which are major eukaryotic transcription factors regulating TNF-α-induced gene expression [2]. Previous studies found that TNF-α-deficient mice showed resistance to skin tumor development [3,4], and these results show that targeting TNF-α signaling could be an important strategy for delaying the promotion of skin tumor.

Cyclooxygenase (COX) is a rate-limiting enzyme for the synthesis of prostaglandins from arachidonic acids. COX-2, one of the key enzymes mediating inflammatory responses, is inducible by tumor promoters, growth factors, and cytokines. COX-2 is reportedly up-regulated in several epithelial cancers, including those of the colon, breast, prostate, and lung, and the selective inhibition of COX-2 has been suggested as a potential strategy for preventing cancers [5,6]. Previous studies have also determined the role of COX-2 in skin carcinogenesis. COX-2 in human and mouse skin cells was up-regulated by acute and chronic ultraviolet B irradiation [7], phorbol 12-myristate 13-acetate (PMA) [8], and TNF-α [9]. Together these findings indicate that COX-2 overexpression in skin epidermis influences the risk of skin carcinogenesis [10,11]. In the JB6 mouse epidermal model, TNF-α reportedly induces neoplastic transformation by up-regulating COX-2 expression [9], but the mechanisms underlying TNF-α-induced COX-2 expression are not fully understood.

Src family kinases (SFKs) are representative nonreceptor protein tyrosine kinases that exhibit intrinsic kinase activity. SFKs are known to be associated with transmembrane receptors including growth factor receptors, hormone receptors, and cytokine receptors [12], and mediate intracellular signaling that induces cell growth, differentiation, survival, adhesion, and migration [13]. The identification of *src* as a proto-oncogene indicates that abnormal signaling via Src kinases is involved in tumorigenesis including epidermal tumors [14], colorectal cancer [15], and human breast tumors [16]. Fyn kinase is a 59-kDa member of SFK, and there are multiple lines of evidence for the correlation between Fyn and skin abnormality. Fyn-deficient mice exhibit an abnormal thickness of epidermis and hyperdifferentiation of keratinocytes [17], and Fyn kinase also mediates keratinocyte migration and squamous carcinoma invasion by disrupting $\alpha_6\beta_4$ integrin [18]. In addition, overexpression of Fyn is involved in neoplastic transformation of fibroblasts and mouse epidermal cells [19,20]. Although TNF-α reportedly induces multiple protein tyrosine kinases including SFKs [21,22], the involvement of Fyn kinase in TNF-α-induced COX-2 expression in skin cells remains to be clarified.

Epidemiological studies have revealed an inverse relationship between a moderate consumption of red wine or berries and the incidence of cancer and cardiovascular diseases [23–25]. There is increasing interest in the chemopreventive effects of resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a non-flavonoid polyphenol present in red wine and berries as a major active ingredient. However, the resveratrol content of red wine is relatively low (1.0 mg/l), with flavonoids such as anthocyanins (281 mg/l) and quercetin (18.8 mg/l) being present at much higher levels [26]. Despite this, the focus has remained on the health benefits of resveratrol, which has hindered clarification of the potent beneficial effects of other phenolic substances from red wine and berries.

The present study investigated the potential inhibitory effect of delphinidin (3,3′,4′,5,5′,7-hexahydroxyflavylium, Fig. 1A), which is a major flavonoid of the anthocyanidin class, on TNF-α-induced COX-2 up-regulation in JB6 P+ mouse epidermal (JB6 P+) cells, and the underlying mechanisms. Here we report that delphinidin inhibited COX-2 expression by

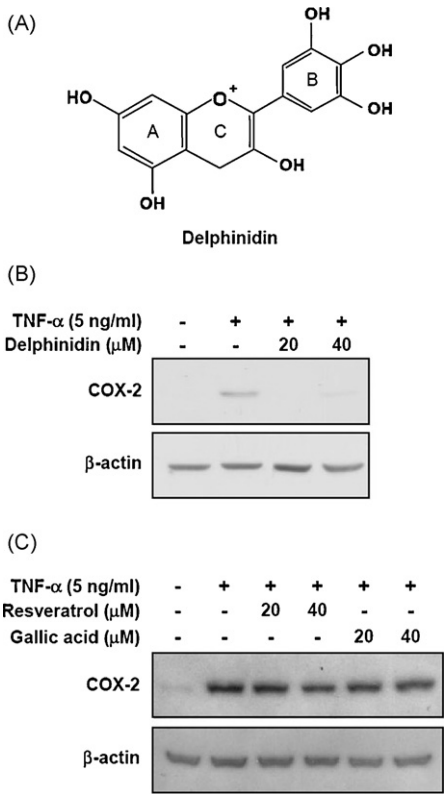

Fig. 1 – Effects of delphinidin, resveratrol, and gallic acid on TNF-α-induced COX-2 expression in JB6 P+ cells. (A) Chemical structure of delphinidin. (B) Delphinidin down-regulated TNF-α-induced COX-2 expression in JB6 P+ cells. JB6 P+ cells were treated with delphinidin at the indicated concentrations (20 or 40 μM) for 30 min before being exposed to 5 ng/ml TNF-α for 4 h. The cells were lysed, and the expression of COX-2 was analyzed by Western blotting as described in Section 2. (C) Resveratrol and gallic acid did not suppress TNF-α-induced COX-2 expression in JB6 P+ cells. JB6 P+ cells were treated with resveratrol or gallic acid at the indicated concentrations (20 or 40 μM) for 30 min before being stimulated by 5 ng/ml TNF-α for 4 h.

directly binding with Fyn kinase and inhibiting its activity, and subsequently suppressing TNF-α-induced signal transduction pathway, while two other phytochemicals (resveratrol and gallic acid) had no effects. This study also suggests that Fyn kinase is mainly involved in the TNF-α-induced COX-2 up-regulation. These data reveal the possibility that the health benefits of red wine and berries are caused by anthocyanidins including delphinidin, rather than by resveratrol or gallic acid.

2. Materials and methods

2.1. Chemicals

Delphinidin, resveratrol, and gallic acid were purchased from Sigma–Aldrich (St. Louis, MO). Recombinant mouse TNF-α was purchased from BD Biosciences (San Jose, CA). PP2 was obtained from Calbiochem (San Diego, CA). Eagle's minimal essential medium (MEM), fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin were purchased from GIBCO BRL (Grand Island, NY). The antibody against COX-2 was purchased from Cayman Chemical (Ann Arbor, MI), and that against β-actin was obtained from Sigma–Aldrich. Antibodies against phosphorylated extracellular-signal regulated kinase 1/2 (ERK1/2) (Thr202/Tyr204), total ERK1/2, and total mitogen- and stress-activated protein kinase 1 (MSK1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated p90 ribosomal S6 kinase (p90RSK) (Thr359/Ser363), total p90RSK, phosphorylated p38 (Tyr180/Tyr182), total p38, phosphorylated c-Jun N-terminal kinase (JNK) (Thr183/Tyr185), total JNK, phosphorylated Akt (Ser473), total Akt, and phosphorylated MSK1 (Ser376) were obtained from Cell Signaling Biotechnology (Beverly, MA). Active Fyn protein and the antibody against Fyn were obtained from Upstate Biotechnology (Lake Placid, NY). CNBr-Sepharose 4B and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech (Piscataway, NJ), and the protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). G418 and the luciferase assay substrate were purchased from Promega (Madison, WI).

2.2. Cell culture

The JB6 P+ cell line was cultured in monolayers at 37 °C in a 5% CO_2 incubator in MEM containing 5% FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin. The JB6 P+ was stably transfected with a COX-2, AP-1, and NF-κB luciferase reporter plasmid or siRNA-Fyn, and maintained in MEM supplemented with 5% FBS containing 200 μg/ml G418. Stable transfection lines of JB6 P+ cells were kindly provided by Dr. Zigang Dong (University of Minnesota).

2.3. Luciferase assay for COX-2 promoter activity, and AP-1 and NF-κB transcription activity

Confluent monolayers of JB6 P+ cells stably transfected with a COX-2, AP-1, or NF-κB luciferase reporter plasmid were trypsinized, and 8×10^3 viable cells suspended in 100 μl of 5% FBS/MEM were added to each well of a 96-well plate. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2. When the cells reached 80–90% confluence, they were starved by culturing them in 0.1% FBS/MEM for 24 h. The cells were then treated for 30 min with each chemical before being exposed to 5 ng/ml TNF-α for 24 h in the case of COX-2 activity and for 3 h in the case of AP-1 and NF-κB activities. Cells were then disrupted with 100 μl of lysis buffer [0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM dithiothreitol (DTT), and 2 mM EDTA], and the luciferase activity was measured using a luminometer (Microlumat Plus LB 96 V, Berthold Technologies, Bad Wildbach, Germany).

2.4. Western blot analysis

Cells were cultured for 48 h and then starved in MEM containing 0.1% FBS for 24 h. The cells were then treated with chemicals for 30 min before being exposed to 5 ng/ml TNF-α for different time periods. Cell lysates were scraped and treated with lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 0.1 mM PMSF, 10% glycerol, and a protease inhibitor cocktail tablet] for 40 min on ice followed by centrifugation at 16,000 × *g* for 10 min. The protein concentration of the supernatant was determined using a dye-binding protein assay kit (Bio-Rad Laboratories) as described in the manufacturer's manual. Lysate protein (40 μg) was subjected to 10% SDS-PAGE and electrophoretically transferred to a PVDF membrane (Millipore, Bedford, MA). After blotting, the membrane was blocked in 5% fat-free dry milk for 1 h and then incubated with the specific primary antibody for 2 h at room temperature. Protein bands were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) after hybridization with the HRP-conjugated secondary antibody.

2.5. Direct Fyn kinase assays

The direct Fyn kinase assays were performed in accordance with the instructions provided by Upstate Biotechnology. In brief, every reaction contained 6.25 μl of assay buffer [200 mM Tris–HCl (pH 7.5), 0.4 mM EGTA, and 0.4 mM sodium orthovanadate] and a magnesium acetate–ATP cocktail buffer [2.5 mM HEPES (pH 7.4), 50 mM magnesium acetate, and 0.5 mM ATP]. For Fyn kinase, the Src substrate peptide was included at 250 μM. Ten nanograms of an active Fyn protein and 10 μl of diluted [γ-^{32}P]ATP solution were incubated at 30 °C for 10 min with the above assay buffer and substrate peptide, and then 15-μl aliquots were transferred onto p81 paper and washed three times with 0.75% phosphoric acid for 5 min and once with acetone for 5 min. The radioactive incorporation was determined using a scintillation counter. The effects of delphinidin (0–40 μM) or PP2 (10 μM) were evaluated by separately incubating each compound with the reaction mixtures at 30 °C for 10 min. Each experiment was performed three times.

2.6. Direct and cell-based pull-down assays

Active Fyn protein (0.2 μg) or a JB6 P+ cellular supernatant fraction (500 μg) was incubated with delphinidin–Sepharose 4B beads (100 μl, 50% slurry) or Sepharose 4B beads (as a

negative control) in a reaction buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 μg/ml bovine serum albumin, 0.02 mM PMSF, and 1× protease inhibitor mixture]. After incubation with gentle rocking overnight at 4 °C, the beads were washed five times with 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], and proteins bound to the beads were analyzed by immunoblotting. For making delphinidin–Sepharose 4B beads, delphinidin is immobilized into CNBr-activated Sepharose 4B beads following the modification of manufacturer's instructions. Briefly, dissolve delphinidin in coupling buffer (0.1 M $NaHCO_3$, pH 8.3; 0.5 M NaCl) and mix with the beads which are previously activated with 1 mM HCl. Wash with the coupling buffer and block any remaining active groups with 0.1 M Tris–HCl buffer (pH 8.0). Finally, wash alternately with 0.1 M acetate buffer (pH 4.0) and 0.1 M Tris–HCl buffer (pH 8.0) containing 0.5 M NaCl to remove excess of uncoupled delphinidin. During this procedure, one of the OH groups of delphinidin makes the link with beads.

2.7. ATP and delphinidin competition assays

Recombinant Fyn (0.2 μg) was incubated with 100 μl of delphinidin–Sepharose 4B or Sepharose 4B beads in a reaction buffer (see previous section) for 12 h at 4 °C, and ATP was added at either 10 or 100 μM to a final volume of 500 μl. After 30 min of incubation, the samples were washed and the proteins were detected by Western blotting.

2.8. Molecular modeling

The homology model structure of the full-length Fyn kinase was kindly provided by Dr. Dubravko Jelic (GlaxoSmithKline Research Center). Insight II (Accelrys, San Diego, CA) was used for the docking study and structure analysis of the model.

2.9. Statistical analysis

Where necessary, data are expressed as mean and SD values, and Student's *t* test was used for single statistical comparisons. A probability cutoff of $p < 0.05$ was used as the criterion for statistical significance.

3. Results

3.1. Delphinidin inhibits TNF-α-induced COX-2 expression in JB6 P+ cells

To investigate the possibility that delphinidin protects against TNF-α-induced skin carcinogenesis, we first examined whether delphinidin attenuates TNF-α-induced COX-2 expression in JB6 P+ cells. TNF-α-induced COX-2 expression in JB6 P+ cells was strongly inhibited by delphinidin (Fig. 1B) but not by resveratrol (a member of stilbene class) or gallic acid (a member of benzoic acid class) (Fig. 1C). Resveratrol slightly inhibited TNF-α-induced COX-2 expression only at 40 μM, but its inhibitory effect was lesser compared to the effect of delphinidin.

3.2. Delphinidin attenuates TNF-α-induced COX-2 promoter activity and AP-1 and NF-κB transcription activities in JB6 P+ cells

To further confirm that the inhibitory effect of delphinidin on TNF-α-induced COX-2 up-regulation is attributable to transcriptional regulation, we next examined whether delphinidin affects the COX-2 promoter activity induced by TNF-α in JB6 P+ cells. COX-2 promoter activity was determined by using JB6 P+ cells stably transfected with a COX-2 luciferase plasmid. Delphinidin inhibited TNF-α-induced COX-2 promoter activity in a dose-dependent manner (Fig. 2A), which indicated that COX-2 expression was down-regulated by delphinidin from the transcriptional level. The COX-2 gene is known to contain AP-1 and κB regulatory elements in the 5′-flanking region [27], and TNF-α can activate transcription factors such as AP-1, NF-κB, and nuclear factor of activated T cells (NFAT) [9]. To elucidate the mechanism underlying transcriptional regulation of the COX-2 gene, we further examined the activities of AP-1 and NF-κB, both of which are major transcriptional factors involved in COX-2 expression. JB6 P+ cells stably transfected with an AP-1 or NF-κB luciferase plasmid were used to detect TNF-α-induced transactivation of AP-1 and NF-κB. Delphinidin at 40 μM suppressed TNF-α-induced AP-1 and NF-κB activities by 82% and 44%, respectively (Fig. 2B and C). The inhibition of AP-1 activity by delphinidin is more efficient than that of NF-κB activity, when compared the treatment of delphinidin at the same concentration; $p = 0.004$ at 10 μM, $p = 0.010$ at 20 μM, and $p = 0.022$ at 40 μM of delphinidin treatment. This *p* value is calculated by comparing the percentage of AP-1 inhibition versus NF-κB inhibition by treating the same concentration of delphinidin. These results indicate that delphinidin regulates TNF-α-induced COX-2 expression at the transcriptional level by suppressing AP-1 activity more strongly than NF-κB activity.

3.3. Delphinidin inhibits the phosphorylation of upstream kinases involved in COX-2 expression

To elucidate the mechanisms underlying how delphinidin regulates TNF-α-induced COX-2 expression, we next examined the effects of delphinidin on the TNF-α-induced phosphorylations of JNK, p38 MAP kinase, Akt, p90RSK, MSK1, and ERK protein kinases. Delphinidin inhibited the TNF-α-induced phosphorylations of JNK, p38 MAP kinase, and Akt in a dose-dependent manner (Fig. 3A). Delphinidin also inhibited the TNF-α-induced phosphorylations of p90RSK, MSK1, and ERK in JB6 P+ cells (Fig. 3B). These results suggest that delphinidin regulates the upstream kinases of MAP kinases involved in TNF-α-induced COX-2 expression in JB6 P+ cells.

3.4. Delphinidin inhibits Fyn kinase activity by directly binding with Fyn kinase

Previous studies found that SFKs play pivotal roles in TNF-α signaling by acting as adaptor proteins associated with TNFRs [21,28,29], and TNF-induced ERK phosphorylation is reportedly regulated by SFKs [30]. Therefore, SFKs have the potential to act as upstream kinases of MAP kinases. Because Fyn kinase

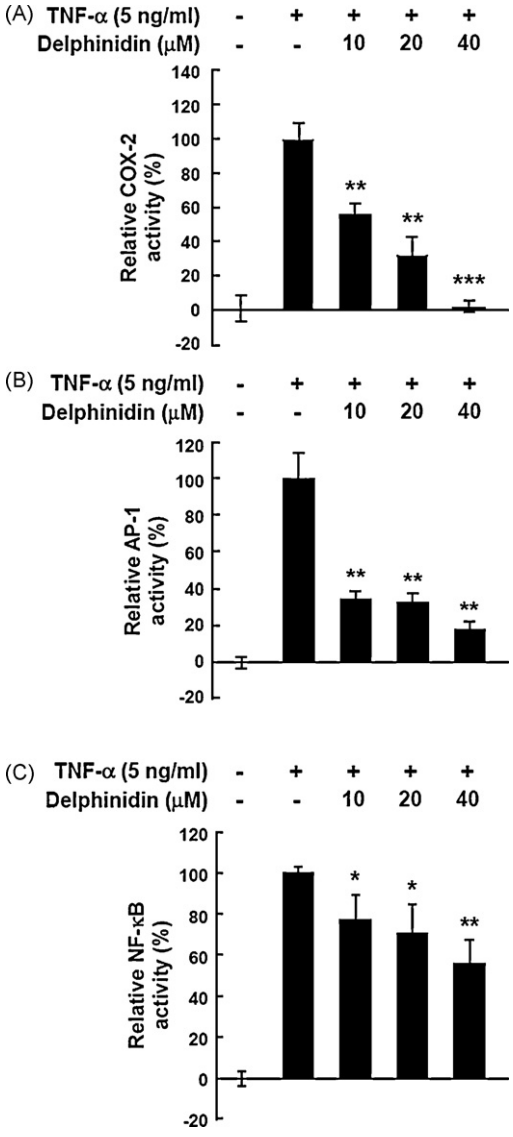

Fig. 2 – Effects of delphinidin on TNF-α-induced COX-2 promoter activity and AP-1 and NF-κB transcription activities. Delphinidin inhibited the TNF-α-induced COX-2 promoter activity (A), and AP-1 (B) and NF-κB (C) transcription activities in JB6 P+ cells. JB6 P+ cells, which were stably transfected with COX-2, AP-1, or NF-κB luciferase reporter plasmids, were pretreated with delphinidin for 30 min at the indicated concentrations before being exposed to 5 ng/ml TNF-α for 24 h in the case of COX-2 activity and for 3 h in the case of AP-1 and NF-κB activities. The relative activity was measured by the luciferase assay as described in Section 2. Data are mean and SD values of COX-2, AP-1, and NF-κB luciferase activities calculated from three independent experiments. Asterisks indicate significant differences compared to groups exposed to TNF-α only (* shows $p < 0.05$, ** shows $p < 0.01$, and * shows $p < 0.001$).**

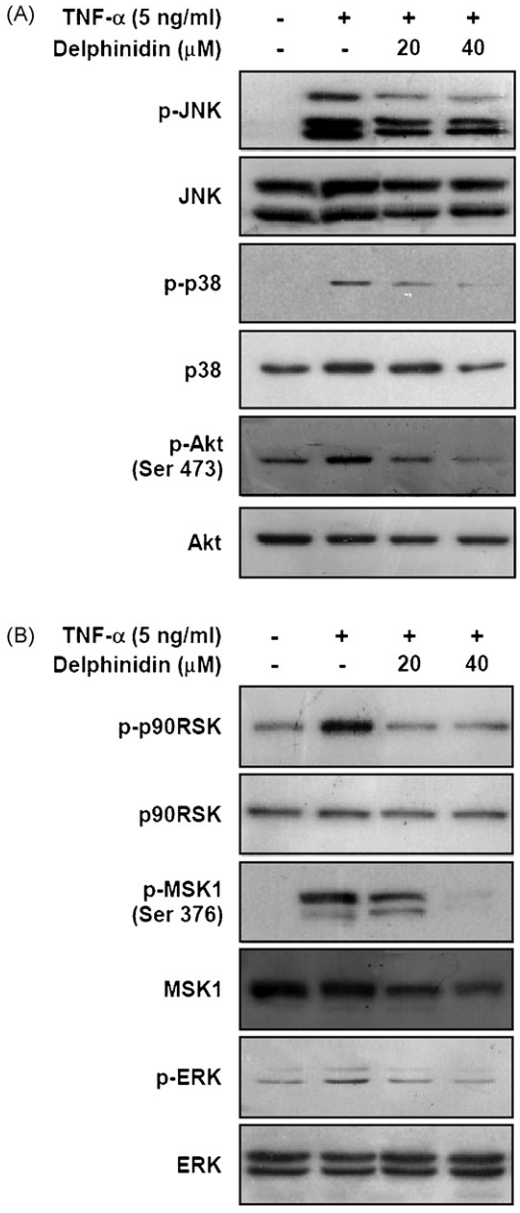

Fig. 3 – Effects of delphinidin on the TNF-α-induced phosphorylation of upstream kinases involved in COX-2 expression in JB6 P+ cells. (A) Delphinidin inhibited the TNF-α-induced phosphorylations of JNK, p38 MAP kinase, and Akt. JB6 P+ cells were treated with delphinidin (20 or 40 μM) for 30 min before being stimulated by 5 ng/ml TNF-α for 15 min. The cells were lysed, and the levels of phosphorylated and total JNK, p38 MAP kinase, and Akt proteins were determined by Western blotting as described in Section 2 using specific antibodies against the proteins. (B) Delphinidin inhibited the TNF-α-induced phosphorylations of p90RSK, MSK1, and ERK. JB6 P+ cells were treated with delphinidin (20 or 40 μM) for 30 min before being stimulated by 5 ng/ml TNF-α for 15 min. The cells were lysed, and the levels of phosphorylated and total p90RSK, MSK1, or ERK proteins were determined by Western blotting as described in Section 2 using specific antibodies against the proteins.

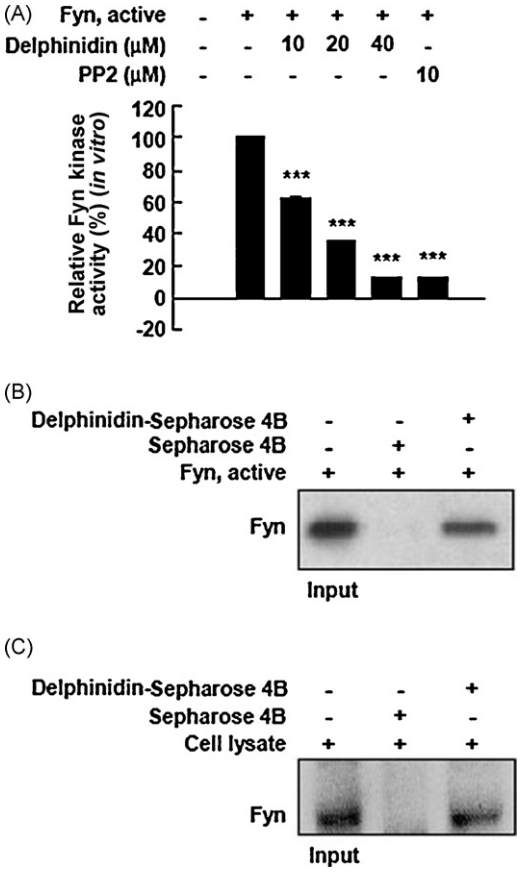

Fig. 4 – Effects of delphinidin on Fyn kinase activity and its direct binding with Fyn kinase. (A) Delphinidin directly inhibited Fyn kinase activity. A direct Fyn kinase assay was performed as described in Section 2, and Fyn kinase activity was expressed as a percentage relative to that for treatment with active Fyn protein alone. Data are mean and SD values calculated from three independent experiments. Asterisks indicate significant differences between groups treated with active Fyn and delphinidin together, and the group treated with an active Fyn alone (* shows $p < 0.001$). (B) Delphinidin directly bound with Fyn kinase protein. The Fyn-kinase–delphinidin binding was confirmed by immunoblotting using an antibody against Fyn: lane 1 (input control), Fyn protein standard; lane 2 (control), Fyn pulled down using Sepharose 4B as described in Section 2; and lane 3, Fyn pulled down using delphinidin–Sepharose 4B beads as described in Section 2. (C) Delphinidin bound with TNF-α-activated Fyn kinase protein in JB6 P+ cells. The Fyn-kinase–delphinidin binding in JB6 P+ cells was confirmed by immunoblotting using an antibody against Fyn: lane 1 (input control), whole-cell lysates from JB6 P+ cells; lane 2 (control), lysates of JB6 P+ cells precipitated with Sepharose 4B beads as described in Section 2; and lane 3, whole-cell lysates from JB6 P+ cells precipitated with delphinidin–Sepharose 4B beads as described in Section 2.**

(an SFK) has also been shown to be activated by TNF-α [22], we next used a direct Fyn kinase assay to determine the effect of delphinidin on Fyn kinase activity. The results indicated that delphinidin inhibited Fyn kinase activity in a dose-dependent manner, with the inhibitory effect of delphinidin at 40 μM being similar to that of PP2 (a well-known Fyn kinase inhibitor) at 10 μM (Fig. 4A). To determine the underlying mechanism, we further determined whether delphinidin can directly interact with Fyn kinase. Pull-down assays revealed that delphinidin directly bound with an active Fyn protein (Fig. 4B, lane 3) but not with Sepharose 4B beads alone (Fig. 4B, lane 2). The input lane (Fig. 4B, lane 1) comprised 50 ng of active Fyn protein as a marker to ensure that the detected band was Fyn protein itself. Cell-based pull-down assays also revealed that delphinidin bound with TNF-α-induced Fyn kinase in JB6 P+ cells (Fig. 4C, lane 3). These results demonstrate that delphinidin suppresses Fyn kinase activity by directly binding with Fyn kinase protein and subsequently inhibiting downstream signals.

3.5. *Fyn kinase is involved in TNF-α-induced COX-2 expression in JB6 P+ cells*

To elucidate whether the inhibition of Fyn kinase activity by delphinidin suppresses COX-2 expression, we next investigated the role of Fyn kinase as an upstream mediator of TNF-α signaling involved in COX-2 up-regulation in JB6 P+ cells. TNF-α-induced COX-2 expression (Fig. 5A) and COX-2 promoter activity (Fig. 5B) were blocked by treatment with PP2 at the indicated concentrations, which indicated that TNF-α-induced COX-2 expression was mediated by Fyn kinase activation in JB6 P+ cells. To further confirm the role of Fyn kinase in COX-2 expression induced by TNF-α in JB6 P+ cells, we compared the levels of COX-2 expression between siRNA-Fyn JB6 P+ cells and mock-transfected JB6 cells. The TNF-α-induced COX-2 expression was significantly lower in siRNA-Fyn JB6 P+ cells than in mock-transfected JB6 cells (Fig. 5C), as was JNK phosphorylation (Fig. 5D). These findings demonstrate that Fyn kinase mainly down-regulates TNF-α-induced JNK phosphorylation, and subsequently inhibits COX-2 expression in JB6 P+ cells.

3.6. *Delphinidin binds with Fyn kinase noncompetitively with ATP*

To elucidate whether delphinidin binds with Fyn kinase in an ATP pocket of its kinase domain, we next performed pull-down assays with ATP at various concentrations. The binding ability of delphinidin with Fyn kinase did not vary with the ATP concentration (Fig. 6A), indicating that delphinidin inhibits Fyn kinase activity noncompetitively with ATP.

4. Discussion

Considerable attention has been focused on the health-promoting effects of red wine and berries and their non-flavonoid polyphenol compound resveratrol, but the underlying molecular mechanisms, molecular targets, and active ingredients have remained unknown. Anthocyanins are natural polyphenolic compounds that furnish many fruits

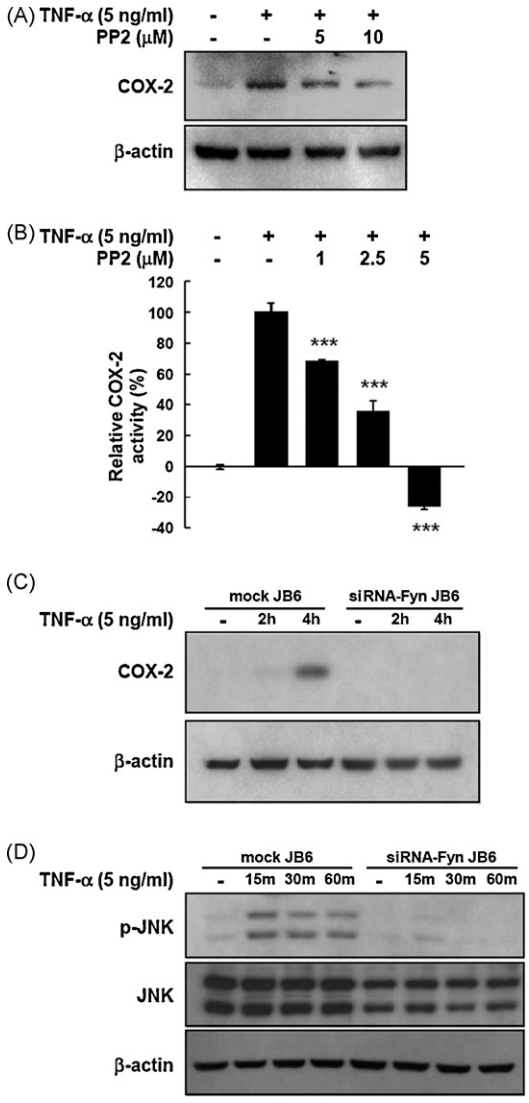

Fig. 5 – The role of Fyn kinase in TNF-α-induced COX-2 expression in JB6 P+ cells. (A) TNF-α-induced COX-2 expression was attenuated by a Fyn kinase inhibitor in JB6 P+ cells. The cells were pretreated with PP2 (5 or 10 μM) for 30 min before being stimulated by 5 ng/ml TNF-α for 4 h. The cells were then lysed, and the expression level of COX-2 was determined by Western blotting as described in Section 2. (B) TNF-α-induced COX-2 promoter activity was attenuated by a Fyn kinase inhibitor in JB6 P+ cells. The JB6 P+ cells, which were stably transfected with COX-2 luciferase reporter plasmids, were pretreated with PP2 (1, 2.5 or 5 μM) for 30 min before being exposed to 5 ng/ml TNF-α for 24 h. The relative activity was measured by the luciferase assay as described in Section 2. Data are mean and SD values of the COX-2 promoter activity calculated from three independent experiments. Asterisks indicate significant differences between groups treated with TNF-α and delphinidin together, and the group exposed to TNF-α alone (* shows $p < 0.001$). (C) siRNA-Fyn inhibited TNF-α-induced COX-2 expression. Mock and siRNA-Fyn-transfected JB6 cells were exposed to TNF-α for the indicated time periods, and the expression of COX-2 was analyzed by Western blotting as described in Section 2. (D) siRNA-Fyn inhibited the TNF-α-induced JNK phosphorylation. Mock-transfected JB6 cells and siRNA-Fyn JB6 cells were exposed to TNF-α for the indicated time periods, and the levels of phosphorylated and total JNK were determined by Western blotting as described in Section 2 using specific antibodies against the proteins.**

and vegetables with a blue-red color, including red wine and berries. Delphinidin, an aglycone of anthocyanin that has a diphenylpropane-based polyphenolic ring structure, is also regarded as one of the most potent cancer chemopreventive agents. Delphinidin is an active component of red wine [31], and inhibited lipopolysaccharide-induced COX-2 expression in mouse macrophage cells [32]. Delphinidin protects human keratinocytes from UVB-induced apoptosis [33] and inhibits PMA-induced JB6 P+ cell transformation by regulating the ERK and JNK pathways [34]. Therefore, we further determined whether delphinidin has the potential to inhibit TNF-α-induced inflammatory responses in JB6 P+ model. The TNF-α-induced COX-2 expression in JB6 P+ cells was suppressed by delphinidin, showing even much higher inhibitory effects than resveratrol and gallic acid which are two other well-known phytochemicals present in many fruits and vegetables.

The promoter region of the COX-2 gene contains binding sites for various transcriptional factors, including AP-1, NF-κB, CREB (cyclic AMP response element binding protein), and NFAT. Numerous intracellular signal transduction pathways including those involving MAP kinases are known to regulate the activation of transcription factors and subsequently affect the target-gene expression levels [35,36]. To determine whether the inhibition of phosphorylation of MAP kinases by delphinidin contributed to the regulation of COX-2 expression at the transcriptional level, we used luciferase assays to determine the COX-2 promoter activity and AP-1 and NF-κB transcription activities. The results showed that delphinidin regulated COX-2 expression at the transcriptional level by suppressing COX-2 promoter activity, which was due to the inhibition of transactivation of AP-1 and NF-κB. Although previous studies have demonstrated the important roles of AP-1 and NF-κB in JB6 P+ cell tumorigenesis [37], a recent study found that NFAT3 also acts as a critical transcription factor in regulating TNF-α-induced COX-2 expression in JB6 P+ cells [9]. Therefore, it is possible that delphinidin regulates other transcription factors such as NFAT3 so as to suppress TNF-α-induced COX-2 expression.

To elucidate the inhibitory mechanism of delphinidin on TNF-α-induced COX-2 expression, we further investigated the effects of delphinidin on TNF-α-induced activations of the MAP kinase and phosphatidylinositol-3-kinase (PI3K) signaling pathways. Delphinidin inhibited the phosphorylations of JNK, p38 MAP kinase, Akt, p90RSK, MSK1, and ERK. The role of the TNF-α signaling pathway in skin carcinogenesis has been previously reported in the JB6 P+ model. TNF-α-induced COX-2 expression is reportedly involved in the anchorage-independent cell growth of JB6 P+ cells [9]. However, the mechanisms underlying TNF-α-induced COX-2 expression in JB6 P+ cells are not fully understood. Previously, JNK was reported to play pivotal roles in TNF-α-induced carcinogenesis. In the previous study, it was demonstrated that overexpression of dominant

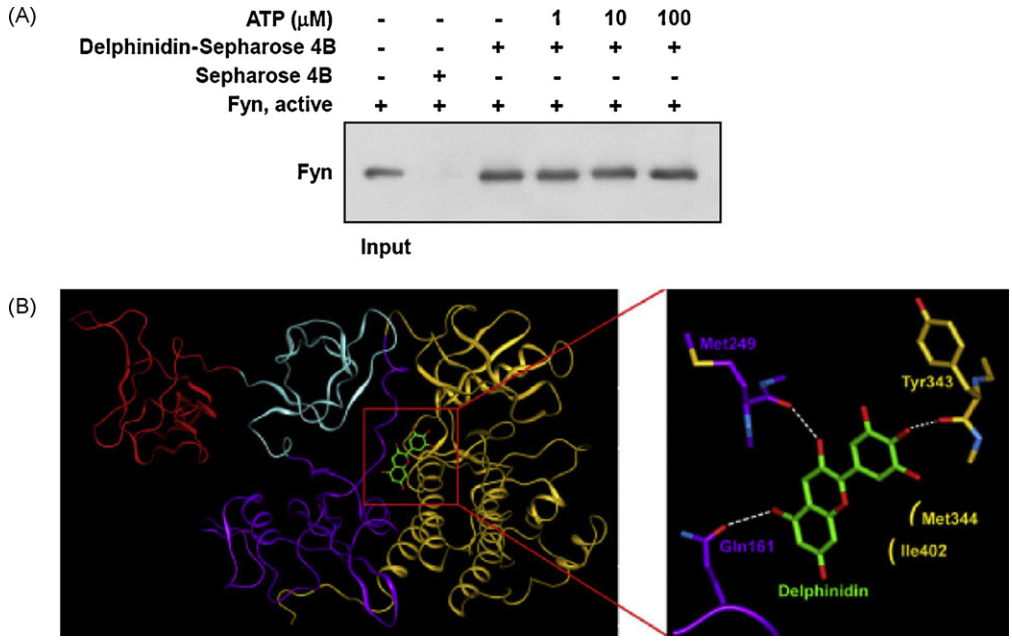

Fig. 6 – Modeling study of Fyn kinase binding with delphinidin. (A) Delphinidin bound with Fyn kinase noncompetitively with ATP. Active Fyn (0.2 μg) was incubated with ATP at the indicated concentrations (1, 10 or 100 μM) and 100 μl of delphinidin–Sepharose 4B beads or 100 μl of Sepharose 4B beads (as a negative control) in a reaction buffer to a final volume of 500 μl. The pulled-down proteins were detected by Western blotting as described in Section 2: lane 1 (input control), Fyn protein standard; lane 2 is the negative control, which indicates that Fyn kinase does not bind with Sepharose 4B; and lane 3 is the positive control, which indicates that Fyn kinase binds with delphinidin–Sepharose 4B. (B) Hypothetical model of the Fyn-kinase–delphinidin complex. The colors indicate the different domains of Fyn kinase, with the N-terminal domain in red, SH3 in cyan, SH2 in violet, and the kinase domain in yellow. Delphinidin (atomic color) binds to the putative allosteric site between the SH2 and domains. The close-up view of the inhibitor interaction at the allosteric site depicts hydrogen bonds as dashed lines and hydrophobic contacts as small curves.

negative JNK dramatically impaired TNF-α-induced JB6 P+ cell transformation even though TNF-α-induced phosphorylations of ERK and p38 MAP kinase were still activated, suggesting that the JNK activation is mainly involved in TNF-α-induced neoplastic transformation. [38]. In addition, MKK4, which can directly phosphorylate JNK, is also essential for TNF-α-induced NF-κB activation [39], supporting the important role of TNF-α-induced JNK activation. Since JNK is predominantly involved in the regulation of AP-1 gene expression induced by TNF-α [40], delphinidin is more effective for the inhibition of AP-1 activity than NF-κB activity in this study through the inhibition of JNK phosphorylation. However, the upstream regulator of JNK in the TNF-α-induced signaling is still unknown.

Many studies have revealed that SFKs mediate the TNF-α-induced signal transduction pathway by interacting with TNFRs. Because TNFRs have no intrinsic tyrosine kinase activity, their association with nonreceptor tyrosine kinases such as SFKs is necessary to induce downstream signals. Among SFKs, only Src, Fyn, and Yes are widely expressed in various tissues [41], and Fyn kinase is already reported to have an oncogenic potential and play key roles in inflammation [19]. Therefore, we focused on demonstrating whether Fyn kinase is involved in the TNF-α-induced inflammation, especially COX-2 expression, in JB6 P+ cells. In the present study, by using PP2 (a pharmacological inhibitor of Fyn kinase) and siRNA-Fyn-JB6 P+ cells, we demonstrated that TNF-α-induced JNK activation and subsequent COX-2 up-regulation are mediated by the activation of Fyn kinase. Our results also demonstrate that Fyn kinase is one of the direct molecular targets of delphinidin for the suppression of TNF-α-induced COX-2 expression. Delphinidin inhibited Fyn kinase activity in a dose-dependent manner, and the inhibitory effect of delphinidin at 40 μM was similar to that of PP2 at 10 μM. Our direct and cell-based pull-down assays demonstrated that delphinidin binds directly to Fyn kinase, which might be responsible for the inhibition of Fyn kinase activity by delphinidin. Epigallocatechin gallate and rosmarinic acid were previously reported to be Fyn kinase inhibitors with a higher affinity to the non-ATP-binding pocket of Fyn kinase than to its ATP-binding pocket [20,42]. Similarly, we found that delphinidin bound with Fyn kinase noncompetitively with ATP. However, since Fyn kinase is highly homologous to Src, it is possible that delphinidin also inhibits Src kinase activity. In the direct Src kinase assay, we confirmed that delphinidin also inhibited Src kinase activity at 20 μM and 40 μM by 14.77% and 7.39%, respectively (unpublished observation). Previously, several studies revealed that TNF-α-induced EGFR activation is dependent on Src kinase [43,44], and delphinidin inhibited EGFR activation [45,46]. Therefore, the inhibition of Src-dependent EGFR activation by delphinidin might be another strategy to suppress TNF-α-induced signaling. Furthermore, since there is a possibility that other kinases besides Fyn can be

recruited to TNFR and remain to be activated even though delphinidin blocked multiple TNF-α-induced phosphorylations of MAPK and PI3K pathway.

To investigate the molecular basis of the Fyn kinase inhibition by delphinidin, we performed a modeling study using the homology model structure of the full-length Fyn [42]. Fyn kinase consists of four domains: an N-terminal membrane-anchoring domain, an SH3 domain, an SH2 domain, and a catalytic kinase domain. Considering the experimental result that delphinidin binds to Fyn kinase noncompetitively with ATP, we docked delphinidin to the putative allosteric site where rosmarinic acid was expected to bind in the homology model structure of Fyn kinase [42]. In the hypothetical structure of the Fyn-kinase–delphinidin complex, delphinidin can make hydrogen bonds with the side chain of Gln161 and the backbone carbonyl group of Met249 in the SH2 domain, and with the backbone carbonyl group of Tyr343 in the catalytic kinase domain (Fig. 6B). Delphinidin can also interact hydrophobically with Ile402 and Met344 of the kinase domain. The interaction between the SH2 domain and delphinidin could influence protein–protein interactions. To prove this, we performed the immunoprecipitation assay to show whether delphinidin inhibits the binding of Fyn kinase with FAK, one of the direct downstream substrates of Fyn kinase, and confirmed that the binding of Fyn with its substrate was attenuated when being treated with delphinidin (data not shown). The interaction with the kinase domain could induce structural distortion of ATP or the substrate binding site, and thus reduce the catalytic activity of Fyn kinase allosterically. Future investigations using X-ray crystallography would help to elucidate the exact binding modes of delphinidin to Fyn kinase. Future study also needs to clarify whether the concentration of delphnidin we used is achievable in human.

In summary, the results reported here indicate that delphinidin inhibits TNF-α-induced COX-2 expression by blocking Fyn kinase activity via direct binding with Fyn. The inhibition of Fyn results in delphinidin suppressing the activations of MAP kinase and PI3K pathways, mainly JNK, and reducing NF-κB and AP-1 activity. Together these results suggest that delphinidin contributes substantially to the cancer chemopreventive potential of red wine and berries.

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