Molecular characterization of cancer chemopreventive effects of anthocyans

（アントシアンの癌化学予防作用の分子機構に関する研究）

De-Xing　HOU

（侯　　德　興）

May　2006

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De-Xing Hou

A Dissertation Presented to the Graduate School of University of Tokyo in Candidacy for the Degree of Doctor of Pharmacology

May 2006

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Abbreviations

AIF = apoptosis-inducing factor

AP-1 = activator protein-1

CAPE = caffeic acid phenylethyl ester

C/EBP = CCAAT/enhancer-binding protein

COX-1 = cyclooxygenase-1

COX-2 = cyclooxygenase-2

CREB = CRE-binding protein

Cy3-Glu = cyanidin 3-glucoside

Cy3-Rut = cyanidin 3-rutinoside

Cy3-Sam = cyanidin 3-sambubioside

DCFH-DA = 2', 7'-dichlorodihydrofluorescein diacetate

$DiOC_6$ (3) = 3,3'-dihexyloxacarbocyanine iodide

DMEM = Dulbecco's minimum essential medium

DMH = 1,2-dimethylhydrazine

DMPO = 5, 5-dimethyl-1-pyrroline-*N*-oxide

DMSO = dimethyl sulfoxide

Dp3-Glu = delphinidin 3-glucoside

Dp3-Rut = delphinidin 3-rutinoside

Dp3-Sam = delphinidin 3-sambubioside

EDTA = ethylenediaminetetraacetic acid

EGCG = epigallocatechin gallate

EGF = epidermal growth factor

EGFR = epidermal growth factor receptor

EGTA = ethyleneglycol-*N*,*N*,*N'*,*N'*-tetraacetic acid

EMEN = eagle's minimum essential medium

ERK = extracellular signal-regulated kinase

ESR = electron spin resonance

FBS = fetal bovine serum

GAPDH = glyceraldehyde-3-phosphate dehydrogenase

HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid

HL-60 = human leukemia cell line

HPLC = high performance liquid chromatography

HRP = horseradish peroxidase

$ID_{(50)}$ = 50% inhibition dose

INF-α = interferon-*alpha*

INF-γ = interferon-*gamma*

iNOS = inducible nitric oxide synthase

INT = *p*-iodonitrotetrazolium violet

JNK = c-Jun NH_2-terminal kinase

LPS = lipopolysaccharide

MAPK = mitogen-activated protein kinase

MAPKK = MAPK kinase

MAKKK = MAPKK kinase

MEK = MAPK/ERK kinase

MMPs = matrix metalloproteinases

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide

NAC = *N*-acetyl-L-cysteine

NF-κB = nuclear factor κB

NO = nitric oxide

NSAID = nonsteroidal anti-inflammatory drug

P3G = pelargonidin 3-*O*-*beta*-D-glucoside

PARP = poly(ADP)ribose polymerase

PGH2 = prosptaglandin H_2

PGHS-1 = prostaglandin endoperoxide H synthase-1

PGHS-2 = prostaglandin endoperoxide H synthase-2

PhIP = 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

PMSF = phenylmethylsulfonyl fluoride

PVDF = polyvinylidene fluoride

ROS = reactive oxygen species

RT-PCR = reverse-transcriptional polymerase chain reaction

SAPK = stress-activated protein kinase

SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEK = SAPK/ERK kinase

SOD = superoxide dismutase

TBARS = thiobarbituric acid-reactive substance

TNF-α = tumor necrosis factor-*alpha*

TPA = 12-*O*-tetradecanoylphorbol-13-acetate

TRE = TPA-response elements

UV = ultra violet

YGM-3 = cyanidin 3-(6, 6'-caffeoylferuloylsophoroside)-5-glucoside

YGM-6 = peonidin 3-(6, 6'-caffeoyl- feruloylsophoroside)-5-glucoside

ΔΨm = mitochondrial membrane potential

Abstract

Anthocyan encompasses “anthocyanin” for the glycoside and “anthocyanidin” for the aglycon. Anthocyans are flavanols, which occur ubiquitously in the plant kingdom and confer bright red or blue coloration on many fruits and vegetables such as berries, red grapes, purple sweet potato, and red cabbages. Based on food composition data, we consume considerable amounts of anthocyans from crops, fruits, and vegetable-based diet although the range is from several milligrams to hundred milligrams, depending on the nutrition customs. Epidemiological investigations have indicated that the high consumption of colored fruits and vegetables such as strawberries or bilberry is associated with a reduced risk of human cancer and coronary heart disease. Recent studies indicated that anthocyans have strong free radical scavenging and antioxidant activities, and show inhibitory effects on the growth of some cancer cells. Animal experiments showed that oral intake of anthocyans from purple sweet potato and red cabbage suppressed rat colon carcinogenesis. In addition, anthocyans can be directly absorbed and distributed to the blood in human and rats after consumption of dietary anthocyans. These facts suggest that anthocyans may have cancer chemopreventive effects. However, the mechanism behind the effect is little known. In the present study, the chemopreventive effects of anthocyans were characterized at cellular and molecular levels through targeting several key steps involved in carcinogenesis such as cell transformation, inflammation and cell proliferation.

Molecular characterization of the inhibitory effects of anthocyans on cell transformation in mouse epidermal cells

Activator protein-1 (AP-1) is a transcription factor and has been shown to play a critical role in promoting carcinogenesis. In mouse epidermal cell line JB6, tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) can induce AP-1 activity and neoplastic transformation by activating the cascades of mitogen-activated protein kinases (MAPK). Delphinidin, cyanidin, petunidin and their glycosides could inhibit TPA-induced AP-1 transcriptional activity and cell transformation. Structure-activity studies indicated that the ortho-dihydroxyphenyl structure at the B-ring of aglycon seems essential for the inhibitory action because pelargonidin, peonidin and malvidin, having no such ortho-dihydroxyphenyl structure, failed to show the inhibitory effects in both AP-1 activity and cell transformation. The results from signal transduction analysis indicated that delphinidin blocked phosphorylation of extracellular signal-regulated kinase (ERK) at early times, and phosphorylation of c-Jun NH_2-terminal kinase (JNK) at later times, but not p38 phosphorylation at any time. Moreover, delphinidin blocked the phosphorylation of MAPK/ERK kinase (MEK, an ERK kinase), SAPK/ERK kinase (SEK, a JNK kinase) and c-Jun (a phosphorylation target of ERK and JNK). The data suggest that the inhibition of TPA-induced AP-1 activity and cell transformation by delphinidin involves the blockage of ERK and JNK signaling cascades. Furthermore, a greater inhibition was observed in combinations of superoxide dismutase (SOD) with anthocyans that have the ortho-dihydroxyphenyl structure on the B-ring of aglycon. Multiplicative model analysis suggested that this greater inhibition between SOD and delphinidin is synergistic, not additive. Thus, the inhibitory effects of anthocyans on AP-1 activation and cell transformation would be due in part to their potent scavenging activity for superoxide radicals and in part to blocking MAPK.

Molecular characterization of the inhibitory effects of anthocyans on cyclooxygenase-2 expression in lipopolysaccharide (LPS) -evoked macrophages

Cyclooxygenase (COX) is the rate-limiting enzyme for synthesis of dienoic eicosanoids such as prostaglandin(PG) E2. COX exists in two isoforms. COX-1 is expressed constitutively in many types of cells and is responsible for the production of prostaglandins under physiological conditions. COX-2 is induced by pro-inflammatory stimuli, including mitogens, cytokines and bacterial LPS in cells *in vitro* and in inflamed sites *in vivo*. Data indicated that COX-2 is involved in many inflammatory processes and induced in various carcinomas, suggesting that COX-2 plays a key role in tumorigenesis. Interestingly, some antioxidants with chemopreventive effects inhibit the expression of COX-2 by interfering with the signaling mechanisms that regulate the COX-2 gene. Author used mouse macrophage cell line RAW264 to demonstrate the molecular mechanism of anthocyans on the inhibition of COX-2 gene. Anthocyan extracts from bilberry or purified delphinidin and cyanidin inhibited LPS-induced COX-2 expression at protein and transcriptional levels. The data from signal analysis indicated that delphinidin blocked MAPK pathway and NF-κB pathway to suppress COX-2 gene expression because LPS-induced IκB degradation and MAPK activations were blocked by delphinidin. These results indicated that the blockages of MAPK and NF-κB signaling pathway are involved in the inhibition of COX-2 gene expression by anthocyans.

Molecular characterization of apoptosis induction by anthocyans in human leukemia cells (HL-60)

Apoptosis has been reported to pay an important rule in elimination of seriously damaged

cells or tumor cells by chemopreventive and chemotheraptic agents. Apoptotic cells are rapidly recognized and removed by macrophages before cell lysis without inducing inflammation. Therefore, apoptosis-inducing compounds are expected to be ideal anticancer drugs. HL-60 cell line provides a valid model for testing antileukemic or general antitumoral compounds. Delphinidin, cyanidin, petunidin and their glycosides induced apoptosis of HL-60 cells detected by morphological changes and by DNA fragmentation, whereas pelargonidin, peonidin and malvidin showed no induction of apoptosis. Structure-activity studies indicated that the ortho-dihydroxyphenyl structure at the B-ring appears essential for apoptosis actions.

The mechanistic analysis indicated that the apoptosis induction by delphinidin or its glycoside might involve a ROS/JNK-mediated mitochondrial death pathway. Treatment with active anthocyans increased the levels of intracellular ROS, which may be a sensor to activate JNK. Concomitant with the apoptosis, JNK phosphorylation, *c-jun* gene expression, Bid truncation, mitochondrial membrane potential ($\Delta\Psi m$) loss, cytochrome *c* release and caspase activation were observed in the cells treated. Antioxidants such as *N*-acetyl-*L*-cysteine (NAC) and catalase effectively blocked active anthocyans-induced JNK phosphorylation, caspase-3 activation, and DNA fragmentation. Thus, active anthocyans might trigger an apoptotic death program in HL-60 cells through a ROS/JNK-mediated mitochondrial death pathway.

In summary, this study provided the first molecular basis for the cancer chemopreventive effects of anthocyans, demonstrating that anthocyans might have chemopreventive effects on several key steps involved in carcinogenesis.

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Acknowledgments

I thank Prof. Norio Matsuki (Graduate School of Pharmaceutical Sciences, The University of Tokyo) for providing me with a great opportunity, and for many pieces of intellectual guidance along the way. Thanks also to all of the members of thesis committee, Prof. Tatsuro Irimura, Prof. Tetsuo Nagano, Dr. Nobuaki Higashi and Dr. Kenji Kontani (Graduate School of Pharmaceutical Sciences, The University of Tokyo) for spending times to read the dissertation and to give very helpful comments.

In particular, my gratitude goes out to Prof. Makoto Fujii (Faculty of Agriculture, Kagoshima University) and Dr. Kazunari Yokoyama (Tsukuba Institute, The Institute of Physical and Chemical Research (RIKEN)) for their encouraging to challenge along the way.

Thanks to Dr. Nancy Colburn (National Cancer Institute, NIH, USA) for providing the JB6 cell lines, and to Prof. Norihiko Terahara (Minami-Kyushu University) for providing the sample of delphinidin 3-sambubioside. Thanks also to Mr. Takashi Ose, Ms. Keiko Kai, and Mr. Takashi Yanagita and Dr. Takuhiro Uto for their technical assistants in some experiments.

Finally, thanks to my wife and son who are supporting me along the way with love and patience.

Chapter 1 Introduction

1.1 Overview of anthocyans

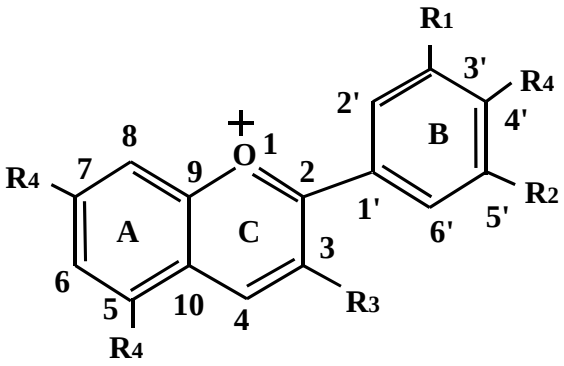
1.1.1 Chemical structure

Anthocyans (in Greek *anthos* means flower, and *kyanos* means blue) are flavanols, which occur ubiquitously in the plant kingdom and confer bright red or blue coloration on many fruits and vegetables. Anthocyan encompasses “anthocyanin” for the glycoside and “anthocyanidin” for the aglycon. They are water-soluble glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts. The differences between individual anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars attached to the molecule, the position of this attachment, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule [1] (Figure 1.1A). Depending on their pH and the presence of chelating metal ions, they are intensely colored in blue, violet or red. Up to date, more than 400 anthocyanins have been found in nature [1-4]. On the other hand, the aglycon is a diphenylpropanoide-based polyphenolic ring structure, and is limited to a few structure variants. Only six kinds of anthocyanidins including delphinidin, cyanidin，petunidin, pelargonidin，peonidin and malvidin are common in fruits and vegetables (Figure 1.1B) although other 11 kinds of anthocyanidins including apigeninidin, aurantinidin, capensinidin, europinidin, hirsutidin, 6-hydroxycyanidin, luteolinidin, 5-methylcyanidin, pulchellidin, rosinidin, and tricetinidin have been also found in plants [1-4].

1.1.2 Food source

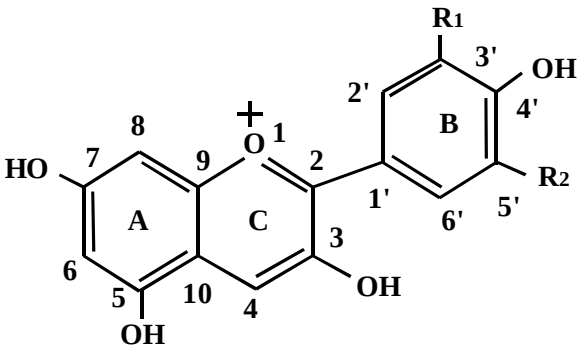
Anthocyans are widely spread in colored crops, fruits and vegetables such as berries, purple sweet potatoes, red grapes and cabbages [2-4]. They are present in very large amounts in some diets. Servings of 200 g of aubergine or black grapes can provide up to

A



R_1 are R_2 are H, OH, or OCH_3
R_3 is a glycosyl or H
R_4 is OH or a glycosyl

B



Anthocyanidin	R_1	R_2
Delphinidin	OH	OH
Cyanidin	OH	H
Petunidin	OCH_3	OH
Pelargonidin	H	H
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3

Figure 1.1. Chemical structure of anthocyanins (A) and anthocyanidins (B).

1500 mg anthocyans and servings of 100 g of berries up to 500 mg. Daily intake of anthocyans in humans has been estimated from several milligrams to grams [5, 6], which depends on the nutrition customs. Mean dietary intake in Finland has been estimated to be 82 mg/day [6]. The American diet can have much as 180-215 mg/day [5, 6]. Recently, daily intake of several grams can be also obtained if an individual is consuming flavonoids supplements, such as bilberry, grape or elderberry extracts [7].

1.1.3 Epidemiological evidence

There are some reports hinting at the potential anti-carcinogenicity of anthocyans. In a cohort of elderly individuals, who consumed large amounts of strawberries, the odds ratio for developing cancer at any site was 0.3, compared to subjects who refrained from high berry consumption [8]. Consumption of colored fruits and vegetables has also been associated with a reduced risk of human breast cancer [9] and colorectal polyp recurrence [10]. Anthocyan-containing foodstuffs have been linked with a decreased risk of coronary heart disease. They have been shown to possess beneficial effects in several parts of the organism [11], including the central nervous system and the eye, and have been suspected to account, at least in part, for the “French paradox”, i.e. the decreased risk of cardiovascular disease despite a high-fat diet in individuals living in France.

1.1.4 Biological activity

1.1.4.1 Antioxidant activity

Due to polyphenolic nature of anthocyans, they are efficient antioxidants in *in vitro* systems [12-14]. The antioxidant potency of anthocyans is modulated by their different chemical structure. Noda *et al.* [12] have evaluated antioxidant activities of three major anthocyanidins including delphinidin, cyanidin and pelarogonidin, using an electron spin resonance (ESR) technique with spin trapping. Anthocyanidins exhibited scavenging

activity against hydroxyl and superoxide radicals. The 50% inhibition dose ($ID_{(50)}$) of delphinidin, cyanidin, and pelargonidin were 2.4, 22, and 456 µM, respectively. These compounds also inhibited H_2O_2-induced lipid peroxidation in the rat brain homogenate [13]. The $ID_{(50)}$ values of delphinidin, cyanidin, and pelargonidin were 0.7, 3.5, and 85 µM, respectively. The results suggest that the hydroxyl groups on the B-ring of anthocyanidins may contribute to the antioxidant activities [13, 14].

Several lines of animal experiments have reported that anthocyans act as potent antioxidants *in vivo* when acute oxidative stress is encountered [15-19]. Ramirez-Toetosa *et al.* [15] investigated the antioxidant potency of anthocyanins in vitamin E-depleted rats. Consumption of the anthocyan-repleted diet significantly improved plasma antioxidant capacity and decreased the vitamin E deficiency-enhanced hydroperoxides and 8-*oxo*-deoxyguanoside concentrations in liver. Thus, dietary consumption of anthocyan-rich foods might contribute to overall antioxidant status, particularly in areas of habitually low vitamin E intake. Using hepatic ischemia-reperfusion as an oxidative stress model, Tsuda *et al.* [16] found that orally administered cyanidin 3-glucoside (Cy3-Glu) significantly suppressed the changes, such as elevation of the liver thiobarbituric acid-reactive substance (TBARS) concentration and the serum activities of marker enzymes (glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and lactate dehydrogenase) for liver injury, caused by hepatic ischemia-reperfusion. The results suggest that orally administered Cy3-Glu functions as a potent antioxidant *in vivo* under oxidative stress.

1.1.4.2 Antimutagenic activity

A variety of mutagens and carcinogens have been found in daily foods. Yoshimoto *et al.* [20–22] observed that the purple-colored sweet potatoes strongly decreased the reverse mutations in *Salmonella typhimurium* TA 98. Furthermore, the purified anthocyanin

compound, 3-(6,6'-caffeylferulylsophoroside)-5-glycoside of cyanidin (YGM-3), showed a powerful antimutagenic capacity, and the capacity was stronger than peonidin derivatives such as 3-(6,6'-caffeylferulylsophoroside)-5-glycoside of peonidin (YGM-6) [22]. The data suggest that the hydroxyl groups on the B-ring of anthocyanidins may contribute to the antimutagenic activities.

1.1.4.3 Anticarcinogenic activity

Some studies have reported the anticarcinogenic activity of anthocyan-rich extracts from various vegetables and fruits. Hagiwara *et al.* [23] performed an animal experiment in 1,2-dimethylhydrazine (DMH)-initiated F344/DuCrj rats. Anthocyans from purple sweet potatoes and red cabbages were given at a dietary level of 5.0% in combination with 0.02% of 2-amino-1-methyl-6-phenylimidazo (4,5-b) pyridine (PhIP), a promoter, in the diet until 36 weeks. Lesion development induced by DMH and PhIP was suppressed by the anthocyans. The marked inhibitory effects on colon carcinogenesis were apparent for the anthocyans comprising cyanidin, not peonidin, as main constituents, suggesting that the inhibition of anthocyans on carcinogenesis may be related to the number of hydroxyl groups on the B-ring. Miyata *et al.* [24] reported that grapefruit juice intake clearly suppressed PhIP-induced DNA damage in rat colon, and suggested that anthocyans may be implicated in the prevention of colon cancer. Other studies reported that anthocyans could inhibit the growth or proliferation of human tumor cells *in vitro* [25-30], such as human liver cancer cell line HepG2 [25], human vulva carcinoma cell line A431[26], and human fibrosarcoma HT-1080 [27]. However, the molecular mechanisms are not clear.

1.1.4.4 Antiinflammatory activity

Using prostaglandin endoperoxide H synthase-1 and -2 isozymes (PGHS-1, and -2) assay for detecting potential antiinflammatory effect *in vitro*, Wang *et al.* [31] found that

anthocyanins and their aglycon, cyanidin, from tart cherries could inhibit the activities of the human PGHS-1 and -2 enzymes, and cyanidin showed better inhibition than aspirin. Seeram *et al.* [32] found that cyanidin showed *in vitro* superior inhibition on the activity of cyclooxygenase, which converts arachidonic acid to prostaglandin H_2 (PGH_2). The inhibitory activity was comparable to that of naproxen, which is a nonsteroidal anti-inflammatory drug (NSAID). Anthocyanins also showed inhibitory effects on production of nitric oxide (NO) [33] and tumor necrosis factor *alpha* (TNF-α) in lipopolysaccharide (LPS) / interferon-*gamma* (IFN-γ)-activated RAW 264.7 macrophages [34].

1.1.5 Bioavailability

Since anthocyans have received attention as important dietary constituents that may contribute to human health benefits, the bioavailability and efficacy of anthocyans are noticed recently. Most of studies indicated that anthocyans can be directly absorbed, and thus should have bioavailability in humans and animals [35-44]. Matsumoto *et al*. [37] have performed an orally administered experiment of anthocyanins in rats and humans. Four components of black currant anthocyanins, delphinidin 3-rutinoside (Dp3-Rut), delphinidin 3-glucoside (Dp3-Glu), cyanidin 3-rutinoside (Cy3-Rut) and Cy3-Glu were found to be directly absorbed and distributed to blood, and excreted into urine as the glycosylated forms. In a pharmacokinetic study of anthocyanins in elderly women, anthocyanins were detected as glycosides in plasma and urine [38-40]. The maximum plasma concentration of total anthocyanins varied from 55.3 to 168.3 nmol/L, with an average of 97.4 nmol/L, and was reached within 71.3 min. The plasma antioxidant capacity in rats was significantly elevated from 58.0 to 89.2 μmol of Trolox equivalent/L of plasma 30 min after the oral administration of acylated anthocyanins in purple-fleshed sweet potatoes [41]. These data indicate that anthocyans are bioavailable for human and animals.

However, the absorption efficiency seems underestimated because previous studies were only based on the measurement of unchanged glycosides of anthocyans *in vivo*, some important metabolites were ignored.

1.2 Carcinogenesis

Carcinogenesis is a multistep processes (Figure 1.2A), as was first described in 1965 by Leslie Foulds, who deduced that there were multiple, pathologically distinguishable stages in the process of cancer induction and tumor progression for many human epithelial cancers [45]. To study the stages of carcinogenesis, mouse skin model has been developed and well established [46]. Based on the model, carcinogenesis can be categorized into three stages: initiation, promotion and progression [45, 46]. Initiation is defined as a mutagenic event in nature and generally results from DNA damage by physical, chemical or viral exposure. Promotion is characterized by transformation of an initiated cell into a population of preneoplastic cells as a result of epigenetic alterations in the cell by chronic exposure to tumor promoters, such as growth factors, hormones or ultraviolet (UV) irradiation. Progression is regarded as a final stage of carcinogenesis, which converts the preneoplastic cells into an invasive and metastatic cell population. These steps are made up of rate-limiting molecular events that occur along signal transduction pathways with attendant alteration of the expression of genes whose products are associated with transformation, inflammation, proliferation, apoptosis and other biological processes (Figure 1.2B). Accumulated data demonstrated that the signaling pathway of mitogen-activated protein kinase (MAPK) is one of most important regulators during carcinogenesis. MAPK, characterized as proline-directed serine/threonine kinase [47-49], is an important cellular signaling component that converts various extracellular signals into intracellular responses through serial phosphorylation cascades [50]. MAPK pathway consists of a three-tiered kinase core where a MAPK kinase kinase (MAPKKK) activates a

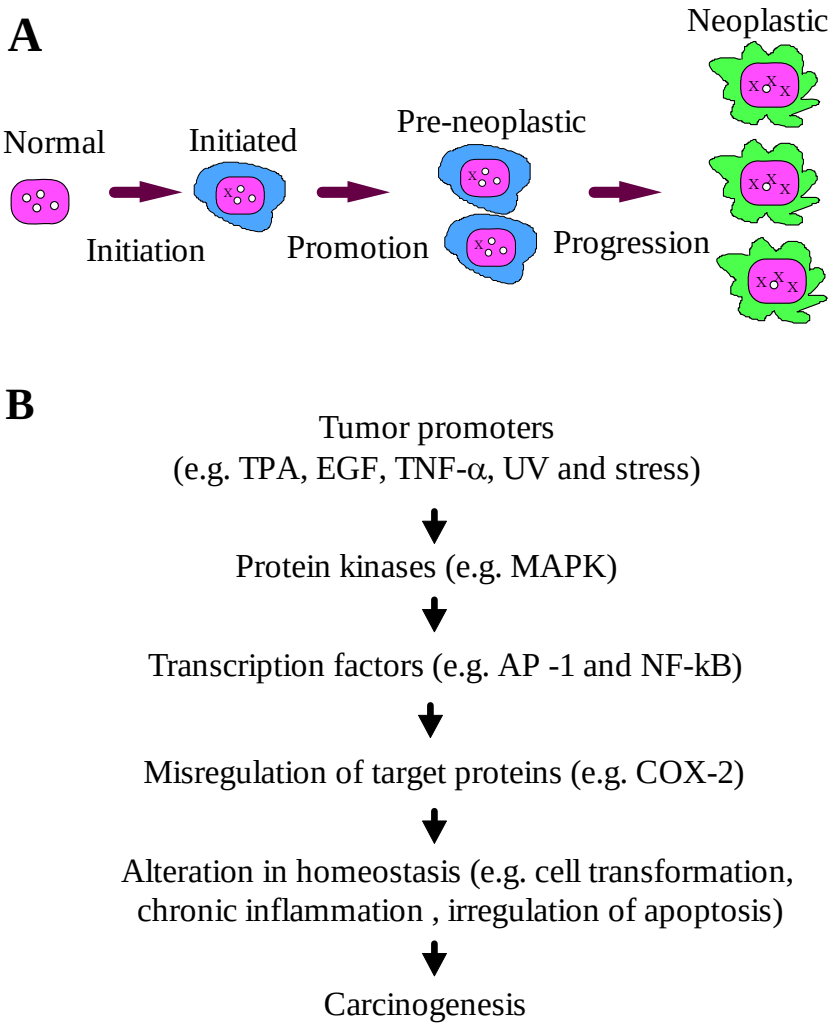


Figure 1.2. Schematic presentation of multistage carcinogenesis. (A) A three-stage process of carcinogenesis, involving tumor initiation, promotion and progression. Rate-limiting events occur during tumor promotion, which yields benign pre-malignant tumors, and during tumor progression to carcinomas that might become invasive or meta-static. **(B)** Extracellular tumor promoters activate signaling cascades, resulting in altered gene transcription in the nucleus. This leads to misregulation of protein expression or activity, and further alteration in homeostasis such as cell transformation, chronic inflammation, and irregulation of apoptosis. Finally, they lead to carcinogenesis.
AP-1: activator protein-1; COX-2: cyclooxygenase-2; EGF: epidermal growth factor; MAPK: mitogen-activated protein kinase; NF-κB: nuclear factor κB; TNF-α: tumor necrosis factor-α; TPA: 12-*O*-tetradecanoylphorbol-13-acetate; UV: ultraviolet.

MAPK kinase (MAPKK) that activates a MAPK (extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 kinase), resulting in activation of a number of genes regarding cell transformation, proliferation, apoptosis and other biological processes such as inflammation. Of which, activator protein-1 (AP-1), nuclear factor κB (NF-κB) and cyclooxygenase-2 (COX-2) are the important targets of MAPK.

AP-1 is a transcription factor that regulates the expression of several genes. Functional activation of the AP-1 transcription complex is implicated in tumor promotion as well as malignant transformation. This complex consists of either homo- or heterodimers of the members of the Jun and Fos family of proteins [51, 52]. The AP-1 mediated transcription of several target genes can also be activated by a complex network of signaling pathways such as MAPK cascade

NF-κB is an inducible transcription factor for genes involved in cell survival, cell differentiation, and inflammation that are linked to cancer. NF-κB is a heterodime protein of p65/p50 subunit and binds to a common sequence motif in the DNA called the κB site [53-55]. In most resting cells, NF-κB is sequestered in the cytoplasm by binding to the inhibitory IκB proteins which blocks the nuclear localization sequences of NF-κB. NF-κB is activated by a variety of stimuli such as carcinogens, inflammatory agents, tumor promoters including cigarette smoke, phorbol esters, okadaic acid, H_2O_2 and TNF. These stimuli promote dissociation of IκB through phosphorylation, ubiquitination and its ultimate degradation in the proteasomes. This process unmasks the nuclear localization sequence of NF-κB, facilitating its nuclear entry, binding to κB regulatory elements and activating transcription of target genes.

COX-2 is the gene of cyclooxygenase-2 (COX-2), which is the key enzyme in the biosynthesis of the prostaglandins. Tumor promoters, growth factors, cytokines and bacterial LPS stimulate COX-2 transcription [56]. Multiple lines of evidence indicated that COX-2 is involved in many inflammatory processes and overexpressed in various

carcinomas, suggesting that COX-2 is a key factor in tumorigenesis [57].

1.3 Cancer chemoprevention

Cancer chemoprevention, a relatively new and promising strategy to prevent cancer, is defined as the use of either natural or synthetic substances or their combination to block, reverse or retard the process of carcinogenesis [58, 59]. Accumulating evidence from both

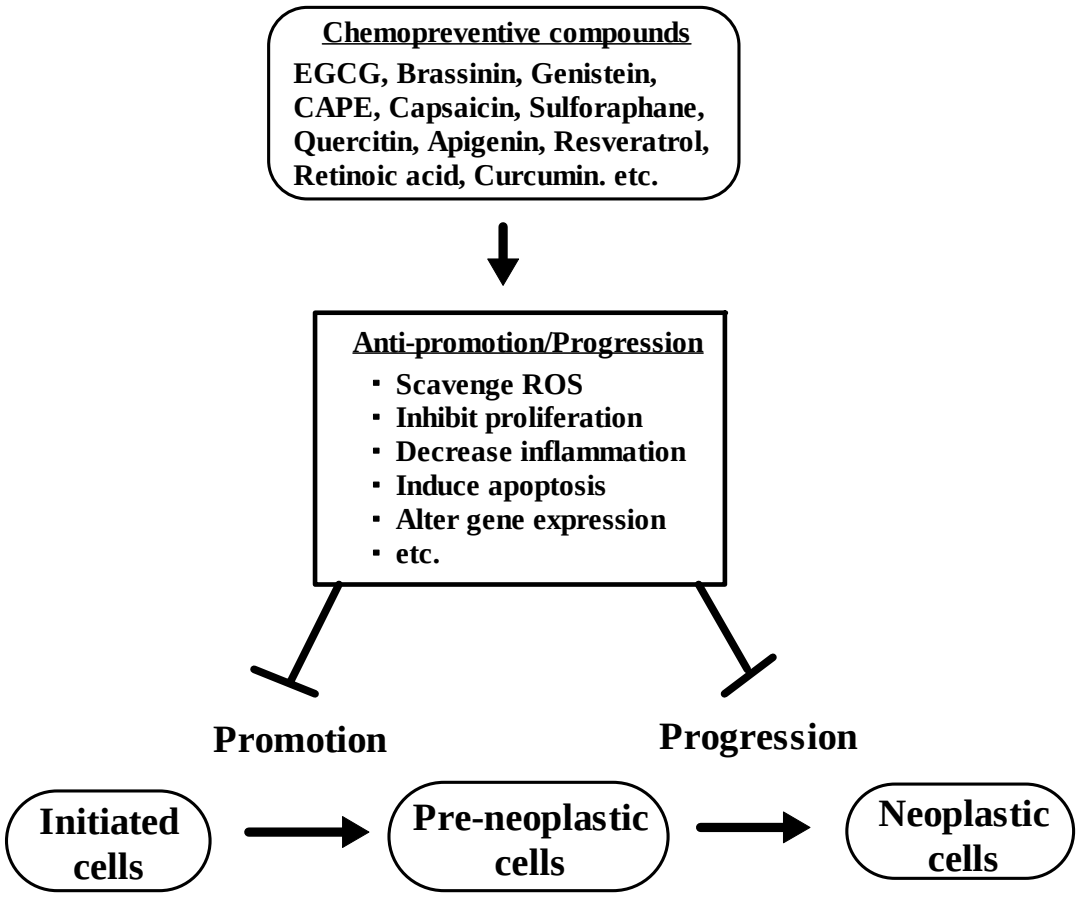


Figure 1.3. Schematic presentation of cancer chemoprevention by chemopreventive compounds. The chemopreventive compounds including natural or synthetic substances can scavenge ROS, inhibit cell proliferation, decrease inflammation, and /or induce apoptosis to block promotion and/or progression. Therefore, they play important roles in cancer prevention. EGCG: epigallocatechin gallate; CAPE: caffeic acid phenylethyl ester; ROS: reactive oxygen species.

population-based and laboratory studies indicate an inverse relationship between regular consumption of fruits and vegetables and the risk of cancer in general. A recent report published by American Institute for Cancer Research regarding dietary prevention of cancer indicates that about 7-31% of all cancer worldwide could be reduced by diets high in fruits and vegetables [60]. Attention has recently been focused on non-nutritive phytochemicals present in plant-based diet as potential chemopreventive agents. It is now estimated that more than 1000 different phytochemicals possess chemopreventive activities [61]. Examples are curcumin from turmeric, gingerol from ginger, epigallocatechin gallate (EGCG) from green tea, brassinin from cruciferous vegetables, genistein from soya, capsaicin from hot chili pepper, sulforaphane from broccoli, resveratrol from grapes, caffeic acid phenylethyl ester (CAPE) from honey bee propolis, etc. [62-64]. It is becoming clear that these dietary components exert pleiotropic effects on cancer chemoprevention, including antitransformation, antiinflammation and apoptosis induction, by targeting a number of cancer-related molecules, such as MAPK signaling pathway and oncogenic genes [65-67] (Figure 1.3).

1.3.1 Targets for anti-promotion

Tumor promotion is a stepwise process occurring with comparatively low frequency, and requires the chronic action of tumor promoters. In many major cancer targets, human cancer development requires 20 ~ 40 years or more [68]. Thus, tumor promotion is considered a rate-limiting step. Although the promotion in human may be more complex, the mouse *in vivo* model provides an excellent example to investigate papilloma formation in response to tumor promoters such as TPA and EGF [68, 69], and the mouse epidermal cell line, JB6, allowed a detailed investigation of the molecular events specific to tumor promotion [70, 71]. Many studies using these two models have identified dietary components that act through diverse mechanisms to suppress tumor promotion. For

instance, many natural products, such as retinoic acid [72, 73], isoflavone genistein [74], EGCG [75], resveratrol [76], and curcumin [77] were demonstrated to suppress tumor promotion by targeting a number of signaling transduction pathways such as MAPK with attendant activation of cancer-related genes.

1.3.2 Targets for decreasing inflammation

Tumor promoters can elicit the production of proinflammatory cytokines (such as TNF and several interleukins) and nonprotein factors (such as NO) involved in inflammation and carcinogenesis [78]. Of critical importance to carcinogenesis is the prostaglandin synthesis [79, 80], which is regulated by COX-2. The COX-2 gene has been referred to as an inducible immediate early gene product by tumor promoters such as such as TPA and LPS [81]. Thus, the identification of COX-2 inhibitors is considered to be a promising approach to prevent cancer. Many chemopreventive agents have been found to have anti-inflammatory properties by down-regulating COX-2 expression [82, 83].

1.3.3 Targets for inducing apoptosis

Apoptosis plays important roles in many biological processes including carcinogenesis. Apoptosis represents an important cellular protective mechanism against neoplastic transformation, whereby eliminating a damaged cell or repressing the outgrowth of transformed cells that have been improperly induced to divide even in the absence of mitotic stimuli. Since disruption of apoptosis promotes survival and outgrowth of damaged or initiated cells, suppression of this physiologic phenomenon may result in non-genotoxic carcinogenesis. Therefore, induction of apoptosis in precancerous or malignant cells is considered as another promising strategy of cancer chemoprevention [84].

Many chemotherapeutic agents have been found to induce apoptotic cell death [85, 86]. Similarly, many chemopreventive agents can also induce apoptosis. These agents

include retinoic acid [87], sulindac [88], curcumin [88], perilly alcohol [89], phenethylisothiocyanate [88, 90], EGCG [91], apigenin [92], quercetin [93], silibinin [94], genestin [95], silymarin [96], and resveratrol [76]. They targeted cellular signaling events leading to apoptosis. Apoptosis induced by some of these agents is reported to be at least partially responsible for their chemopreventive activities.

In summary, accumulated studies indicate anthocyans have wide food source, considerable bioavailability, and biological activities including antioxidation, antimutagenicity, antiinflammatory, and antiproliferation of some cancer cells. These facts suggest that anthocyans might be potential chemoprevention agents, and that mechanisms on chemopreventive effects need to be considered at molecular level. Most of cancer chemoprevention-related compounds, such as components of tea or red wine, act to prevent tumor promotion by targeting signal transduction pathways to attenuate the expression of AP-1, and/or COX-2, or by inducing cell cycle arrest and apoptosis. Therefore, the preset study is to characterize the chemopreventive effects of anthocyans by targeting those well-accepted cellular/molecular mechanisms that can at least partially explain the effectiveness of natural compounds as chemopreventive agents. The contents include that (1) anthocyans inhibit neoplastic transformation through the inhibition of AP-1 activation; (2) anthocyans suppress inflammation by blocking COX-2 overexpression; and (3) anthocyans inhibit proliferation/or growth of tumor cells by inducing apoptosis.

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Chapter 2 Molecular characterization of the inhibitory effects of cell transformation by anthocyans in mouse epidermal cells

2.1 Introduction

Extensive studies indicate that anthocyans have strong free radical scavenging and antioxidant activities [1-5], those have been demonstrated to play important roles in prevention against mutagenesis and carcinogenesis [6]. Anthocyans also show inhibitory effects on the growth or proliferation of some cancer cells [7-11]. Recent study reported that oral intake of anthocyans from purple sweet potato and red cabbage suppressed rat colon carcinogenesis induced by DMH and PhIP [12]. However, the molecular mechanism is not clear. Thus, the mechanisms for anthocyans application in anticarcinogenesis are considered to elucidate at molecular level.

Carcinogenesis is a multistage process that encompasses three independent steps: initiation, promotion, and progression [13, 14]. During these steps, a number of critical gene regulation events occur. Thus, understanding the molecular basis of carcinogenesis is important for prevention of carcinogenesis. Accumulated studies have suggested that the transcription factor AP-1 plays an important role in promoting carcinogenesis [15, 16]. AP-1 is a dimeric protein typically composed of the products of the *jun* and *fos* oncogene families [16]. AP-1 dimers bind to the promoter regions on DNA that contain TPA response elements (TRE) to activate the transcription of genes involved in cell proliferation [16], transformation [17, 18], and apoptosis [19]. A variety of stimuli, such as phorbol esters [17, 18, 20], UV radiation [21], growth factors [22] and oxidative agents [23], can stimulate AP-1 activity by activating MAPK, such as ERK, JNK, and p38 kinase. Increased AP-1 activity has been shown to be involved in the tumor promotion and

progression of various types of cancers, such as skin [24, 25], lung [26] and breast cancer [27]. *In vivo* mouse data also demonstrate that AP-1 activity is required for tumor promotion [28, 29].

JB6 mouse epidermal cells provide a cell culture-based model for studying tumor promotion [15] (Figure 2.1). Observations first recorded in the JB6 model have been validated in other culture models, including human keratinocytes [30, 31], as well as *in vivo* models using transgenic mice [28]. In mouse epidermal cells, tumor promoters such as TPA, EGF and TNF-α induce AP-1 activity and neoplastic transformation in promotion-sensitive (P^+), but not promotion-resistant (P^-) JB6 cell lines by activating MAPK including ERK, JNK or p38 kinase [15, 17, 18]. The induced AP-1 activity and neoplastic transformation can be blocked by chemopreventive agents, such as retinoids [17, 32], pyrrolidine dithiocarbamate [33], tea polyphenols [34, 35], and glycoside compounds [36]. Many of these inhibitors have been shown to be active not only in the JB6 transformation model but also in mouse skin tumor promotion *in vivo.* Thus, mouse epidermal JB6 cells provide a validated model to screen cancer chemopreventive agents, and to elucidate their mechanisms at the molecular level.

Based on the anticarcinogenic effects of anthocyans and the role of AP-1 in carcinogenesis, the effects of the representative anthocyanins and anthocyanidins on TPA-induced AP-1 activity and cell transformation were investigated in mouse JB6 cells. The results demonstrated that the hydroxyl group on the B-ring is critical for the inhibition of cell transformation, and that active delphinidin blocked activation of ERK and JNK signaling pathways leading to AP-1 activation and cell transformation.

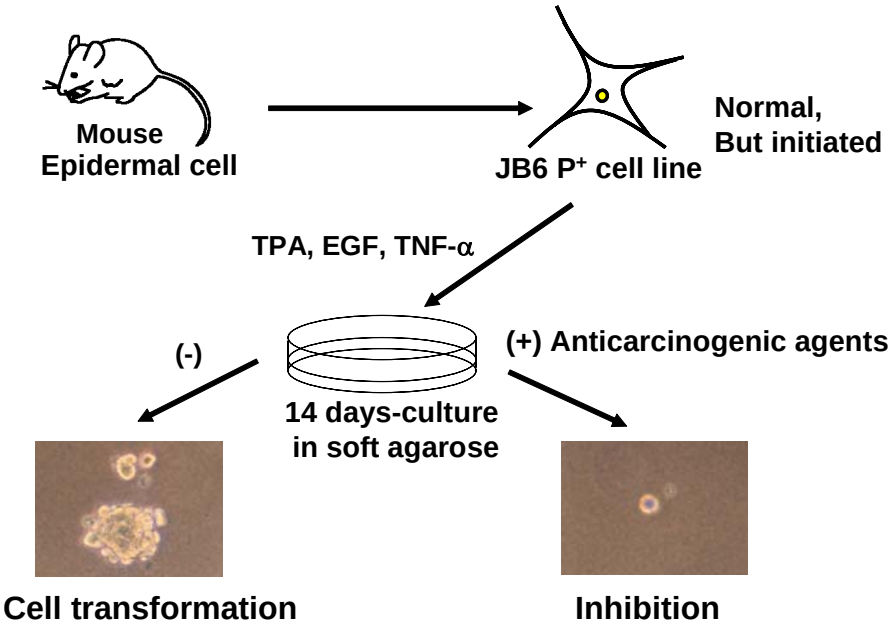


Figure 2.1. Mouse epidermal JB6 cell line provides a cell culture-based model for studying tumor promotion. The JB6 cell line was established from epidermal cells of newborn mouse. Upon induction by TPA, EGF or TNF-α, the tumor promotion-sensitive (P^+) JB6 cells proceed irreversibly to an anchorage-independent growth phenotype in soft agar culture for two weeks. The induced AP-1 activity and neoplastic transformation can be blocked by chemopreventive agents. Therefore, mouse epidermal JB6 cells provide a validated model to screen cancer chemopreventive agents, and to elucidate their mechanisms at the molecular level. TPA: 12-*O*-tetradecanoylphorbol-13-acetate; EGF: epidermal growth factor; TNF-α: tumor necrosis factor alpha.

2.2 Materials and methods

2.2.1 Reagents and cell culture

Cyanidin 3-sambubioside (Cy3-Sam) and delphinidin 3-sambubioside (Dp3-Sam) were isolated from the dried calices of *Hibiscus sabdariffa L.* [37]. Cyanidin 3-(6, 6'-caffeoylferuloylsophoroside)-5-glucoside (YGM-3) and peonidin 3-(6, 6'-caffeoyl-feruloylsophoroside)-5-glucoside (YGM-6) were isolated from purple sweet potatoes as

described previously [38] (Figure 2.2). The purity of those samples is over 99%. Delphinidin chloride, cyanidin chloride, pelargonidin chloride, peonidin chloride and malvidin chloride purified by high performance liquid chromatography (HPLC) were obtained from Extrasynthese (Genay, France) (Figure 1.1B), and the purity is 99.7, 98.7, 99.9, 99.4 and 95.4%, respectively. Petunidin chloride was prepared by acid hydrolysis of petanin (petunidin 3-*p*-coumaroyl- rutinoside-5-glucoside) isolated from purple potato [39], and purified by HPLC (purity is over 99%). Anthocyanins are dissolved in distilled water, and anthocyanidins were dissolved in dimethyl sulfoxide (DMSO, final concentration was 0.2%). Antibodies against phospho-MEK1/2, phospho-SEK1, phospho-ERK1/2, phospho-JNK, phospho-p38 kinase, phospho-c-Jun (Ser73), MEK1/2, SEK1, ERK1/2, JNK, p38 kinase, and c-Jun (Ser73) were from Cell Signaling Technology (Beverly, MA). Luciferase assay substrate was obtained from Promega (Madison, WI). Fetal bovine serum (FBS) was from Equitech-Bio (Kerrville, TX). TPA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT), *p*-iodonitrotetrazolium violet (INT), superoxide dismutase (SOD), D-mannitol and catalase were from Sigma (St. Louis, MO).

The JB6 P^+ mouse epidermal cell line, Cl41 [20], and its AP-1-luciferase reporter stable transfectant P^{+11} cells [32] were gifts from Dr. Nance Colburn (NIH, USA) and cultured at 37°C, 5% CO_2 in Eagle essential minimum medium (EMEM) containing 5% FBS, 2 mM L-glutamine and 25 μg/ml gentamicin.

2.2.2 Anchorage-independent transformation assay

The inhibitory effects of anthocyanins and anthocyanidins on TPA-induced cell transformation were investigated in the parental JB6 Cl41 cells or AP-1-luciferase stable transfectant JB6 cells (P^{+11}) [40]. There is no marked difference in TPA-induced

transformation between both cell lines (data not shown). Cells (1×10^4) were suspended in 2 ml of 0.38% BME agar medium over 3 ml of 0.5% BME agar medium containing 10% FBS, 20 ng/ml TPA with anthocyanins or anthocyanidins. The cultures were maintained in a 37°C, 5% CO_2 incubator for 14 days, and the anchorage-independent colonies after staining with INT were scored with a computerized image analyzer. The inhibitory efficiency of TPA-induced cell transformation by anthocyanins or anthocyanidins is expressed as a percentage of the transformation frequency when the cells were treated with TPA alone.

2.2.3 Luciferase assay for AP-1-dependent transactivation

AP-1-luciferase stable transfectant JB6 cells (P^{+11}) [40] were used to assay AP-1-dependent transactivation. Viable cells (2×10^4) were plated in a 48-well dish for 24 h before each experiment. The cells were starved by being cultured in 0.1% FBS-EMEM for another 18 h to eliminate the influence of FBS on AP-1 activity, and then treated with or without anthocyanidins for 30 min before they were exposed to 20 ng/ml TPA for 24 h. For antioxidant agents, the cells were treated with SOD, catalase or D-mannitol alone or with SOD plus anthocyanidins for 30 min, respectively, before the cells were exposed to 20 ng/ml TPA for 24 h. The cells were extracted with lysis buffer, and the luciferase activity was measured with a luminometer (Berthold) according to the supplier's recommendations. AP-1 activity is expressed as fold induction relative to the control cells without TPA treatment [41, 42].

2.2.4 Cell survival assay

The cell survival rate was measured by a MTT assay [41]. HL-60 cells (2×10^4/well) were incubated with different concentrations of anthocyanins or athocyanidins in 96-well plates for 48 h. MTT solution (5 mg/ml) was then added to each well and incubated for another 4 h. The resulting MTT-formazan product was dissolved by the addition of 0.04 N HCl-isopropanol solutions. Absorbance was measured at 595 nm with a microplate reader. The cell viability was expressed as the optical density ratio of the treatment to control.

2.2.5 Western blotting analysis

After the cells (1.5×10^6) were cultured in 10-cm dish for 24 h, the cells were starved in serum-free for another 4 h to eliminate the influence of FBS on MAPK activation [40]. The cells were then treated with or without anthocyanidins for 30 min before they were exposed to 20 ng/ml TPA for the different times. The harvested cells were lysed and the supernatants were boiled for 5 min. Protein concentration was determined, using dye-binding protein assay kit (Bio-Rad, Hersules, CA) as described in manufacture's manual. Lysate protein of 40 μg was run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to PVDF membrane (Amersham Pharmacia Biotech, England). After blotting, the membrane was incubated with specific primary antibody overnight at 4°C, and further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected by ECL system with a Lumino Image Analyzer. The relative amount of proteins associated with specific antibody was quantified, using the Imager Gauge Software (Fuji Photo Film).

2.2.6 Statistical analysis

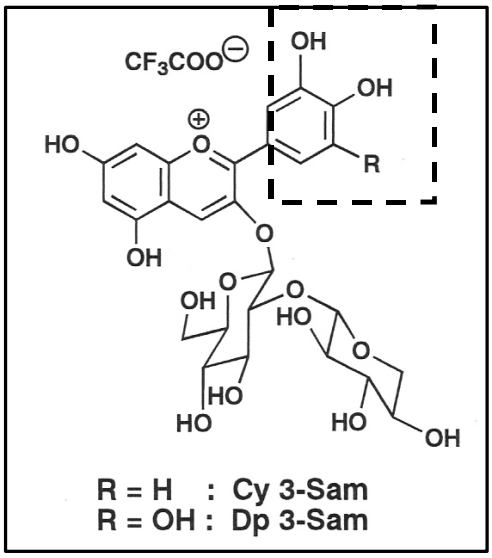
Difference between the treated and the control was analyzed by Student's *t*-test. A

probability of $P<0.05$ was considered significant. The multiplicative models for the interaction between two agents were used to determine the effect of a combination of SOD with delphinidin [43, 44]. The additive model predicts the effect of a combination to be equal to the effect of its constituents, and an observed effect of the combination higher than predicted by the additive model indicates synergism [43].

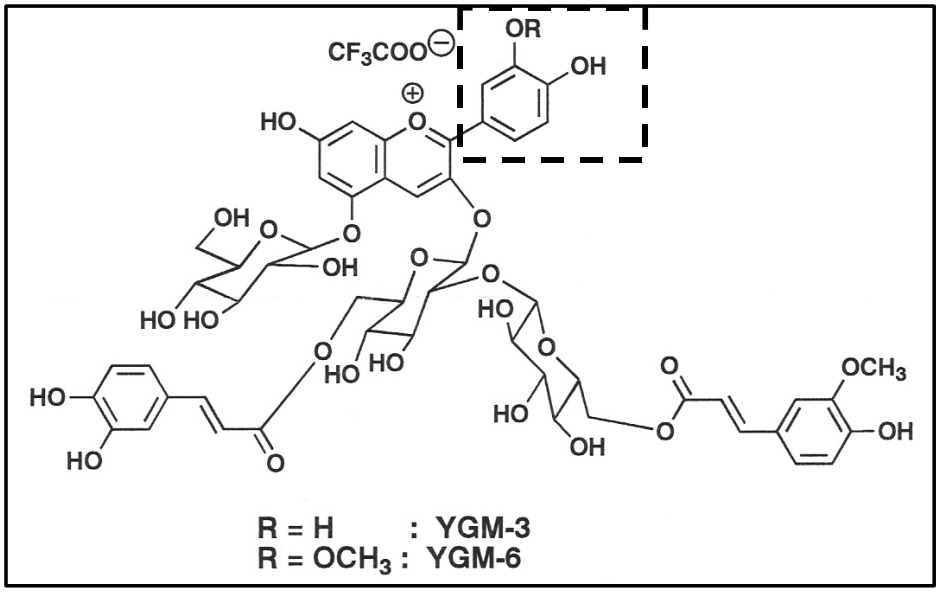
2.3 Results

2.3.1 The effects of anthocyanins on TPA-induced JB6 cell transformation

Cells (1×10^4) were exposed to 20 ng/ml TPA in soft agar for 14 days; 1000-2000 transformed colonies were induced. TPA-induced cell transformation was significantly inhibited by Dp3-Sam, Cy3-Sam and YGM-3, but not by YGM-6 (Figure 2.2) ($P<0.05$) at the concentration range from 5-20 μg/ml (Figure 2.3). The inhibitory actions by Dp3-Sam, Cy3-Sam and YGM-3 were not caused by their cytotoxicity because the concentration range that inhibited cell transformation did not affect cellular viability as measured by MTT assay (data not shown). It is noticed that YGM-3 and YGM-6 have same glucoside with different anthocyanidin (cyanidin for YGM-3, and peonidin for YGM-6). The results indicated that the inhibitory effects of anthocyanins appear to be related with anthocyanidin structure. The data from screening 18 kinds of anthocyanins indicate that the inhibitory effects of anthocyanins on TPA-induced JB6 cell transformation depend on the structure of anthocyanidins (data not shown). Thus, six kinds of anthocyanidins, which represent the aglycons of the most abundant anthocyanins, were chose to investigate the structure-activity relationship and molecular mechanisms.



Cy 3-Sam: Cyanidin 3-sambubioside
Dp 3-Sam: Delphinidin 3-sambubioside



YGM-6: Peonidin 3-(6, 6'-caffeoylferuloylsophoroside)-5-glucoside
YGM-3: Cyanidin 3-(6, 6'-caffeoylferuloylsophoroside)-5-glucoside

Figure 2.2. Chemical structure of anthocyanins used.

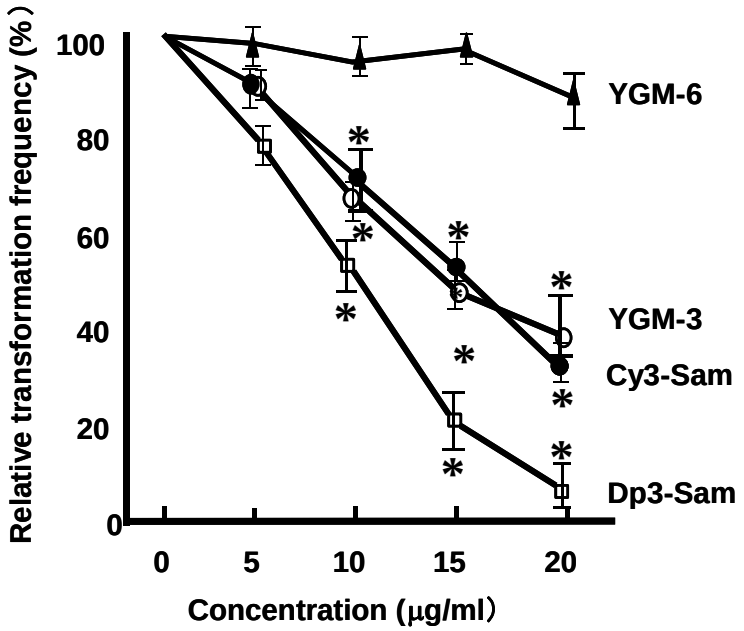


Figure 2.3. The effect of anthocyanins on TPA-induced JB6 cell transformation. JB6 Cl41 cells (1×10^4) were exposed to 20 ng/ml TPA with or without the indicated concentrations of anthocyanins on soft agar medium. The cell colonies were scored with a computerized image analyzer after 14-day incubation in a 37°C, 5% CO_2 incubator. The inhibitory efficiency of cell transformation by anthocyanins is expressed as a percentage of the transformation frequency when the cells were treated with TPA alone. Each value represents the mean ± SD of 4-5 separate experiments. *P<0.05, significantly different from TPA alone.

2.3.2 The effects of anthocyanidins on TPA-induced JB6 cell transformation and AP-1 activity

Six kinds of anthocyanidins (Figure 1.1B) were used to investigate the inhibitory effects of anthocyanidins on TPA-induced JB6 cell transformation and AP-1 activity. TPA-induced cell transformation was significantly inhibited by delphinidin, petunidin and cyanidin, but not by pelargonidin, peonidin and malvidin (P<0.05) at the concentration range from 5-20 μM (Figure 2.4A). Previous studies have suggested that AP-1 transactivation is required for TPA-induced cell transformation in mouse JB6 cells [17, 32,

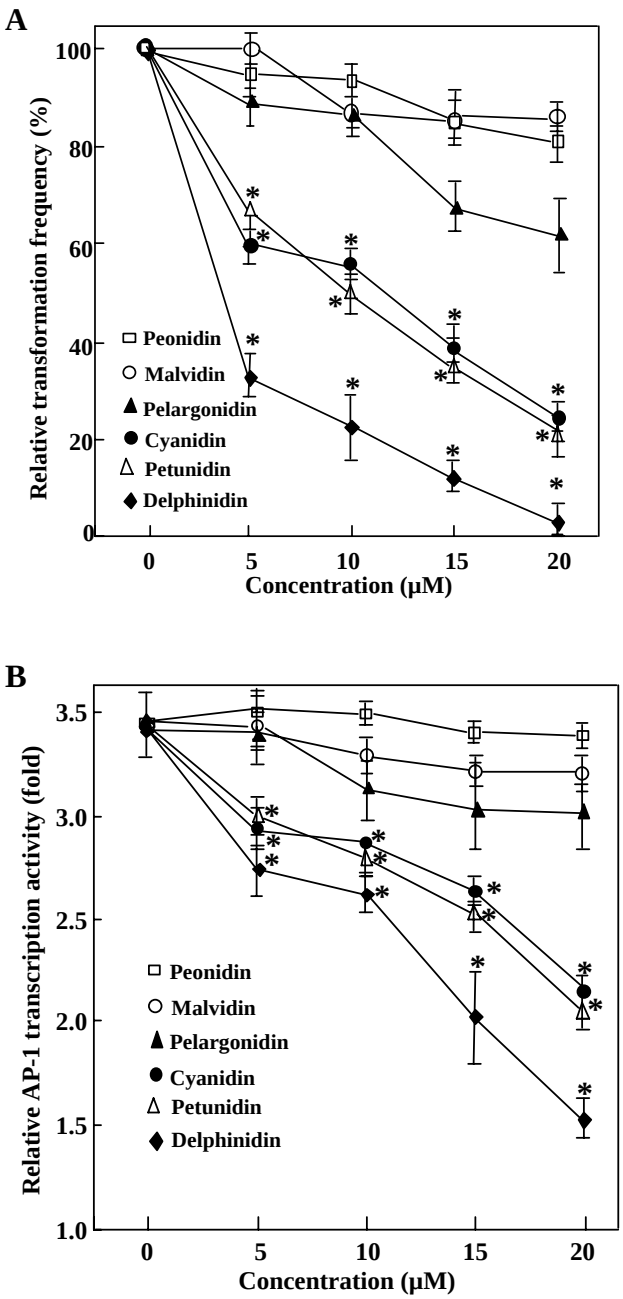


Figure 2.4. The effects of anthocyanidins on TPA-induced JB6 cell transformation (A) and AP-1 activation (B). The inhibitory efficiency of six kinds of anthocyanidins on cell transformation was estimated as described in Figure 2.3. For report gene assay, AP-1 luciferase reporter plasmid stable transfectant JB P^{+11} cells (2×10^4) were seeded into each well of 48-well plates. The cells were treated with or without the indicated concentrations of anthocyanidins for 30 min before they were exposed to 20 ng/ml TPA for 24 h. The luciferase activity was assayed, and AP-1 activity is expressed as fold induction to the control cells without TPA treatment. Each value represents the mean ± SD of 4-5 separate experiments. **P*<0.05, significantly different from TPA alone.

45]. Thus, the effects of those anthocyanidins on TPA-induced AP-1 activity were examined, using a reporter gene assay. The treatments of delphinidin, petunidin and cyanidin, but not pelargonidin, peonidin and malvidin, markedly inhibited TPA-induced AP-1 activity at the same concentration range (Figure 2.4B; $P<0.05$). The inhibitory actions by delphinidin, petunidin and cyanidin were not caused by their cytotoxicity, because the concentration range that inhibited cell transformation and AP-1 activity did not affect cellular viability as measured by MTT assay (data not shown). These results indicate that inhibition of AP-1 activity by delphinidin, petunidin and cyanidin is important in their inhibitory action against TPA-induced cell transformation.

The ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins (Figure 1.1B) may be essential for the inhibitory action because pelargonidin, peonidin and malvidin, having no such ortho-dihydroxyphenyl structure, failed to show the inhibitory effects.

2.3.3 Delphinidin blocks TPA-induced ERK and JNK phosphorylation, but not p38 phosphorylation

Because delphinidin showed the strongest potent inhibition in AP-1 transactivation, delphinidin was used to further investigate the effects on MAPK pathways. MAPK pathways including ERK, JNK and p38 kinase influence AP-1 transactivation by increasing the abundance of AP-1 components and/or altering the phosphorylation of their subunits [46, 47]. Previous studies indicated that ERK activation is earlier event occurring at 0.5 h - 4 h following TPA treatments, while activation of JNK and p38 kinase is a later event occurring at 6 h - 24 h in JB6 cells [47]. Thus, the times at 2 h and at 12 h were set to target ERK, and JNK or p38 kinase, respectively. As shown in Figure 2.5A, TPA induced markedly phosphorylation of ERK at 2 h and phosphorylation of JNK and p38 kinase at

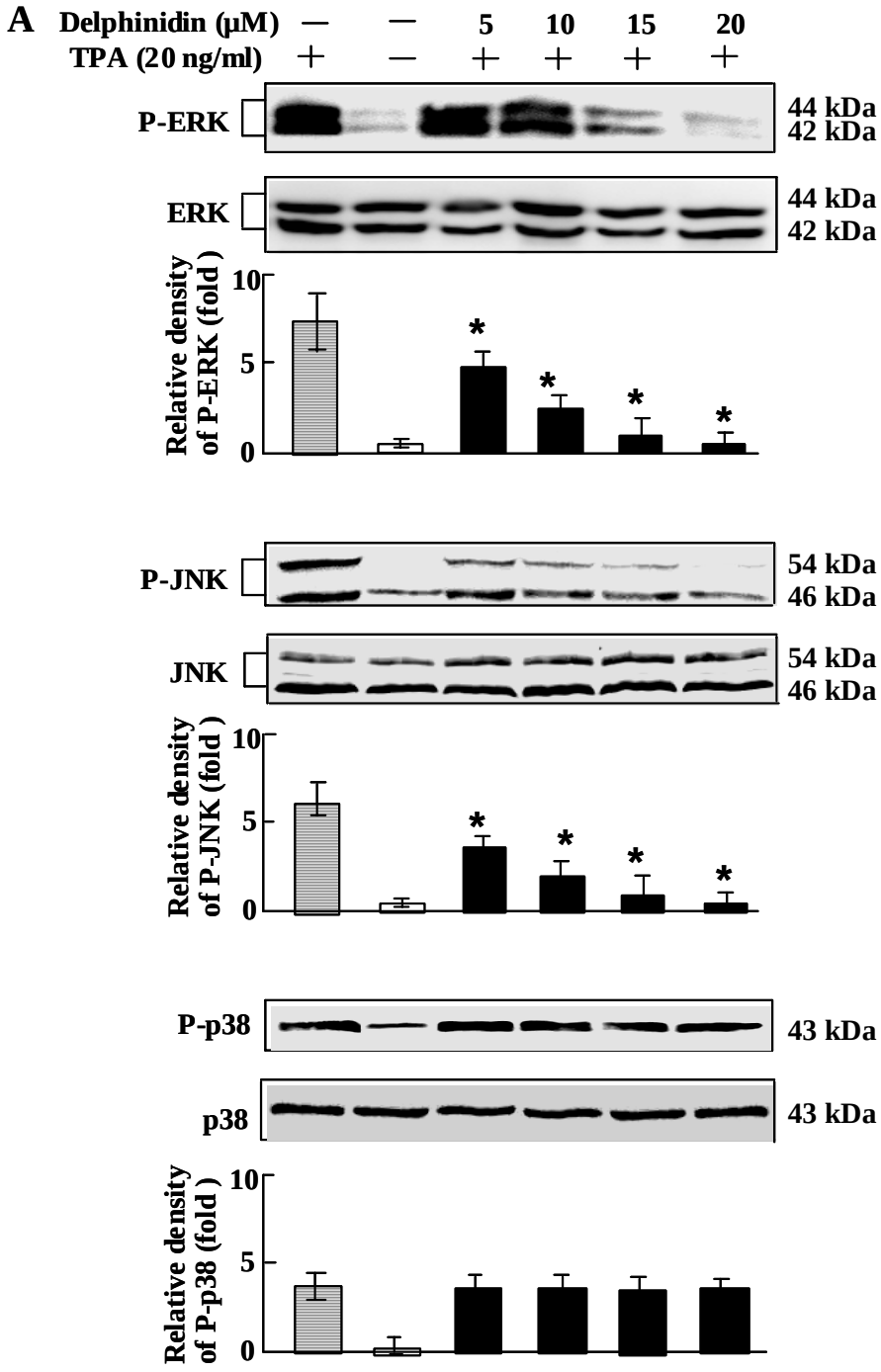


Figure 2.5. The effects of delphinidin (A) and peonidin (B) on TPA-induced MAPK phosphorylation. After JB6 cells were starved in serum-free medium for 4 h. The cells

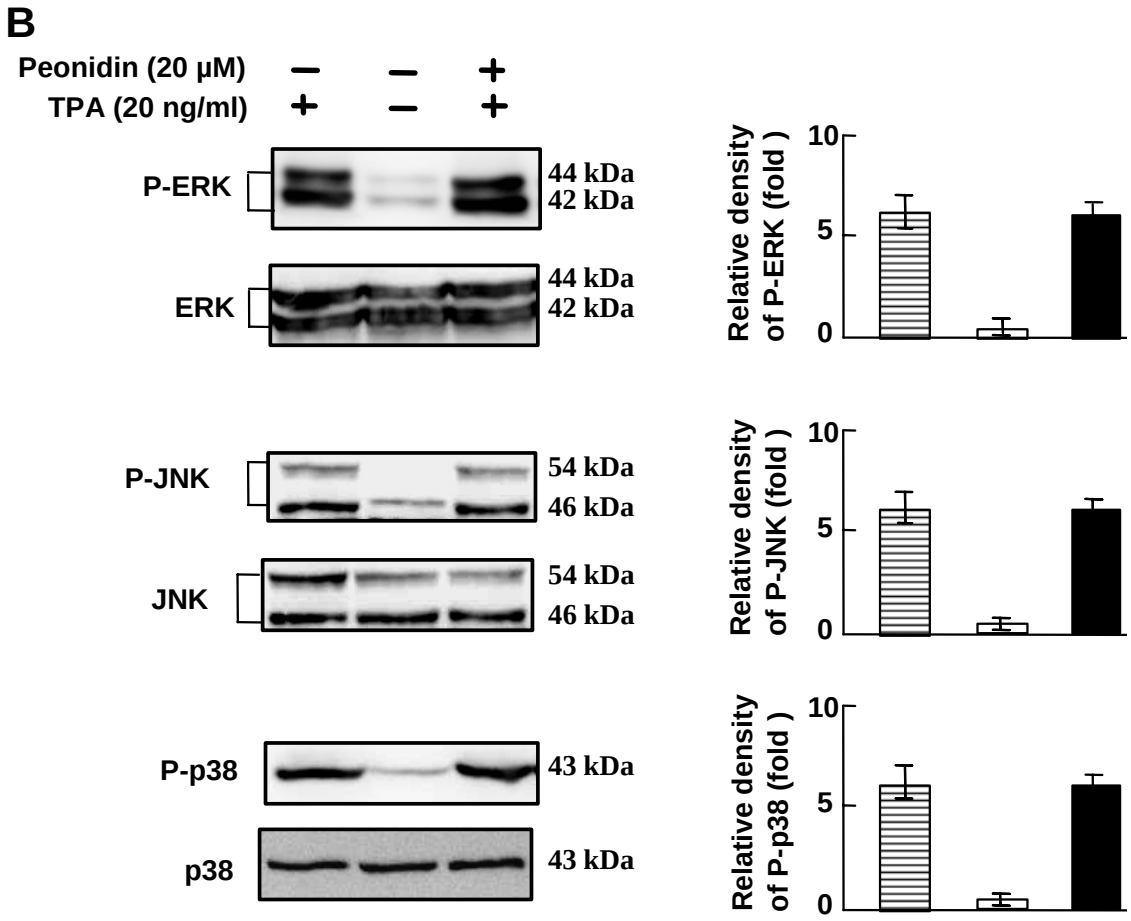


Figure 2.5 (continued)

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were treated with 20 μM delphinidin or peonidin for 30 min, and then exposed to 20 ng/ml TPA for 2 h to target ERK, and for 12 h to target JNK and p38 kinase. The proteins of total and phosphorylated MAPK were detected with the indicated specific antibodies as described in Materials and methods. Histograms show the densitometric analysis of phosphorylated protein normalized to total MAPK. The data represents the mean ± SD of 3-4 separate experiments, and the figure is a representative of those experiments each with similar results. * $P<0.05$, significantly different from TPA alone.

12 h (lane 1). Delphinidin suppressed TPA-induced phosphorylation of ERK and JNK, respectively, in a dose-dependent manner (lane 3-6). However, the phosphorylation of p38 kinase was not blocked by delphinidin. The results suggest that the mechanism by which delphinidin inhibits AP-1 transactivation may involve early inhibition of ERK phosphorylation and later inhibition of JNK phosphorylation, but does not involve targeting p38 kinase. As a control, the effect of peonidin, which showed no inhibition on cell transformation (Figure 2.4A) and AP-1 activity (Figure 2.4B), on MAPK was also examined. The data showed that peonidin did not block TPA-induced phosphorylation of JNK, ERK and p38 kinase (Figure 2.5B). These results further supported that the suppression of MAPK activation by anthocyanidins were associated with their inhibitory effects on AP-1 activation and cell transformation.

2.3.4 Delphinidin blocks the activation of JNK and ERK signaling cascades

MAPK/ERK kinase (MEK) is a protein kinase that phosphorylates and activates ERK [48-50]. Thus, the effect of delphinidin on the phosphorylation of MEK was further investigated. As shown in Figure 2.6A, delphinidin suppressed TPA-induced MEK1/2 phosphorylation at the same time- and dose-range that blocked TPA-induced ERK phosphorylation. On the other hand, SAPK/ERK kinase 1(SEK1) is another protein kinase that phosphorylates and activates JNK [51-53]. Delphinidin blocked TPA-induced SEK1 phosphorylation at the dose range that blocked TPA-induced JNK phosphorylation (Figure 2.6B). Moreover, the phosphorylation of c-Jun, which is a JNK target, was markedly blocked by delphinidin at the same concentration range while the total amount of c-Jun protein was not changed (Figure 2.6C). Thus, it appears that the inhibition of AP-1 activity by delphinidin is mediated by blocking ERK and JNK signaling cascades.

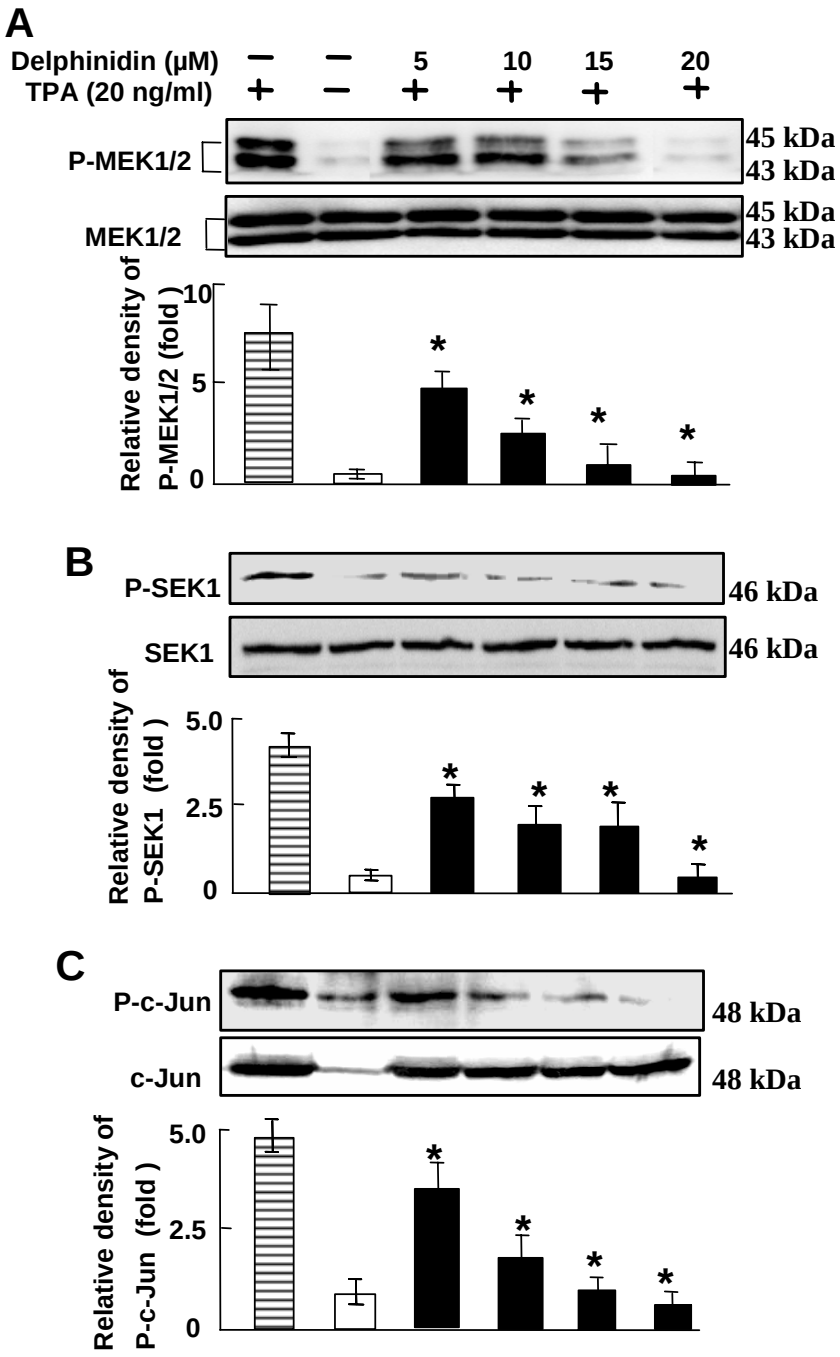

Figure 2.6. Delphinidin blocks TPA-induced phosphorylation of MEK1/2 (A), SEK1 (B) and c-Jun (C). Cell treatment and Western blotting analysis were done as described in Figure 2.5. Histograms show the densitometric analysis of phosphorylated protein normalized to total protein. The data represents the mean ± SD of 3-4 separate experiments, and the figure is a representative of those experiments each with similar results. * $P < 0.05$, significantly different from TPA alone.

2.3.5 UO126 and SP600125 suppress the activation of ERK and JNK and cell transformation

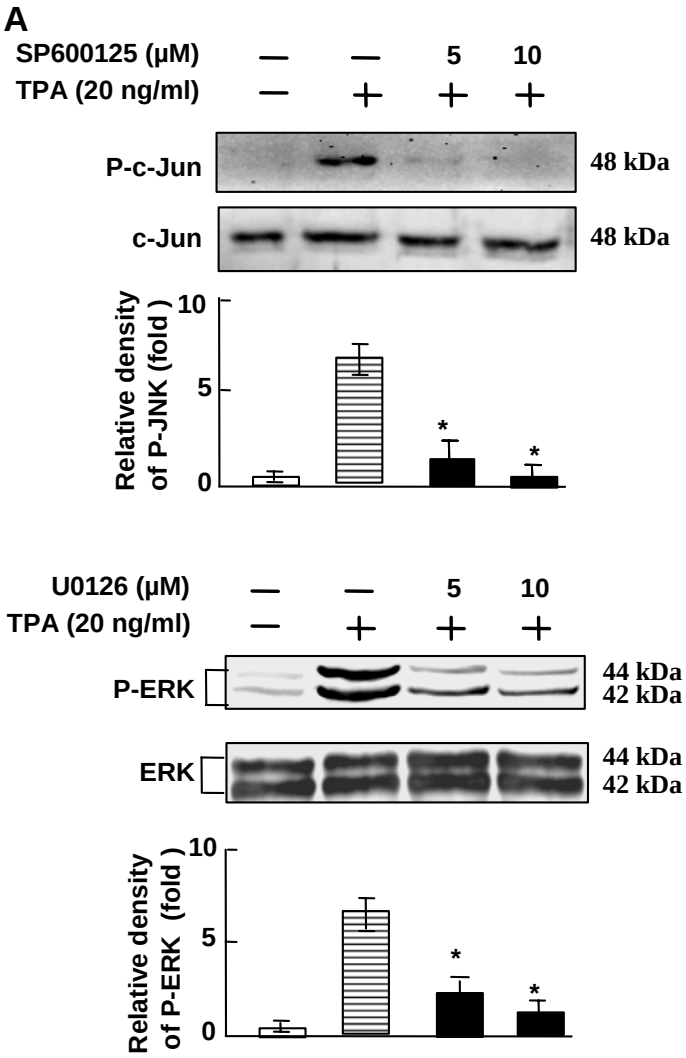

Figure 2.7. UO126 and SP600125 suppress phosphorylation of ERK and c-Jun (A), and cell transformation (B). Cell treatment and Western blotting analysis were done as described in Figure 2.5. In brief, the cells were treated with UO126 or SP600125 (5-10 μM) for 30 min, and then exposed to 20 ng/ml TPA for 2h or 12 h, respectively. Histograms show the densitometric analysis of phosphorylated protein normalized to total protein. The data represents the mean ± SD of 3-4 separate experiments, and the figure is a representative of those experiments each with similar results. * $P < 0.05$, significantly different from TPA alone.

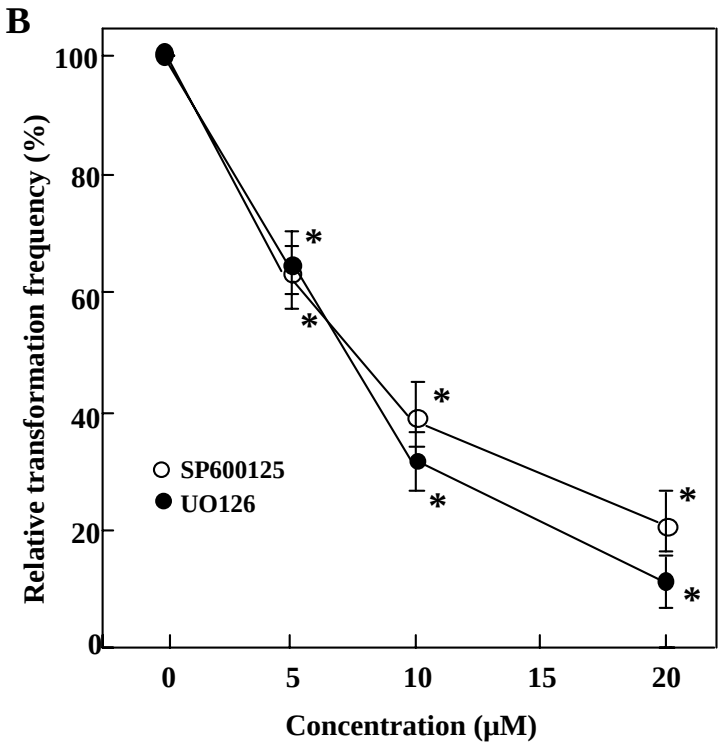

Figure 2.7 (continued)

For cell transformation, JB6 Cl41 cells (1×10^4) were exposed to 20 ng/ml TPA with or without the indicated concentrations of UO126 or SP600125 on soft agar medium. The inhibitory efficiency of UO126 or SP600125 on cell transformation was estimated as described in Figure 2.3. * $P < 0.05$, significantly different from TPA alone.

The results indicate that delphinidin might inhibit AP-1 transactivation and cell transformation by an early inhibition of ERK activation and later inhibition of JNK activation. To further confirm whether ERK and JNK activation is essential to cell transformation, two specific inhibitors (SP600125 for JNK and UO126 for MEK1/2) were used to challenge to block TPA-induced activation and cell transformation. It is known that UO126 is a selective MEK1/2 inhibitor that inhibits the phosphorylation of ERK1/2 [54], and SP600125 is a selective JNK inhibitor that inhibits the phosphorylation of c-Jun [55].

As shown in Figure 2.7, both inhibitors could effectively block TPA-induced phosphorylation of ERK and c-Jun (Figure 2.7A) and cell transformation (Figure 2.7B), respectively. These results demonstrated that both ERK and JNK cascades are critical in mediating cell transformation.

2.3.6 Synergy effect between delphinidin and SOD in inhibiting AP-1 activity

Several reports suggest that TPA treatment in JB6 cells is associated with the generation of ROS that further promotes neoplastic transformation [56, 57]. To identify which species of TPA-induced ROS is required for AP-1 activation in JB6 cells, three kinds of ROS eliminating enzymes or agents including SOD (a scavenger of superoxide anion), catalase (a decomposer of hydrogen peroxide) and D-mannitol (a scavenger of hydroxyl radical) [56] were used. In the concentration range of the agents that did not affect cellular viability as measured by MTT assay (data not shown), TPA-induced AP-1 activity was significantly blocked by SOD, but not by catalase or D-mannitol (Figure 2.8A; $P<0.05$). The results indicate that TPA-induced superoxide anion may contribute to AP-1 activation. Because either anthocyanidin (Figure 2.4B) or SOD alone could inhibit AP-1 activity, the cells were further treated with combinations of the two agents. A greater inhibition was observed in combinations of SOD with delphinidin, cyanidin and petunidin, but not with pelargonidin, peonidin and malvidin (Figure 2.8B), suggesting that the ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins may be essential for this action. To distinguish whether the combination inhibition is additive or synergistic, the multiplicative model [43, 44] was applied to estimate the effect of combinations of SOD with delphinidin, a stronger inhibitor, at varying concentrations (Figure 2.8C). The results obtained with 200 U/ml SOD indicate the actual AP-1 activity observed is less than that

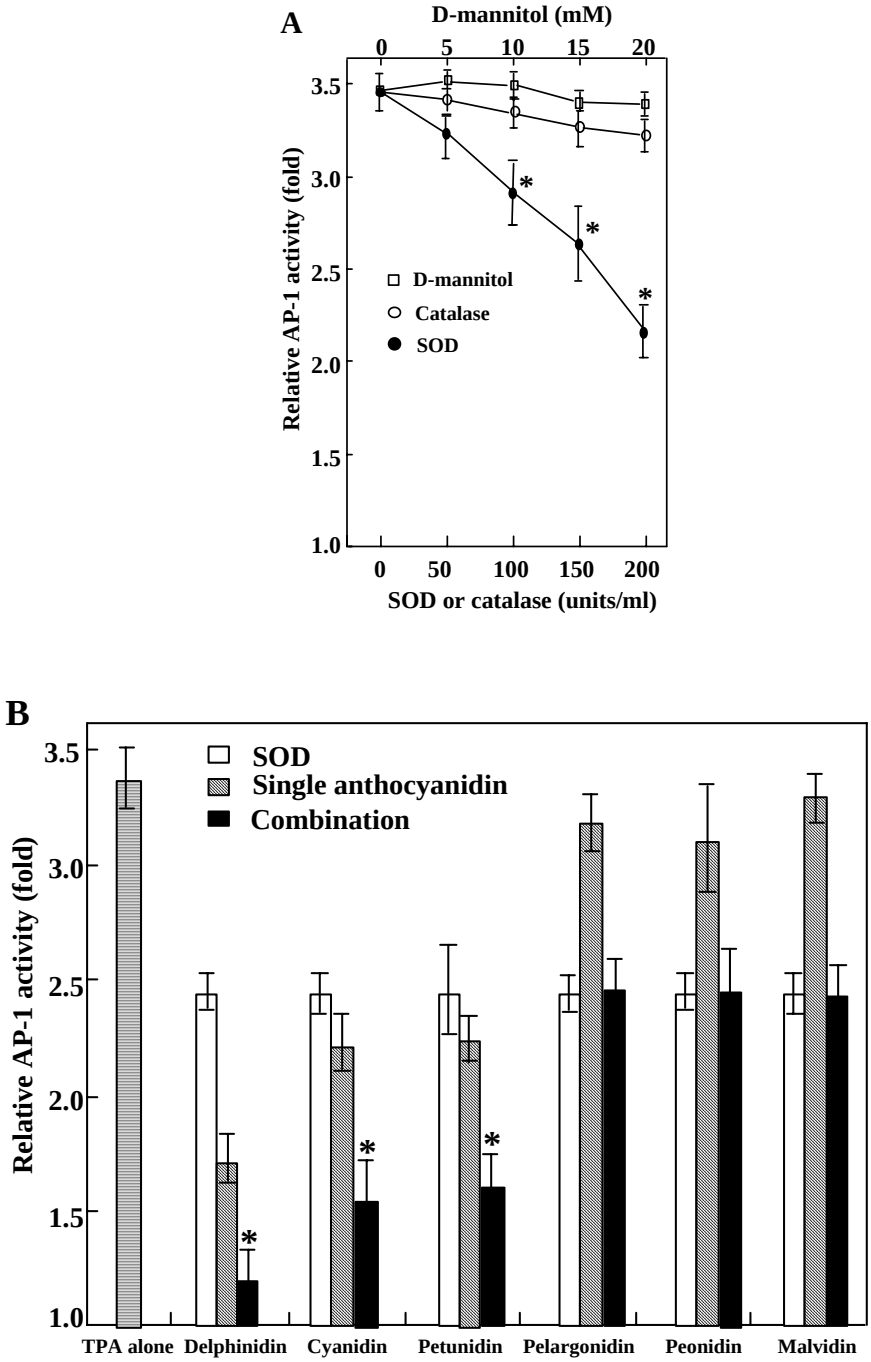

Figure 2.8. Synergistic inhibition between delphinidin and SOD. JB P^{+11} cell culture and luciferase activity assay were done as described in Figure 2.4B. Each value represents the mean ± SD of 3-6 separate experiments. **(A)** SOD, but not catalase or D-mannitol, inhibits TPA-induced AP-1 activity. The cells were treated with or without SOD, catalase and D-mannitol for 30 min at the indicated concentrations before they were exposed to 20 ng/ml TPA for 24 h. * $P < 0.05$, significantly different from TPA alone.

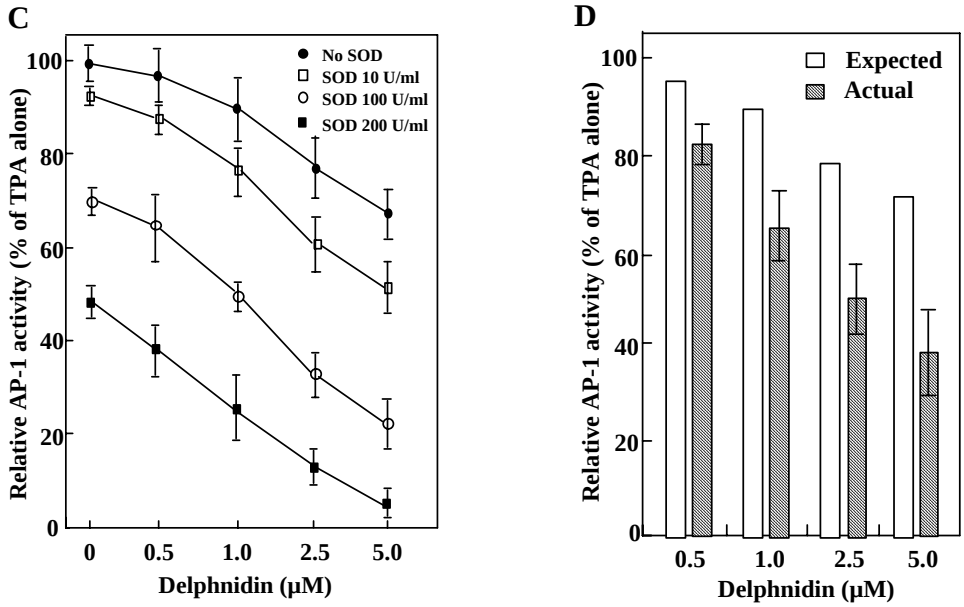

Figure 2.8 (continued)

(B) Delphinidin, cyanidin and petunidin, but not pelargonidin, peonidin or malvidin, show greater inhibition of TPA-induced AP-1 activity with SOD. The cells were treated by six anthocyanidins (5 μM) with or without SOD (200 U/ml) for 30 min before they were exposed to 20 ng/ml TPA for 24 h. * $P<0.05$, significantly different from SOD or anthocyanidin alone. **(C)** Delphinidin synergistically inhibits AP-1 activity with SOD. The cells were treated with the indicated concentrations of delphinidin in the presence or absence of various doses of SOD. Results represent the mean ± SD of three experiments as a percentage of AP-1 activity in the presence of TPA alone. **(D)** Comparison between the actual AP-1 activity (*shaded bars*) and the expected value for additive effects (*open bars*) in combinations of them.

expected from additive effects calculated according to the multiplicative model (Figure 2.8D). Similar results were also obtained with 10 and 100 U/ml SOD (data not shown). Thus, these results suggest that delphinidin and SOD appear to act in a synergistic manner to inhibit TPA-induced AP-1 activity.

2.4 Discussion

Anthocyans are known to exist in red fruits and vegetables. This study reports a molecular evidence for anticarcinogenesis by active anthocyans. The data showed that active anthocyans inhibited TPA-induced MAPK, AP-1 activation and cell transformation. The ortho-dihydroxyphenyl structure on the B-ring of anthocyanin aglycon appears essential for the inhibitory action.

To elucidate the molecular events of anthocyans in the inhibition of AP-1 activity and cell transformation, delphinidin showing the strongest potent inhibition was used to investigate the effects on signaling pathways mediating AP-1 activity. Although many mechanisms may be involved in the up- and down-regulation of AP-1 activity, MAPK including ERK, JNK and p38 kinases are known to be common signaling pathways mediating AP-1 activity [16, 46]. The results indicate that delphinidin blocked ERK phosphorylation at early times and JNK phosphorylation at later times, but not p38 phosphorylation at any time (Figure 2.5A). Moreover, the phosphorylation of MEK1/2 (an ERK kinase), SEK (a JNK kinase) and c-Jun (a phosphorylation target of ERK and JNK) were also blocked in delphinidin-treated cells (Figure 2.6). These data suggest that the inhibition of TPA-induced AP-1 activity by delphinidin may involve the blockage of activation of ERK and JNK signaling cascades. Many previous reports indicate that ERK and/or JNK are able to induce AP-1 activity and cell transformation [18, 48, 58, 59]. Some chemopreventive agents including tea polyphenols [34, 35] and glycoside compounds [36] inhibit AP-1 transactivation by blocking JNK activation. Recent studies have suggested that ERK also play a critical role in TPA-induced AP-1 activity and cell transformation in JB6 cell lines [18, 60]. For instance, stable expression of dominant negative ERK in tumor promoter-sensitive cell line, JB6 CI41, blocks TPA-induced cell transformation [60]. In the

present study, delphinidin blocked the TPA-induced activation of both ERK and JNK in different time fashion. Furthermore, two specific inhibitors (UO126 for MEK1/2 and SP600125 for JNK) could effectively block TPA-induced phosphorylation of ERK and c-Jun, and cell transformation at the same dose, respectively (Figure 2.7). Consistent with this finding, the data indicate that the inhibition of both ERK and JNK cascades are critical in diminishing TPA-induced cell transformation. Moreover, both ERK and JNK cascades were blocked by delphinidin, suggesting more upstream effectors may sever as the target(s) of delphinidin. Accumulated evidence has indicated that the upstream effector of ERK cascade is Ras/Raf, and that of JNK cascade is Rac/Rho [61, 62]. Of which, MEKK1/3 may sever as a crosstalking between the two cascades for stress response [62]. It is interested to clarify the upstream target(s) of the anthocyanidins in next study.

Another important question is how do the potent anthocyanidins block TPA-induced AP-1 activation. TPA treatment in JB6 might generate ROS that further promotes neoplastic transformation [56, 57]. TPA-induced cell transformation in soft agar could be 90% blocked by the addition of SOD, but not by catalase or GSH-Px [56]. In the present study, TPA-induced AP-1 activation was blocked by SOD, but not by catalase and mannitol, implicating superoxide anion in the process of activating AP-1. This requirement of superoxide for AP-1 activation has also been shown to occur in transgenic mice *in vivo* during skin tumor promotion [63]. Recent report has indicated that anthocyanidins can also scavenge superoxide radicals when they encountered the radicals in an *in vitro* ESR system [64]. The $ID_{(50)}$ for superoxide radicals was, respectively, 2.2, 22, and 456 μM of delphinidin, cyanidin and pelargonidin [64]. Thus, the effect of combinations of SOD with anthocyanidins was further investigated in this study. A greater inhibition was observed in combinations of SOD with anthocyanidins those have the ortho-dihydroxyphenyl structure

on the B-ring of anthocyanidins (Figure 2.8B), suggesting that this structure may contribute to the inhibitory action. Furthermore, the results from multiplicative model analysis suggested that this greater inhibition between SOD and delphinidin is synergistic, not additive. These findings together with other reports suggest that the inhibitory effects of anthocyanidins on AP-1 activation are due, in part, to their potent scavenging activity for superoxide radicals and, in part, to blocking MAPK. Both targets may be important in the cancer prevention activity of anthocyanidins although the mechanistic relations between them are needed to clarify. It is noteworthy that delphinidin inhibits AP-1 activity by blocking ERK at early times and JNK activation at later times in the present study. SOD selectively inhibits the TPA-induced activation of protein kinase Epsilon and prevents subsequent activation of JNK2 in response to TPA, thereby delaying AP-1 activation and inhibiting mouse skin tumor promotion [63]. Thus, the signaling pathways blocked by delphinidin or SOD may differ in part.

It has been reported that anthocyans have strong free radical scavenging and antioxidant activities [1-5], those have been suggested to play an important role in prevention against mutagenesis and carcinogenesis. Moreover, oral intake of anthocyans from purple sweet potato and red cabbage could suppress rat colon carcinogenesis induced by DMH and PhIP [12]. Thus, the results from this study with other reports suggest a possibility that moderate consumption of anthocyans through the intake of the products such as bilberry extracts may be linked with cancer prevention. Further epidemiological study on this aspect is required.

In summary, the molecular mechanisms of the antitumor promotion action by anthocyans were investigated, using mouse JB6 cell model. Of which, only those having an ortho-dihydroxyphenyl structure on the B-ring of aglycon suppressed TPA-induced cell

transformation and AP-1 activity. The potent anthocyanidins may block TPA-induced ERK and JNK signaling cascades leading to activation of AP-1. These findings provide the first molecular basis for the anticarcinogenic action of anthocyans.

2.5 Abstract

To investigate the molecular mechanism of anthocyans as anticarcinogenic agents, representative anthocyanins and their anthocyanidins were used to examine their effects on cell transformation in mouse JB6 cells, a validated model for screening cancer chemopreventive agents and elucidating the molecular mechanisms. Of anthocyanins and anthocyanidins tested, only those with an ortho-dihydroxyphenyl structure on the B-ring suppressed TPA-induced cell transformation and AP-1 activity, suggesting that the ortho-dihydroxyphenyl may contribute to the inhibitory action. Delphinidin, but not peonidin, blocked the phosphorylation of protein kinases in ERK pathway at early times and JNK signaling pathway at later times. p38 kinase was not inhibited by delphinidin. Furthermore, two MAPK specific inhibitors (SP600125 for JNK and UO126 for MERK1/2) could specifically block the activation of c-Jun and ERK and cell transformation. Those results demonstrated that active anthocyans contribute to the inhibition of cell transformation by blocking activation of the MAPK pathway. These findings provide the first molecular basis for the anticarcinogenic action of anthocyanidins.

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Chapter 3 Molecular characterization of the inhibitory effects of cyclooxygenase-2 expression by anthocyans in LPS-evoked macrophage cells

3.1 Introduction

COX is a rate-limiting enzyme for synthesis of dienoic eicosanoids such as prostaglandin E2. COX exists in two isoforms [1, 2]. COX-1 is expressed constitutively in many types of cells and is responsible for the production of prostaglandins under physiological conditions. COX-2 is induced by pro-inflammatory stimuli, including mitogens, cytokines and bacterial LPS in macrophages [2] and epithelial cells [3, 4]. Accumulated data indicate that COX-2 is involved in many inflammatory processes and induced in various carcinomas, suggesting that COX-2 plays a key role in inflammation and tumorigenesis [5, 6] (Figure 3.1). Interestingly, some antioxidants with chemopreventive effects inhibit the expression of COX-2 by interfering with the signaling mechanisms that regulate the COX-2 gene [7]. Thus, COX-2 gene has been used as a biomarker to investigate the cancer chemopreventive effects of phytochemicals, and the identification of COX-2 inhibitor is considered to be a promising approach to protect against inflammation and tumorigenesis.

In the COX-2 gene, four transcription factors including NF-κB, CCAAT/enhancer-binding protein (C/EBP), AP-1 and CRE-binding protein (CREB) have been identified to bind the *cis*-acting elements in the promoter of COX-2 and regulate the transcription [5, 6] (Figure 3.2). NF-κB is a transcription factor involved in LPS-mediated induction by many cytokines and inflammatory products [8, 9]. NF-κB is inactivated in the cytosol by binding to IκB, and become active through translocation to the nucleus preceded by LPS-induced proteolytic degradation of IκB [10, 11] (Figure 3.3). C/EBP is generally considered to regulate COX-2 production mainly with the two C/EBP family members, C/EBPδ and β

[12-14]. Finally, AP-1 and CREB are essential for both basal and induced COX-2 transcription [15, 16] (Figure 3.2).

LPS is an endotoxin from gram-negative bacteria, which provokes a wide variety of immunologic responses through inducing the activation of variety proteins. Several lines of evidence indicates that LPS stimulates COX-2 production through activating MAPK including ERK, JNK and p38 kinase [14, 17].

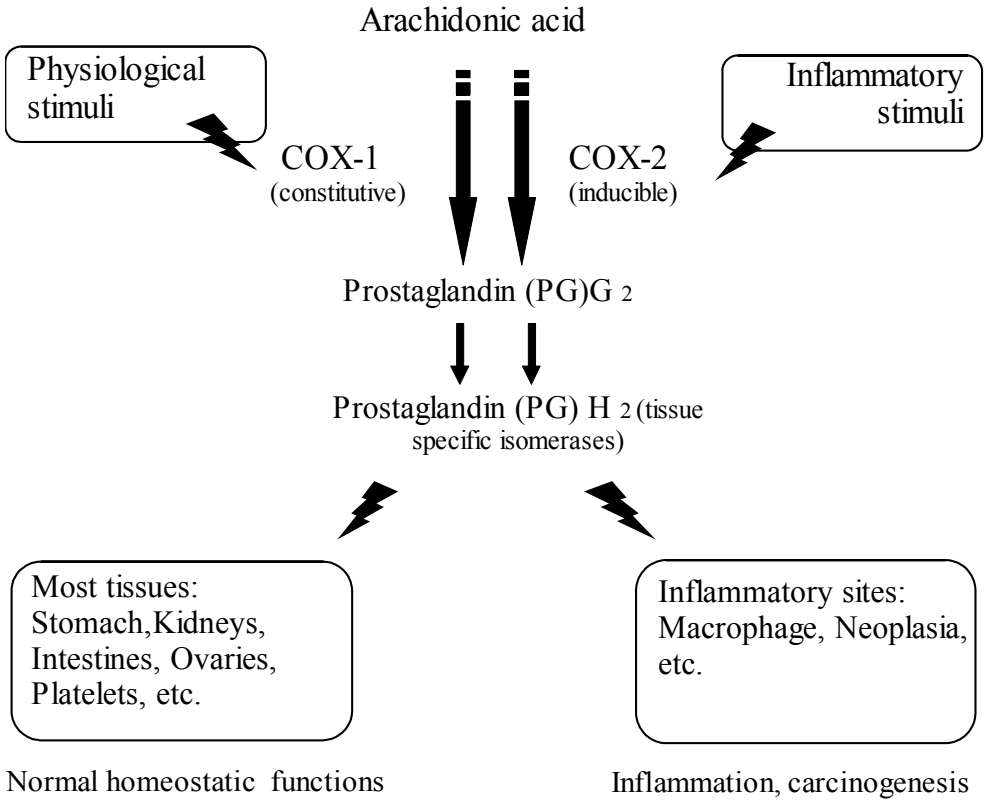

Figure 3.1. Cyclooxygenase in physiology and disease. COX, existing in two isoforms, is a rate-limiting enzyme for synthesis of prostaglandin G2 from arachidonic acid. COX-1 is expressed constitutively in many types of cells and is responsible for the production of prostaglandins under physiological conditions. Thus, COX-1 plays a critical role in keeping normal homeostatic function. COX-2 is induced by pro-inflammatory stimuli, including mitogens, cytokines and bacterial LPS in macrophages and epithelial cells. Therefore, COX-2 plays a key role in inflammation and carcinogenesis.

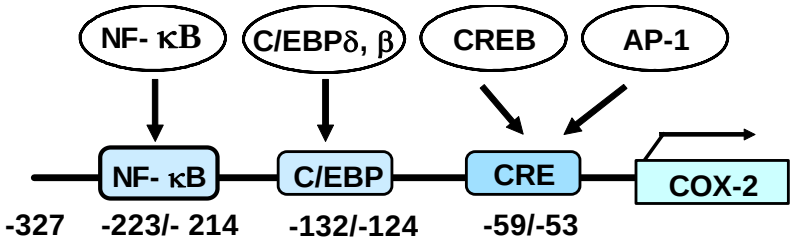

Figure 3.2. The *cis*-acting elements and transcriptional factors in the promoter of COX-2. There are three *cis*-acting elements present in the core region of COX-2 promoter including NF-κB, C/EBP and CRE sites. The transcription factors including NF-κB, C/EBP, CREB and AP-1 bind those sites, respectively, and regulate COX-2 transcription. AP-1: activator protein-1; C/EBP: CCAAT/enhancer-binding protein; CREB: CRE-binding protein; NF-κB: nuclear factor κB.

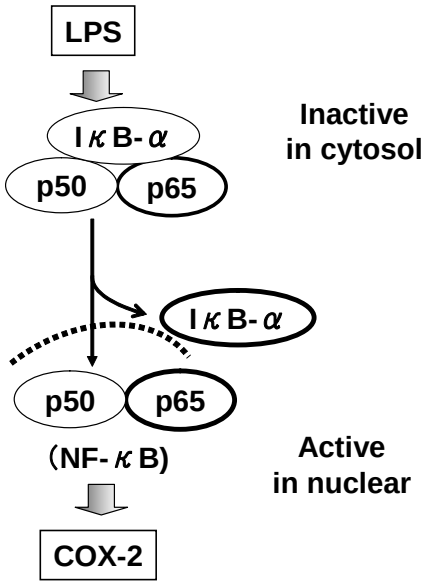

Figure 3.3. IκB degradation and NF-κB activation. NF-κB is inactivated in the cytosol by binding to IκB, and become active through translocation to the nucleus preceded by degradation of IκB, which can be induced by a number of factors such as LPS. NF-κB: nuclear factor κB; LPS: lipopolysaccharide.

Some previous studies have suggested that anthocyanins and anthocyanidins may have anti-inflammatory effects. Wang *et al.* [18] found that anthocyanins and their aglycon, cyanidin, from tart cherries could inhibit the enzyme activities of COX-2. Seeram *et al.* [19] found that cyanidin showed superior inhibition on the cyclooxygenase activity *in vitro*. However, the underlying mechanisms are not well understood.

In the present study, mouse macrophage cell line RAW264, which can be stimulated with LPS to mimic a state of infection and inflammation, was used to demonstrate the molecular mechanism of inhibitory actions of anthocyans on COX-2 expression. The data showed that the inhibitory effects of anthocyans on COX-2 expression depended on the ortho-dihydroxyphenyl structure on the B-ring, and delphinidin with this structure inhibited LPS-induced COX-2 expression by blocking the signaling cascades of MAPK with the attendant activations of NF-κB, C/EBPδ and AP-1.

3. 2 Materials and methods

3.2.1 Materials and cell culture

Bilberry powder was obtained from Indena company, and anthocyans were extracted in a water/methanol (1:1) solution containing 2% HCl as described previously [20]. Anthocyanidins (delphinidin chloride, cyanidin chloride, pelargonidin chloride, peonidin chloride and malvidin chloride) purified by HPLC were obtained from Extrasynthese (Genay, France), and dissolved in DMSO (final concentration was 0.2%). LPS (*Escherichia coli* Serotype 055:B5), and MTT were from Sigma (St. Louis, MO). SB203580 was from Calbiochem (Nottingharm, UK), and SP600125 were from Biomol Research Lab. (Polymouth Meeting, PA). U0126 and antibodies against phospho-ERK1/2, phospho-JNK, phospho-p38 kinase, phospho-c-Jun (Ser73), phospho-CREB, ERK1/2, JNK, p38 kinase, c-Jun, CREB and IκB-α were purchased from Cell Signaling Technology

(Beverly, MA). Antibodies against COX-2, COX-1, C/EBPβ, C/EBPδ and p65 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Murine macrophage-like RAW264 cells were obtained from RIKEN BioResource Center Cell Bank (Cell No. RCB0535), Japan, and cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS.

3.2.2 RNA extraction RT-PCR

RNA extraction and RT-PCR were performed as described previously [21]. In brief, RAW264 (1 ×10^6 cells) were pre-cultured in 6-cm dish for 24 h, and then starved by being cultured in serum-free medium for another 2.5 h to eliminate the influence of FBS. The cells were treated with the concentration range of 25-100 μM delphinidin for 30 min before exposure to 40 ng/ml LPS for 6 h**.** Cellular RNA was extracted with an ISOGEN RNA isolation kit (Nippon Gene, Tokyo) as described in manufacture. The oligonucleotide primers used to amplify mouse COX-2 were 5'-CAG CAA ATC CTT GCT GTT CC-3' and 5'-TGG GCA AAG AAT GCA AAC ATC-3' [22]. The oligonucleotide primers used to amplify mouse COX-1 were 5'-ACT GGC TCT GGG AAT TTG TG-3' and 5'-AGA GCC GCA GGT GAT ACT GT-3' [23]. The RT-PCR was done by one-step reaction with Read-to-Go RT-PCR beads (Amersham Pharmacia Biotech) as described previously (21). Briefly, RNA (250 ng) was used for reverse-transcription into cDNA at 42°C for 30 min using oligo (dT) 12-18 primers. Amplifications were done at 95°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec with GenAmp PCR System 2400 machine (Perkin-Elmer). Template- and cycle-dependence of the PCR products were confirmed, and the available cycle numbers of PCR for COX-2 and COX-1 were determined as 30 cycles, respectively. The PCR products were separated on 2% agarose gel, and digitally imaged after staining ethidium bromide. The bands were quantified by Imager Gauge Software (Fuji Photo Film).

The mRNA level in the control culture is arbitrarily set to 1.0, the basal level for subsequent mRNA comparisons.

3.2.3 Nuclear protein extraction

Nuclear extracts were prepared as described previously [17]. Briefly, RAW264 cells (1×10^6) were pre-cultured in 6-cm dish for 24 h, and then starved by being cultured in serum-free for another 2.5 h to eliminate the influence of FBS. The cells were treated with delphinidin for 30 min before exposure to 40 ng/ml LPS for 4 h. Harvested cells were lysed by incubation in buffer A (10 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) on ice for 15 min, and then centrifuged at 15,000 rpm for 10 min at 4°C. The nuclear pellets were resuspended in Buffer B (20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM sodium ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) for 15 min at 4 °C, and then centrifuged at 13,500 $\times g$ for 15 min at 4°C. The supernatants containing nuclear extracts were stored at -80°C until using.

3.2.4 Western blotting

Western blotting assay was performed as described previously [24]. In brief, RAW264 (1×10^6 cells) were pre-cultured in 6-cm dish for 24 h, and then starved by being cultured in serum-free for another 2.5 h to eliminate the influence of FBS. The cells were treated with delphinidin or other inhibitors for 30 min before exposure to 40 ng/ml LPS for the different times. Cellular lysates were boiled for 5 min. Protein concentration was determined using dye-binding protein assay kit (Bio-Rad Hercules, CA) according to the manufacturer's manual. Equal amounts of lysate protein (~40 μg) were run on 10%

SDS-PAGE and electrophoretically transferred to PVDF membrane (Amershan Pharmacia Biotech). After blotting, the membrane was incubated with specific primary antibody overnight at 4 °C, and further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected by ECL system with a Lumi Vision PRO machine (TAITEC Co., Japan). The relative amount of proteins associated with specific antibody was quantified using the Lumi Vision Imager software (TAITEC Co., Japan).

3.2.5 Statistical analysis

Difference between the treated and the control was analyzed by Student's *t*-test. A probability of $P < 0.05$ was considered significant.

3.3 Results

3.3.1 Anthocyan extracts from bilberry suppress LPS-induced COX-2 expression

RAW264 cells were treated with anthocyans extracts from bilberry at 25-100 μg/ml for 30 min before exposure to 40 ng/ml LPS for 12 h. As shown in Figure 3.4, anthocyans extracts suppressed significantly LPS-induced expression of COX-2 protein from 50 μg/ml, and showed a dose-dependent inhibition in the indicated concentration range. The constitutive protein, COX-1, showed no change in such treatment. The inhibitory action by anthocyans extracts were not caused by their cytotoxicity, because the concentration range that suppressed COX-2 expression did not affect cellular viability as measured by MTT assay (data not shown). These results indicate that anthocyans extracts from bilberry may be potential inhibitors for COX-2 expression. HPLC data showed that there were 15 kinds of glycosides containing aglycons of delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin in the anthocyans extracts from bilberry (data not shown). Thus,

five kinds of anthocyanidins were chose to investigate the structure-activity relationship in next experiment.

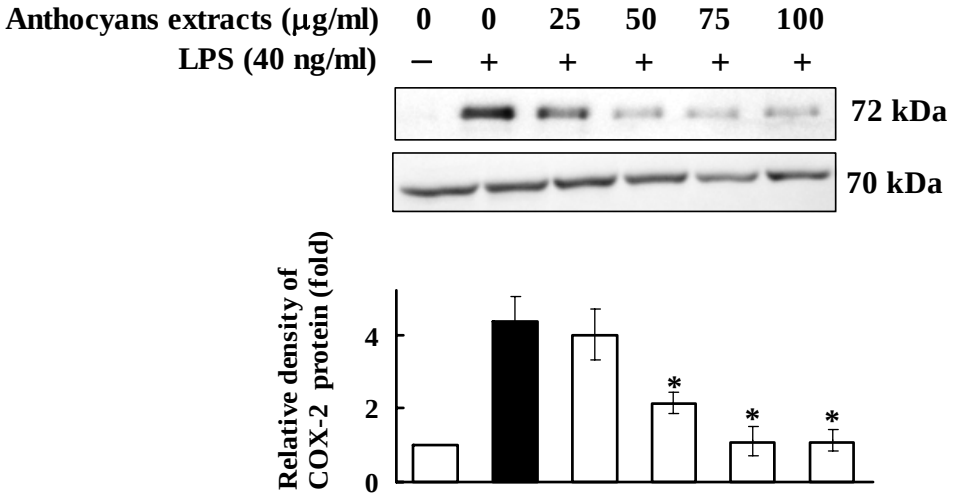

Figure 3.4. Anthocyan extracts from bilberry suppress COX-2 expression in LPS-activated RAW264 cells. After RAW264 cells (1×10^6 cells) were starved in serum-free medium for 2.5 h, the cells were treated with 25-100 μg/ml of anthocyans extracts for 30 min, and then exposed to 40 ng/ml LPS for 12 h. Cellular lysate was applied on 10% SDS-PAGE. The proteins of COX-2 and COX-1 were detected with corresponding specific antibodies, and visualized by chemiluminescence's ECL kit. The relative amounts of the proteins were quantified using Imager Gauge Software (Fuji Photo Film). Histograms show the densitometric analysis of COX-2 protein normalized to COX-1 protein. The data represent the mean ± SD of three separate experiments, and the picture is a representative of those experiments each with similar results. *$P < 0.05$, significantly different from LPS alone.

3.3.2 The effects of anthocyanidins on LPS-induced COX-2 expression

RAW264 cells were treated with typical five kinds of anthocyanidins (Figure 1.1B) at 25-100 μM for 30 min before exposure to 40 ng/ml LPS for 12 h. The results showed that LPS-induced COX-2 protein was suppressed by addition of over 50 μM of delphinidin or cyanidin, but not suppressed by addition of ever 100 μM of pelargonidin, peonidin or

malvidin. Figure 3.5 shows a representative result at 75 μM. LPS-induced COX-2 protein was significantly inhibited by 75 μM of delphinidin or cyanidin, but not by pelargonidin, peonidin or malvidin (P<0.05). The constitutive protein, COX-1, showed no change in such treatment. The ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins appears to be essential for the inhibitory action because pelargonidin, peonidin and malvidin, having no such ortho-dihydroxyphenyl structure, failed to show the inhibitory effect. The inhibitory actions by delphinidin and cyanidin were not caused by their cytotoxicity, because this concentration that suppressed COX-2 expression did not affect cellular viability as measured by MTT assay (data not shown). These results indicate that delphinidin and cyanidin may be potential inhibitors for COX-2 expression.

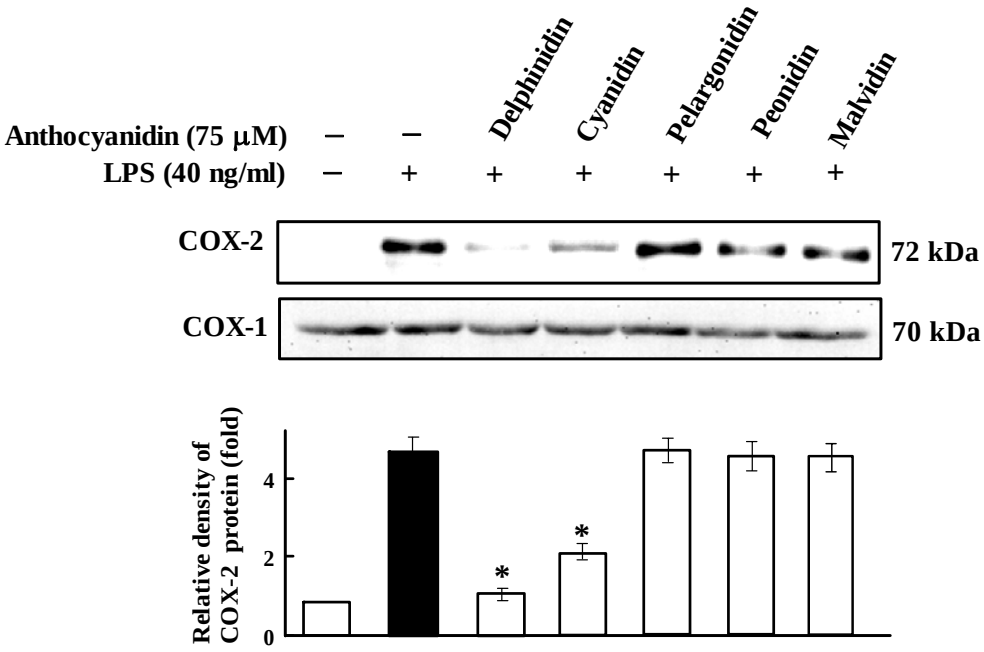

Figure 3.5. The effects of anthocyanidins on COX-2 expression in LPS-activated RAW264 cells. After RAW264 cells (1×10^6 cells) were starved in serum-free medium for 2.5 h, the cells were treated with 75 μM of delphinidin, cyanidin, peonidin, pelargonidin and malvidin for 30 min, respectively, and then exposed to 40 ng/ml LPS for 12 h. The detection and quantification of COX-2 and COX-1 proteins were done as described in Figure 3.4. Histograms show the densitometric analysis of COX-2 protein normalized to COX-1 protein. *P < 0.05, significantly different from LPS alone.

3.3.3 Delphinidin inhibits COX-2 expression at protein and mRNA levels

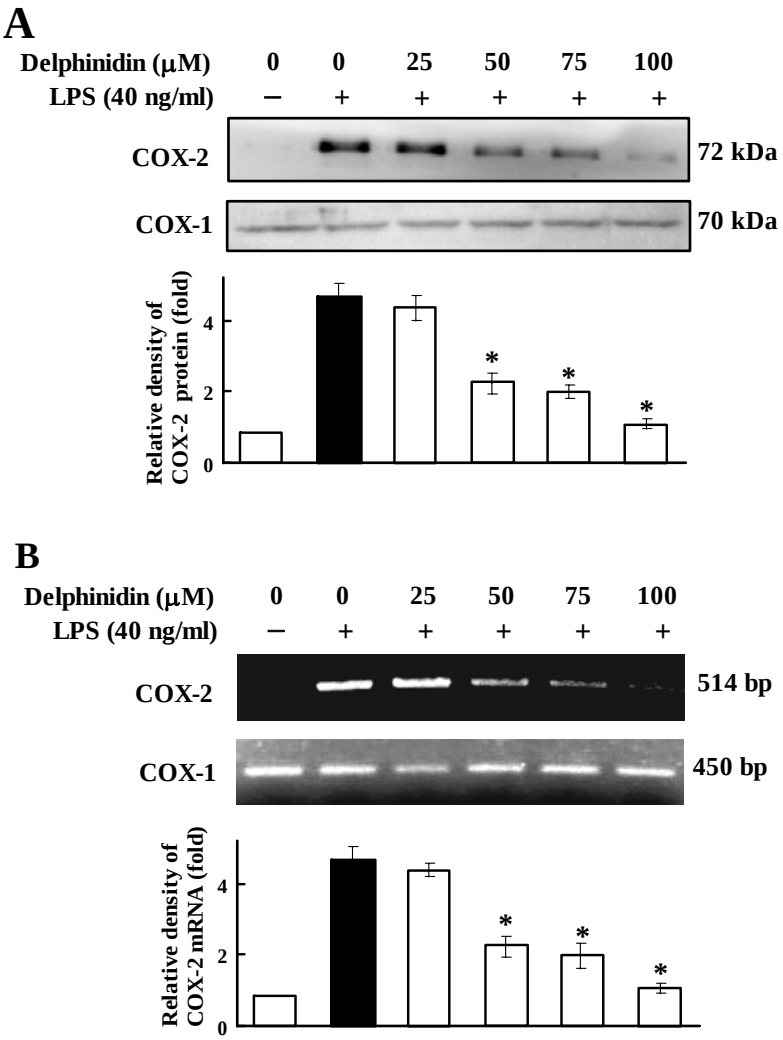

Figure 3.6. Delphinidin causes a dose-dependent inhibition of COX-2 protein (A) and mRNA (B). Cell culture, delphinidin treatment, detection and quantification of COX-2 and COX-1 proteins were done as described in Figure 3.4. Histograms show the densitometric analysis of COX-2 protein normalized to COX-1 protein **(A)**. To detect mRNA expression, the cells were exposed to 40 ng/ml LPS for 6 h. RNA was extracted with ISOGEN RNA isolation kit, and COX-2 and COX-1 mRNA were detected by RT-PCR as described in Material and methods. The RT-PCR products were separated on 2% agarose gel, and digitally imaged after staining with ethidium bromide. Quantification of the bands was performed with an Imager Gauge Software (Fuji Photo Film). Histograms show the densitometric analysis of COX-2 mRNA normalized to COX-1 mRNA **(B)**. The data represent the mean ± SD of three separate experiments, and the picture is a representative of those experiments each with similar results. * $P<0.05$, significantly different from LPS alone

Because delphinidin showed the strongest inhibition on COX-2 expression among five kinds of anthocyanidins, delphinidin was used to investigate the molecular mechanism of inhibitory action in next experiments. As shown in Figure 3.6A, delphinidin showed a dose-dependent inhibition of COX-2 protein, but not COX-1 protein. We also determined mRNA steady-state levels of COX-2 and COX-1 in such treatment by RT-PCR. Delphinidin suppressed the COX-2 mRNA expression in a dose-dependent manner while COX-1 showed no change in such treatment (Figure 3.6B). Thus, the suppression of COX-2 expression by delphinidin is due to transcription regulation.

3.3.4 Effects of delphinidin on the transcriptional factors regulating COX-2 transcription

It has been reported that transcription factors including C/EBP, CREB, AP-1 and NF-κB regulate COX-*2* transcription by binding the responding *cis*-elements in COX-*2* promoter (Figure 3.2) [13-15]. To identify the effects of delphinidin on these transcriptional factors, RAW264 cells were pretreated with indicated concentrations of delphinidin for 30 min before exposure to 40 ng/ml LPS, and these transcription factors were then examined with specific antibodies, respectively.

As shown in Figure 3.7A, delphinidin completely inhibited LPS-induced phosphorylation of c-Jun, which is a major component of AP-1 in c-Jun/c-Fos heterodimer form. However, delphinidin showed no inhibitory effect on LPS-induced phosphorylation of CREB. Delphinidin also blocked LPS-induced translocation of C/EBPδ, but not C/EBPβ, from cytosol to nucleus (Figure 3.7B), which is another important factor in LPS-mediated COX-2 expression [13, 25, 26].

NF-κB is another critical factor for COX-2 induction mediated by LPS or proinflammatory cytokines [8, 9]. Inactivated NF-κB in the cytosol by binding to IκB becomes active through translocation to the nucleus preceded by LPS-induced proteolytic

degradation of IκB [10, 11]. To determine whether delphinidin can directly inhibit degradation of IκB, the level of IκB-α protein was assessed in delphinidin-treated RAW264 cells. First, a time-course experiment was designed to determine the time of LPS-induced IκB-α degradation. LPS caused a degradation of IκB-α protein at 30 min, which was then recovered from 60 min (upper panel in Figure 3.7C). Second, macrophages were pretreated with 25-100 μM of delphinidin for 30 min, and IκB-α protein was detected after exposure

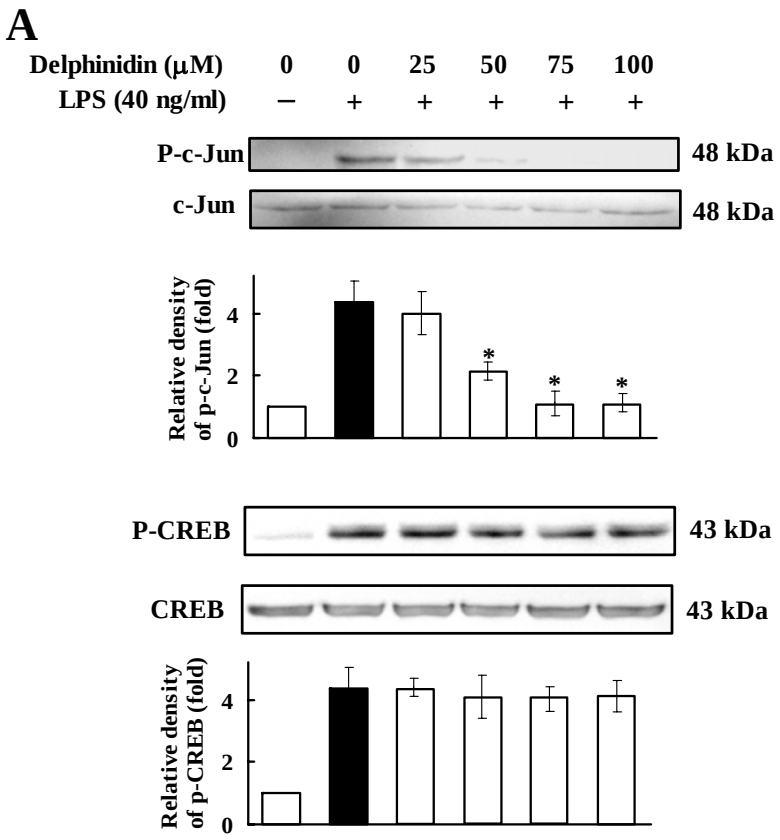

Figure 3.7. Effects of delphinidin on the transcriptional factors regulating COX-2 expression. (A) phosphorylation of c-Jun and CREB. Cell culture and Western blotting analysis were done as described in Figure 3.4. RAW264 cells were treated with 25-100 μM delphinidin for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Phosphorylated c-Jun and CREB were detected with their antibodies, respectively. Histograms show the densitometric analysis of phosphorylated c-Jun and CREB normalized to total c-Jun and CREB, respectively. * $P < 0.05$, significantly different from LPS alone.

to 40 ng/ml LPS for 30 min. Delphinidin significantly suppressed the degradation of IκB-α in the concentrations of 50-100 μM (bottom panel in Figure 3.7C), suggesting that delphinidin may inhibit NF-κB activation by blocking LPS-induced IκB-α degradation. To confirm this, the nuclear translocation of p65, a part of p65/p50 heterodimer, was also examined at the same time. In parallel with IκB-α degradation, LPS resulted in marked p65 translocation from the cytosol to the nucleus after 30 min treatment, and delphinidin

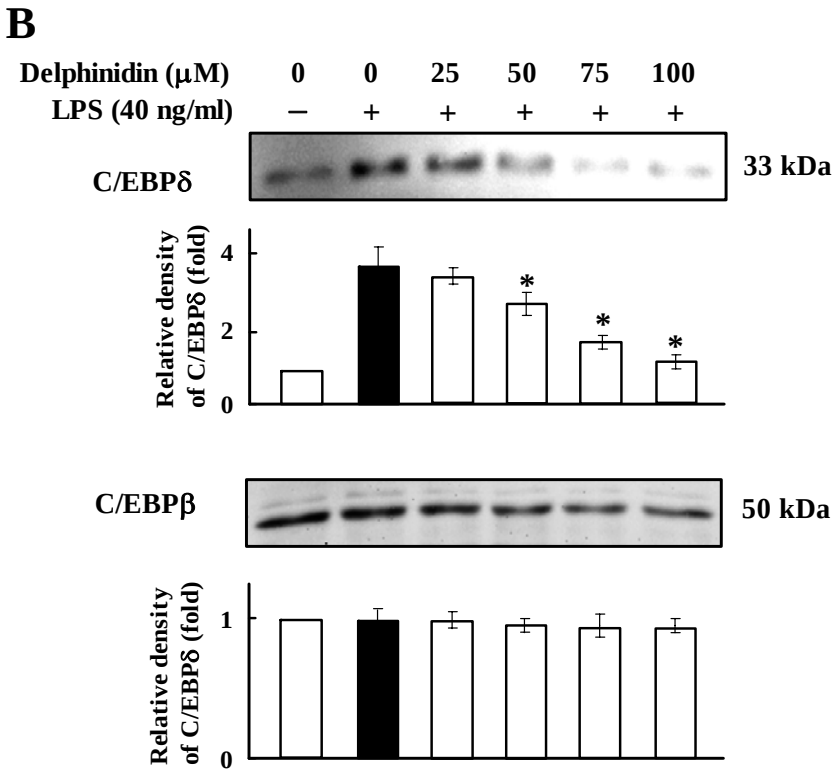

Figure 3.7 (*continued*).

(B) Nuclear translocation of C/EBPδ and C/EBPβ. The cells were treated with 25-100 μM delphinidin for 30 min, and then exposed to 40 ng/ml LPS for 4 h. Nuclear protein was extracted, and nuclear C/EBPδ and β were detected with their antibodies. Histograms show the densitometric analysis of C/EBPδ and C/EBPβ. * $P < 0.05$, significantly different from LPS alone.

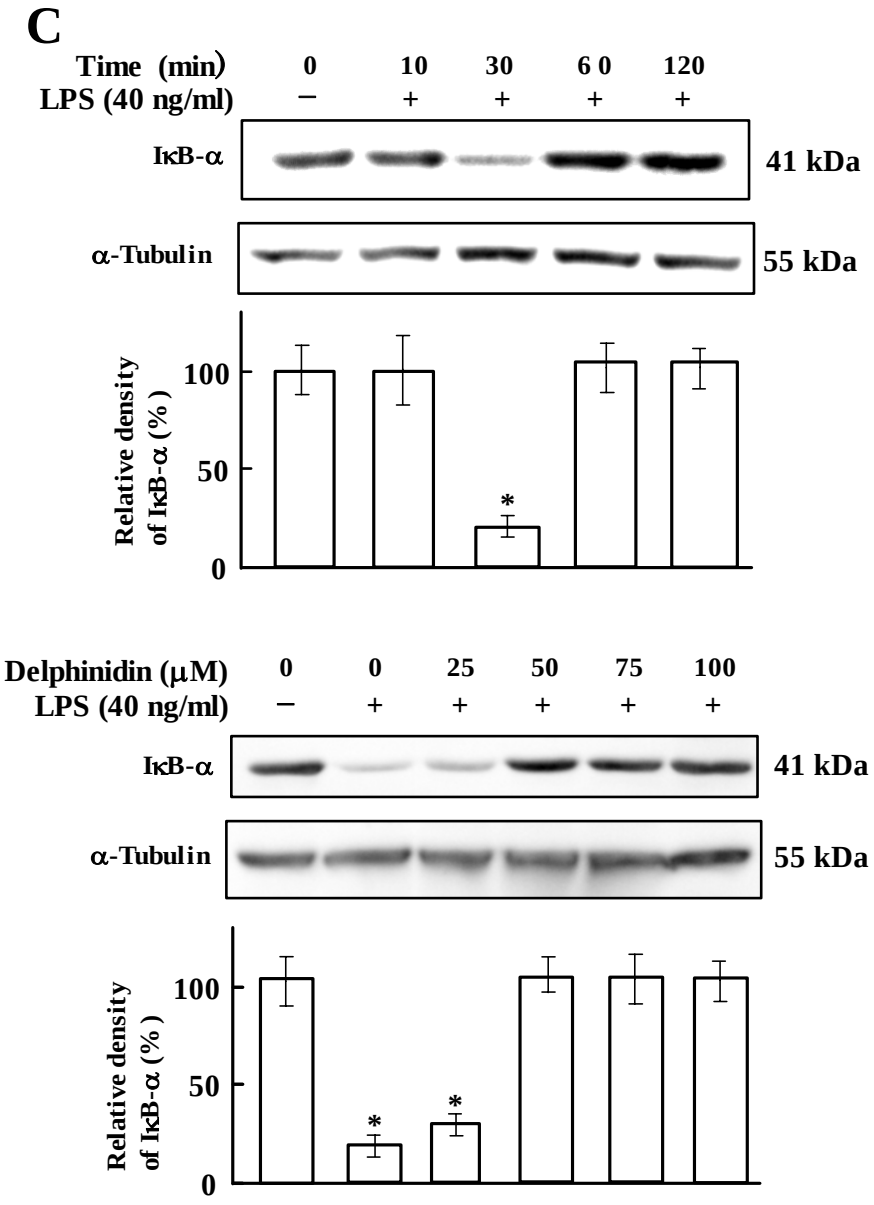

Figure 3.7 (*continued*)

(C) IκB-α degradation. To identify the time of LPS-induced degradation of IκB-α protein, RAW264 cells were treated with 40 ng/ml LPS from 10-120 min. To determine the effect of delphinidin on the degradation of IκB-α protein. The cells were treated with 25-100 μM delphinidin for 30 min before exposure to 40 ng/ml LPS for 30 min. IκB-α protein were detected with its antibody. Histograms show the percentage of IκB-α protein to non-LPS treatment after normalizing to α-tubulin. * $P < 0.05$, significantly different from non-LPS treatment.

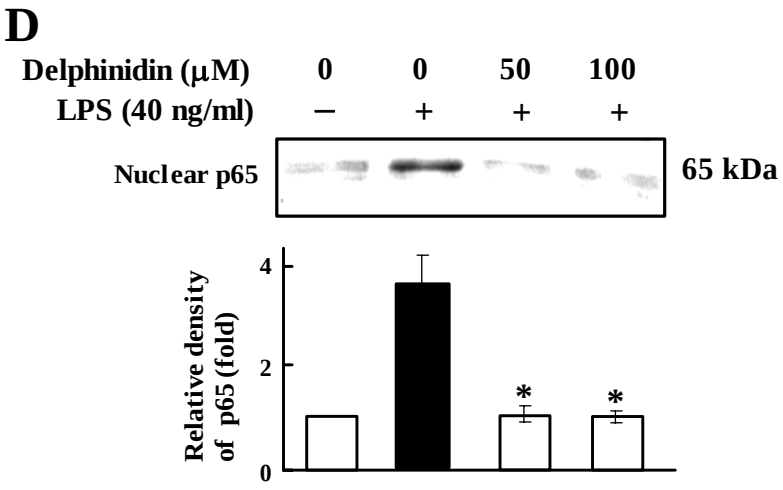

Figure 3.7 (*continued*).

(D) Nuclear translocation of p65. Cell culture and nuclear extraction were performed as Figure 3.7B. Nuclear p65 were detected with p65 antibody. Histograms show the densitometric analysis of p65 in nuclear lysates. * $P < 0.05$, significantly different from LPS alone. The data represent the mean ± SD of three separate experiments, and the picture is a representative of those experiments each with similar results.

(50-100 μM) significantly suppressed LPS-induced nuclear translocation of p65 (Figure 3.7D). These data indicated that delphinidin might inhibit NF-IκB activation by blocking LPS-induced IκB-α degradation and p65 translocation.

Taken together, delphinidin suppressed LPS-stimulated activation of transcription factors including C/EBPδ, AP-1 and NF-κB, but not CREB.

3.3.5 Delphinidin inhibits COX-2 expression by blocking MAPK activation

Accumulated data indicate that LPS induces the activation of MAPK including JNK, ERK and p38 kinase, and subsequently activates the transcription factors with the attendant induction of COX-2 [14, 17]. Thus, the influence of delphinidin on the activation of JNK,

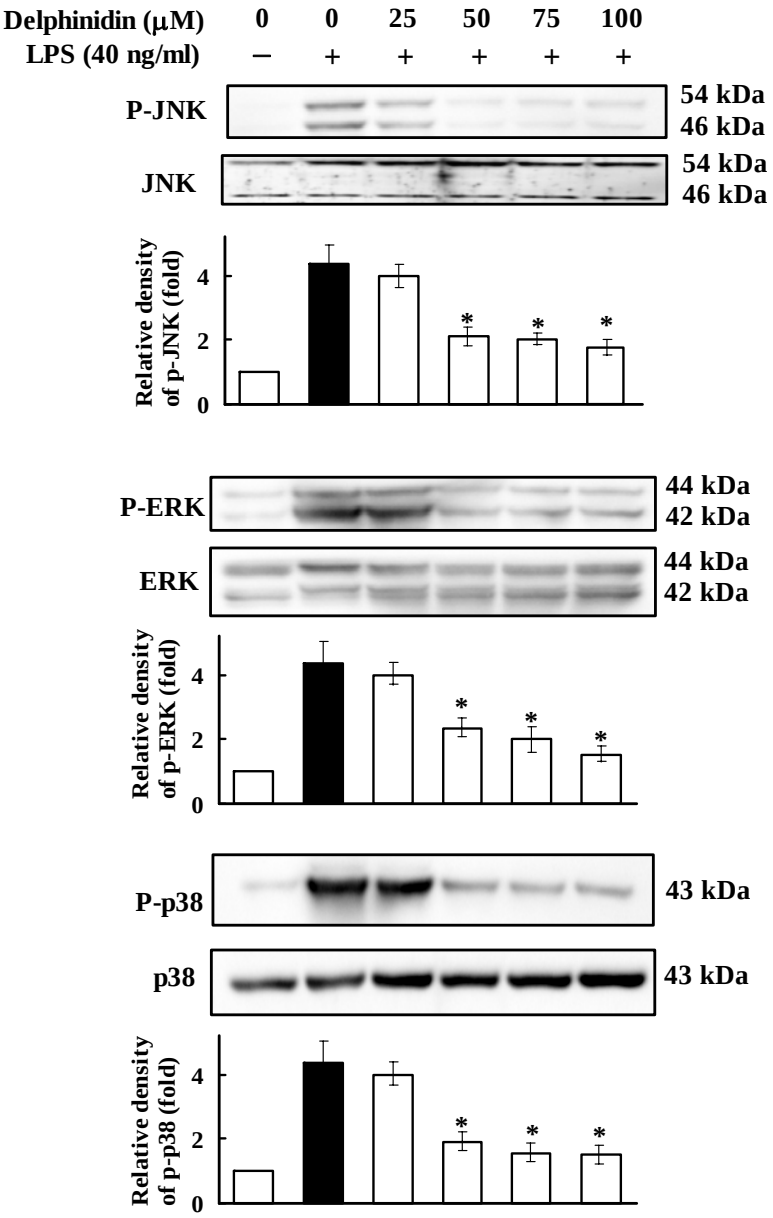

Figure 3.8. Effects of delphinidin on LPS-induced phosphorylation of MAPK. Cell culture and Western blotting were done as described in Figure 3.4. RAW264 cells were treated with 25-100 μM delphinidin for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Total or phosphorylated MAPK were detected with their antibodies, respectively. Histograms show the densitometric analysis of phosphorylated MAPK normalized to total MAPK, respectively. The data represent the mean ± SD of three separate experiments, and the picture is a representative of those experiments each with similar results. * $P < 0.05$, significantly different from LPS alone.

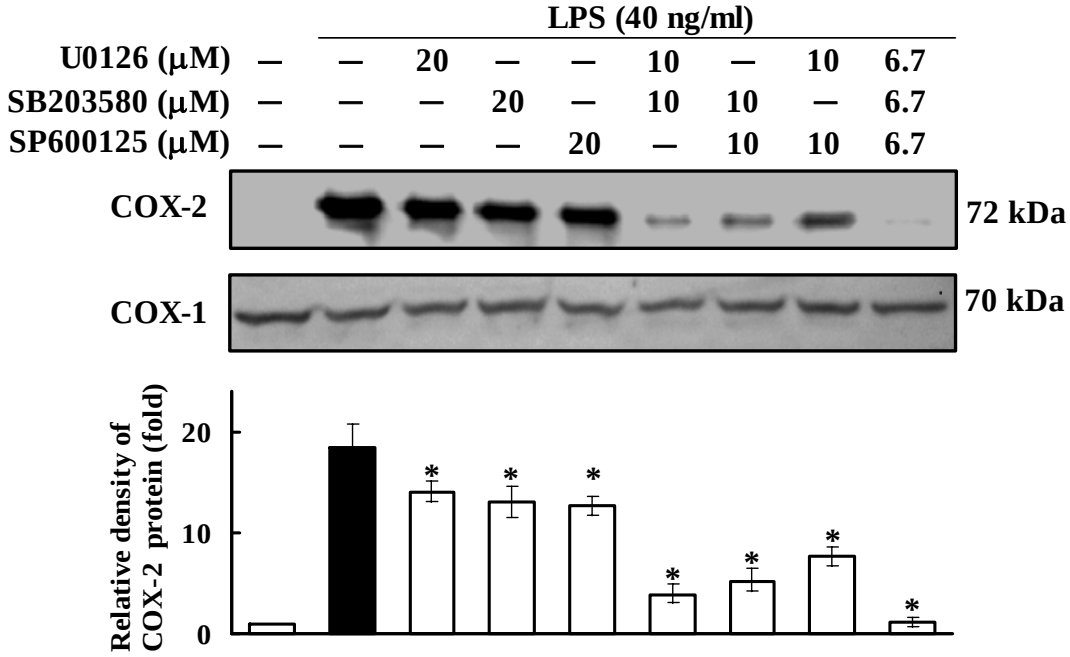

Figure 3.9. Effects of MAPK inhibitors on LPS-induced COX-2 expression. Cell culture and Western blotting were done as described in Figure 3.4. RAW264 cells were treated with the indicated concentrations of U0126, SB203580 and SP600125 for 30 min, and then exposed to 40 ng/ml LPS for 12 h. COX-2 and COX-1 were detected with their antibodies, respectively. Histograms show the densitometric analysis of COX-2 protein normalized to COX-1. The data represent the mean ± SD of three separate experiments, and the picture is a representative of those experiments each with similar results. **P* < 0.05, significantly different from LPS alone.

ERK and p38 kinase was investigated. As shown in Figure 3.8, delphinidin caused a dose-dependent inhibition of LPS-induced phosphorylation of JNK, ERK and p38 kinase in the concentration ranges from 25 to 100 μM, suggesting that delphinidin may suppress COX-2 induction by blocking the activation of MAPK signaling pathways. To confirm this conclusion, MAPK-specific inhibitors (U0126 for MEK1/2, SB203580 for p38 kinase and SP600125 for JNK) were used to challenge to block LPS-mediated COX-2 induction. RAW264 cells were pretreated with the indicated concentrations of the inhibitors for 30 min before exposure to 40 ng/ml LPS for 12 h. As shown in Figure 3.9, COX-2 expression

was partially suppressed by treatment with U0126, SB203580 and SP600125 alone. Treatment with the combination of two inhibitors strongly inhibited COX-2 induction. In particular, treatment with three inhibitors completely inhibited COX-2 induction. These data indicate that there are abundant actions in MAPK pathways with the attendant induction of COX-2. Delphinidin blocked all of three of MAPK pathways to suppress COX expression, suggesting that delphinidin may be a potent inhibitor for COX-2.

3.4 Discussion

Anthocyans have been discussed in relation to antiinflammation activity [15-19]. However, the molecular mechanisms underlying the activity of anthocyans are poorly defined. The present study is the first time to present molecular evidence that anthocyans inhibited COX-2 expression in LPS-activated RAW264 macrophage cells with a structure-activity relationship, and by blocking MAPK-mediated transcription regulation.

Five kinds of the representative anthocyanidins were used to investigate the potency of inhibition of COX-2 expression in the present study. It is noteworthy that the number of hydroxyl groups on the B ring might be associated with their actions. Anthocyanidins that contain single hydroxyl group on the B-ring such as pelargonidin, peonidin and malvidin showed no inhibitory effect. Cyanidin with two hydroxyl groups on the B-ring showed stronger inhibition. Delphinidin, a compound with three hydroxyl groups on the B-ring, exhibited the strongest inhibition in COX-2 expression. These data indicate that ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins is, at least, required to suppress COX-2 expression. Several lines of evidence have shown that the number of hydroxyl groups on the B-ring of anthocyanidins is also associated with the potency of antioxidative [27, 28], prooxidative [29, 30], anti-mutagenesis [31, 32]. For instance, delphinidin and cyanidin, that possess ortho-dihydroxyphenyl structure on the B-ring, showed stronger scavenge activity of superoxide radicals [27], inhibitory effect on

mutagenesis [31, 32]. However, pelargonidin, peonidin and malvidin without such ortho-dihydroxyphenyl structure, failed to show the above activities. Structure-activity studies in flavonoids have indicated that flavonoids with ortho-dihydroxy on the B-ring such as quercetin, rhamnetin, fisetin and luteolin showed stronger inhibition on COX-2 expression [33, 34]. The number of hydroxyl groups on the B-ring appears to be related to a molecular conformation that influences the interactions between flavonoids and enzymes such as tyrosine kinase and protein kinase C, which are involved in the transcriptional activity of COX-2. Indeed, it has been reported that flavonoids, which inhibit tyrosine kinase and protein kinase C, have an ortho-dihydroxy on the B-ring or A- ring [35, 36]. Anthocyans are subclass of flavonoids and also have similar chemical structure on the B-ring. The ortho-dihydroxy structure of anthocyanidins, which is essential for suppressing COX-2 expression, is very similar to those required for the inhibition of tyrosine kinase and protein kinase C. Thus, these findings together with other reports indicate that the biological activity of flavonoids including anthocyanidins appears to be associated with the ortho-dihydroxyphenyl structure on the B-ring.

Multiple lines of evidence have suggested that the inductive effects of LPS on COX-2 induction are mediated by transcriptional factors including AP-1, C/EBP and NF-κB that bind the promoter elements of COX-2 [9, 13-15]. These factors showed a redundancy in the regulating COX-2 transcription in LPS-treated macrophage/monocytic lineage [17]. In the present study, the effects of delphinidin on these transcription factors showed that delphinidin inhibited LPS-induced phosphorylation of c-Jun, a component of AP-1 complex (Figure 3.7A). Delphinidin also blocked LPS-mediated nuclear translocation of C/EBPδ (Figure 3.7B). Interestingly, delphinidin also had inhibitory effect on degradation of IκB-α (Figure 3.7C) and nuclear translocation of p65 (Figure 3.7D). These results are agreement with those from some chemopreventive compounds, such as capsaicin [37], apigenin [38] and diarylheptanoid [39], which inhibit LPS-mediated COX-2 induction by

blocking the degradation of IκB-α in mouse macrophage cells. Taken together, the data indicate that delphinidin may inhibit COX-2 induction by targeting the transcription factors binding to COX-2 promoter, such as NF-κB, AP-1 and C/EBPδ.

MAPK including JNK, ERK and p38 kinase have been reported to play critical roles on LPS-mediated COX-2 induction [26, 27]. These experiments demonstrated that there are abundant actions in MAPK pathways with the attendant induction of COX-2. LPS stimulated the activation of JNK, ERK and p38 kinase, and then sequentially induced COX-2 expression. The blockage of one pathway of MAPK by specific inhibitor is not sufficient to block COX-2 expression. Delphinidin suppressed LPS-induced COX-2 expression by blocking the activation of all of three kinases. Thus, delphinidin may be a potent inhibitor for COX-2, and would belong to the kind of COX-2 inhibitors from some chemopreventive phytochemicals such as diarylheptanoid [39], capsaicin [40] and sesquiterpene lactones [41], that have been reported to inhibit COX-2 by targeting MAPK signaling pathways including ERK, p38 kinase and JNK.

In summary, it is the first time to show that anthocyans inhibited LPS-induced COX-2 expression with a structure-activity relationship. LPS induced COX-2 production by activating MAPK pathways, and delphinidin suppressed LPS-mediated COX-2 expression by blocking MAPK pathways with the attendant activation of NF-κB, C/EBPδ and AP-1. These findings provide the first molecular basis for the anti-inflammatory properties of anthocyans.

3.5 Abstract

The effects of anthocyan extracts and anthocyanidins on the expression of COX-2 were investigated in LPS-activated murine macrophage RAW264 cells. Anthocyan extracts from bilberry showed a dose-dependent suppression of LPS-induced COX-2 expression. Of five anthocyanidins, delphinidin and cyanidin inhibited LPS-induced

COX-2 expression, but, pelargonidin, peonidin and malvidin did not. The structure-activity relationship suggests that the ortho-dihydroxyphenyl structure of anthocyanidins on the B-ring appears to be related with the inhibitory actions. Delphinidin, the most potent inhibitor, caused a dose-dependent inhibition of COX-2 expression at both mRNA and protein levels. Western blotting analysis indicated that delphinidin inhibited the degradation of Iκ-B, nuclear translocation of p65 and C/EBPδ, and phosphorylation of c-Jun, but not CREB. Moreover, delphinidin suppressed the activations of MAPK including JNK, ERK and p38 kinase. MAPK inhibitors (U0126 for MEK1/2, SB203580 for p38 kinase and SP600125 for JNK) specifically blocked LPS-induced COX-2 expression. Thus, the results demonstrated that LPS induced COX-2 expression by activating MAPK pathways, and delphinidin suppressed COX-2 by blocking MAPK-mediated pathways with the attendant activation of NF-κB, AP-1 and C/EBPδ. These findings provide the first molecular basis that anthocyanidins with ortho-dihydroxyphenyl structure may have anti-inflammatory properties through the inhibition of MAPK-mediated COX-2 expression.

3.6 References

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Chapter 4 Molecular characterization of apoptosis induction by anthocyans in human leukemia cells

4.1 Introduction

Several lines of studies have shown that anthocyanins have inhibitory effects on the growth or proliferation of some cancer cells [1-5] and anticarcinogenic effects in 1,2-dimethylhydrazine-initiated F344/DuCrj rats [6]. Thus, it is possible that anthocyanins may also have anticancer effects in tumor cells. However, the molecular mechanism underlying the function is little defined.

Apoptosis has been reported to pay an important rule in elimination of seriously damaged cells or tumor cells by chemopreventive agents [7, 8]. The cells that have undergone apoptosis are typically shown in chromatin condensation, and DNA fragmentation [9]. They are rapidly recognized by macrophages before cell lysis, and then can be removed without inducing inflammation [7, 10]. Therefore, apoptosis-inducing agents are expected to be ideal anticancer drugs. Many therapeutic agents for cancer, such as cisplatin [11, 12], paclitaxel [13, 14], isothiocyanates [15, 16] and adriamycin [17], have been reported to eliminate tumor cells by inducing apoptotic cell death.

Although extracellular stimuli-induced apoptosis may involve multiple mechanisms, several lines of evidence indicate that JNK plays an important role in triggering apoptosis in response to these stimuli [18, 19]. It is known that JNK pathway activation is required for apoptosis induced by UV and gamma radiation [11, 20], ROS [21], ceramide [22, 23], heat shock [10, 24], some anticancer agents [11-14, 17] and chemopreventive agents [15, 16]. Genetic studies in animals also support the role of JNK in an apoptotic death program. The *jnk1*-deficient mice exhibit decreased activation-induced apoptosis [25]. The *jnk1/jnk2*-deficient mice have severe dysregulation of apoptosis in the brain and show embryonically lethal [26]. Moreover, mice whose endogenous *c-jun* serine 63 and 73 (the

major JNK targets) replaced by alanines (Jun AA) are resistant to neuronal apoptosis induced by excitatory amino acid kainate [27].

Accumulated data suggest that a mitochondria-initiated death pathway plays an important role in triggering apoptosis in response to these stimuli [28-30]. In the mitochondria-initiated pathway, mitochondria undergoing permeability transition release apoptogenic protein such as cytochrome *c* or apoptosis-inducing factor (AIF) from mitochondrial intermembrane space into cytosol. Released cytochrome *c* can activate caspase-9, and activated caspase-9 in turn cleaves and activates executioner caspase-3 [31]. After caspase-3 activation, some specific substrates for caspase-3 such as poly (ADP-ribose) polymerase (PARP) are cleaved, and eventually lead to apoptosis [32]. Some data also showed that caspase-8 can directly activate caspase-3, and sequentially induces apoptosis [33, 34]. Furthermore, recent studies have proposed that cellular ROS may mediate the mitochondria-initiated apoptosis [35, 36]. Many stimuli such as TNF-α, anticancer drugs and chemopreventive agents stimulate cells to produce ROS [37-39]. Generated ROS can cause the loss of mitochondrial membrane potential ($\Delta\Psi m$) by activating mitochondrial permeability transition, and induce apoptosis by releasing apoptogenic protein such as cytochrome *c* to cytosol [31, 32].

Based on the functions of anthocyans and the roles of ROS, JNK and mitochondrion in initiating apoptosis, the molecular mechanism of induction of apoptosis by anthocyans was investigated in human leukemia cells (HL-60), which is a valid model for testing antileukemic or general antitumoral compounds [40]. The results indicate that ROS-mediated JNK activation and mitochondrial dysfunction are involved in anthocyan-induced apoptosis.

4.2 Materials and methods

4.2.1 Materials and cell culture

Anthocyanidins (delphinidin chloride, cyanidin chloride, pelargonidin chloride, peonidin chloride and malvidin chloride) purified by HPLC were from Extrasynthese (Genay, France) (Figure 1.1B). Petunidin chloride was prepared by acid hydrolysis of petanin (petunidin 3-*p*-coumaroylrutinoside-5-glucoside) isolated from purple sweet potatoes [41], and purified by HPLC. Dp 3-Sam was isolated from the dried calices of *Hibiscus sabdariffa L.* (Figure 2.2) (42). The antibodies against JNK, phospho-JNK, and caspase-3 were from Cell Signaling Technology (Beverly, MA). The antibodies against cytochrome *c*, Bid and α-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). FBS was from Equitech-Bio (Kerrville, TX). Proteinase K, RNase A, *N*-acetyl-L-cysteine (NAC) and catalase were from Sigma (St. Louis, MO). 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was from Molecular Probes (Eugene, OR). Caspase-8 (IETD-CHO) and caspase-9 (LEHD-CHO) inhibitors were from Calbiochem (Nottingharm, UK). Caspase-3 (DEVD-CHO) inhibitor was from Peptide Institute (Osak, Japan). HL-60 cells were obtained from the Cancer Cell Repository, Tohoku University, Japan, and cultured at 37°C, 5% CO_2 in RPMI-1640 medium containing 10% FBS.

4.2.2 DNA fragmentation assays and morphological analysis of apoptotic cells

For DNA fragmentation assays, the cells ($2x10^6$) were treated with different concentrations of anthocyanidin for 6 h or fixed concentration for varying exposures of time. For various inhibitors, cells were pretreated with various inhibitors at the indicated concentrations for 1 h, and then incubated with delphinidin (100 μM) or Dp 3-Sam (100 μg/ml) for 6 h. Cells were harvested by centrifugation and washed twice in ice-cold PBS. The pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 0.5% SDS) plus 0.2 mg/ml RNase A, and incubated at 50°C for 30 min. Then, proteinase K was added and incubated for another 2 h. The DNA was separated on 2% agarose gel, and

digitally imaged after staining with ethidium bromide. Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with 2.5 μg/ml bisbenzimide Hoechst 33258 fluorochrome, followed by examination on a fluorescence microscope.

4.2.3 RNA extraction and RT-PCR

HL-60 cells (1 x 10^7 cells) were plated in 100-mm tissue culture dishes. After pre-incubation for 24 h, apoptosis induction was carried out for a dose- and time-course by anthocyanidin in 0.1% DMSO as described in the DNA fragmentation assays. Cellular RNA was extracted with an ISOGEN RNA isolation kit (Nippon Gene, Tokyo). The 290-bp target in *c-jun* cDNA [43] was amplified by using the sense primer (5'-CAACATGCT CAGGGAACAGG-3') at positions 261-280 and antisense primer (5'-GGTCCATGC- AGTTCTTGGTC-3') at positions 531-550. As a housekeeping gene, *gapdh* [44] was amplified by using the sense primer (5'-GACCCCTTCATTGAC-CTCAAC-3') at positions 143-162 and antisense primer (5'-CATACCAGGAAATG-AGCTTG-3') at positions 965-984. The RT-PCR was done by one-step reaction with Read-to-Go RT-PCR beads (Amersham Pharmacia Biotech) as described previously [45, 46]. Briefly, RNA (250 ng) was used for reverse-transcription into cDNA at 42°C for 30 min using oligo (dT) 12-18 primers. Amplifications were done at 95°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec with GenAmp PCR System 2400 machine (Perkin-Elmer). Template- and cycle-dependence of the PCR products were confirmed and the available cycle numbers of PCR for *c-jun* and *gapdh* were determined as 33 and 21 cycles, respectively. The PCR products were separated on 2% agarose gel, and digitally imaged after staining ethidium bromide. The bands were quantified by Imager Gauge Software (Fuji Photo Film). The mRNA level in the control culture is arbitrarily set to 1.0, the basal level for subsequent mRNA comparisons.

4.2.4 Extraction of whole cellular protein and fractionation of cytosolic cytochrome *c*

For preparing whole cellular protein, the cells were harvested by centrifugation and rinsed twice with ice-cold PBS. The pellets were lysed with cell lysis buffer containing 10 mM Tris (pH7.5), 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1 μg/ml aprotinin. The lysates were then sonicated for 10-sec twice and centrifuged for 5 min. The supernatants were used as whole cellular protein. For preparing whole cellular protein, the harvested pellets were suspended in 5 vol of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM ethyleneglycol-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), 1 mM DTT, 1 μg/ml aprotinin, 100 μg/ml PMSF, and 250 mM sucrose), and then homogenized for 40 strokes, following centrifugation at 1200 rpm for 10 min at 4˚C. The supernatant was collected and further centrifuged at 60,000 rpm for 60 min at 4˚C to isolate cytosol.

4.2.5 Western blotting analysis

The lysates of whole cellular protein or cytosolic protein were boiled in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromophenol blue) for 5 min. Protein concentration was determined by using dye-binding protein assay kit (Bio-Rad) as described in manufacture manual. Equal amounts of lysate protein were run on 15% SDS-PAGE and electrophoretically transferred to PVDF membrane (Amersham Pharmacia Biotech). The blots were blocked with TBST buffer (500 mM NaCl, 20 mM Tris-HCl (pH 7.4), 0.1% Tween 20) containing 5% nonfat dry milk, and then incubated with specific primary antibody in TBST buffer containing 5% bovine serum albumin overnight at 4˚C. The blots were further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected by ECL kit with a Lumino Image Analyzer (TAITEC).

4.2.6 Intracellular ROS determination

The intracellular formation of ROS was detected by using the fluorescent probe H_2DCF-DA [47]. This compound readily diffuses into cells and is hydrolyzed by intracellular esterase to yield H_2DCF. ROS produced by the cells oxidizes H_2DCF to highly fluorescent compound DCF. Thus, the fluorescence intensity is proportional to the amount of ROS produced by the cells. HL-60 cells were plated in 48-well plate (2×10^5 cells/well). After pre-incubation for 24 h, cells were treated with or without various anthocyanidins at different concentrations or times, and then incubated with or without 20 μM of H_2DCF-DA for 30 min. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission of 530 nm using a fluorescent Mutilabel Counter (Perkin Elmer). The relative amount of intracellular ROS production by anthocyanidins was expressed as the fluorescence ratio of treated to control.

4.2.7 Assay for caspase-3 activity

The activity of caspase-3 was determined by using fluorogenic caspase-3 substrate rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110), according to the manufacturer's instructions (Apo-ONE, Promega). Briefly, cells (2×10^5 cells/ml) were dispensed into the wells of a 96-well plate and treated with Dp3-Sam. 100 μl of homogeneous caspase-3/7 reagent was added to each well, and then gently mixed in a plate shaker at 300-500 rpm for 1 min. After 1 hr incubation at room temperature, the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission of 530 nm with a fluorescent Multilable Counter (PerkinElmer).

4.2.8 Flow cytometric detection of ΔΨm loss

ΔΨm was assessed by a cell permeable marker $DiOC_6$ (3) which specifically accumulates into mitochondria depending on ΔΨm [48]. HL-60 cells (1×10^6/ml) were

treated with Dp3-Sam for various times, and then incubated with 100 nM of $DiOC_6$ (3) for 30 min. The cells were washed with PBS, and resuspended in 1 ml of PBS. The cells (1×10^4) were analyzed at FL1 (530 nm) with a flow cytometer (Beckman Coulter, EPICS XL-MCL).

4.2.9 Statistical analysis

Difference between the treated and the control was analyzed by t-test. A probability of $P<0.05$ was considered significant.

4.3 Results

4.3.1 Apoptosis induction of HL-60 cells by anthocyanidins

To investigate the ability of anthocyanidins to induce apoptosis of cancer cells, HL-60 cells were treated with six kinds of anthocyanidins (Figure 1.1B) at 100 μM for 6 h. Of them, delphinidin, petunidin, and cyanidin induced apoptosis in HL-60 cells detected by morphological changes (Figure 4.1B), and by DNA fragmentation (Figure 4.1A). The ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins appears to be essential for apoptosis induction because pelargonidin, peonidin and malvidin, having no such ortho-dihydroxyphenyl structure, failed to show the apoptosis induction (Figure. 4.1A). Next, delphinidin, a strongest inducer of them, was used for dose- and time-response experiments. As shown in Figure 4.2A, DNA fragmentation was observed at 6 h in a dose-dependent manner with 80-120 μM, and at 100 μM in a time-dependent manner during 4-18 h (Figure 4.2B). The efficacious induction for apoptosis was observed at 100 μM for 6 h in this study.

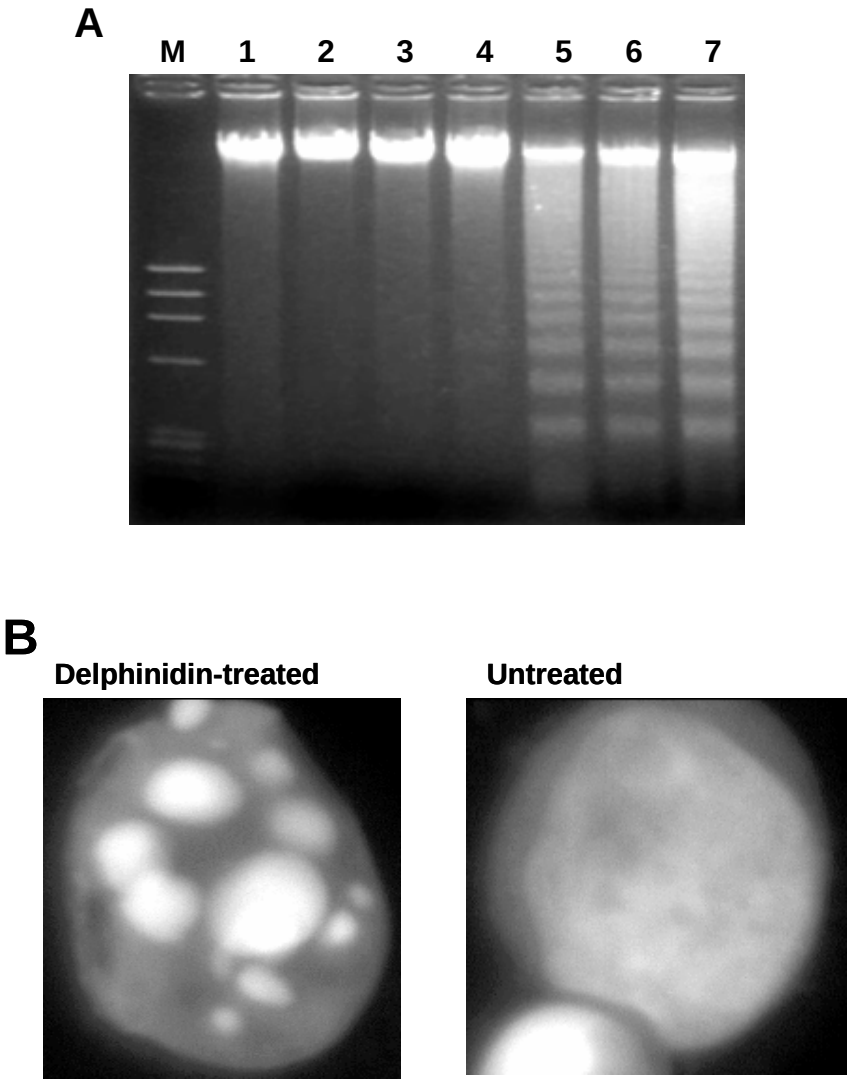


Figure 4.1. Induction of apoptosis in HL-60 cells by anthocyanidins. (A) HL-60 cells ($2x10^6$) were plated on 60-mm dishes and were cultured for 24 h. The cells were then exposed to 100 µM of various anthocyanidins or 0.1% DMSO vehicle for 6 h. Cells were harvested by centrifugation, and DNA was extracted as described in Methods and materials. DNA was separated on 2% agarose gel, and digitally imaged after staining with ethidium bromide. M: DNA marker of øX174/*Hae*III digests; 1, 0.1% DMSO; 2, malvidin; 3, peonidin; 4, pelargonidin; 5, petunidin; 6, cyanidin; 7, delphinidin. **(B)** Morphology of delphinidin-treated HL-60 cells (left) and control cells (right). HL-60 cells were exposed to delphinidin (100 µM) for 6 h, and the morphological changes in the nuclear chromatin of cells were detected by staining with 2.5 µg/ml bisbenzimide Hoechst 33258 fluorochrome, followed by examination under a fluorescence microscope.

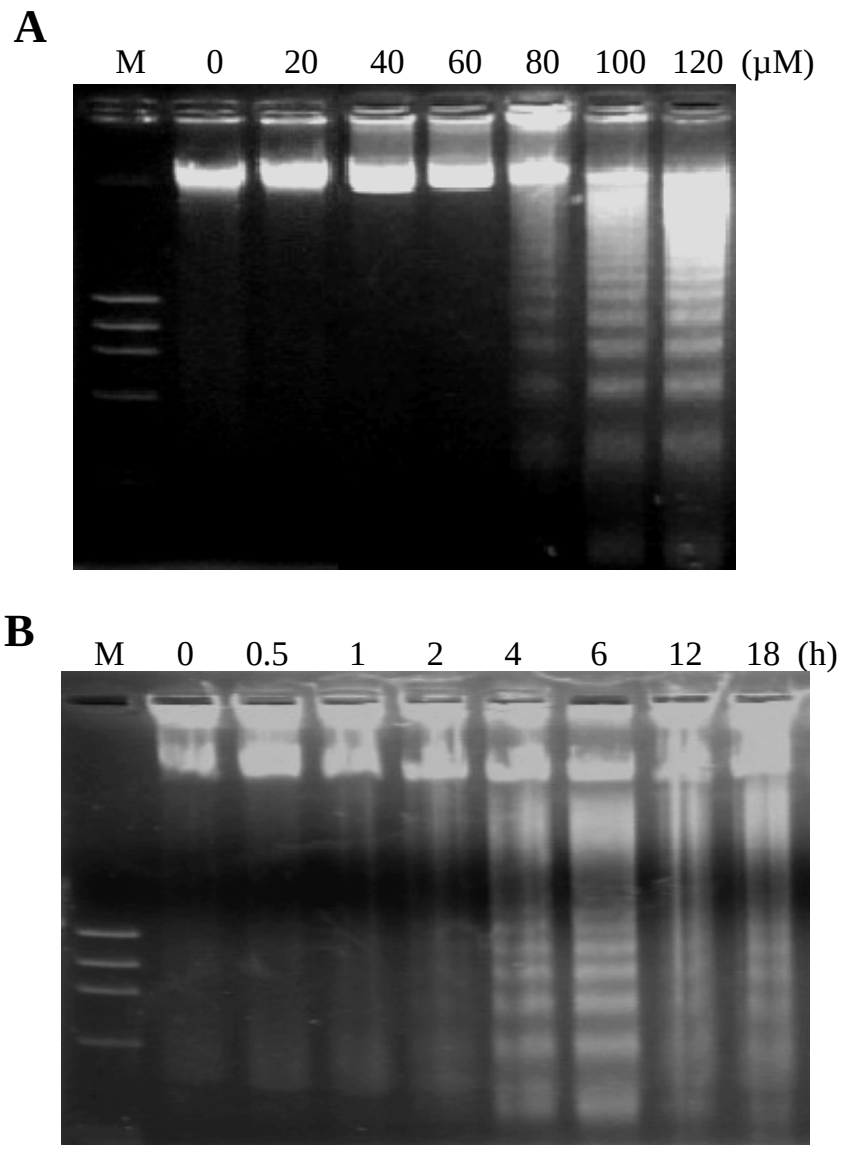


Figure 4.2. Delphinidin induces DNA fragmentation in HL-60 cells. (A) A dose-dependence of DNA fragmentation. Cells were exposed to the indicated concentrations of delphinidin for 6 h. **(B)** A time-course of DNA fragmentation. Cells were exposed to 100 μM delphinidin for the indicated times. The cells were then harvested by centrifugation, and DNA was extracted as described in Materials and methods. DNA was separated on 2% agarose gel, and digitally imaged after staining with ethidium bromide. M: DNA marker of øX174/*Hae*III digests.

4.3.2 *c-jun* expression

Because recent studies suggest that *c-jun* expression may be related to apoptosis [23, 35, 36], the change of *c-jun* mRNA levels in the cells treated with delphinidin was examined, using RT-PCR technique. As shown in Figure 3, delphinidin induced *c-jun* mRNA expression in a time- and dose-dependent manner. Within 6 h of exposure, delphinidin induced markedly *c-jun* mRNA expression at the concentrations from 80 to, at least, 120 μM (Figure 4.3A). Exposure to 100 μM delphinidin resulted in *c-jun* mRNA expression beginning at 1 h and reaching a maximum at 4 h (Figure 4.3B). Furthermore, actinomycin D (a RNA transcription inhibitor) completely inhibited delphinidin-induced *c-jun* expression, and cycloheximide (a protein synthesis inhibitor), which itself slightly increased *c-jun* mRNA, superinduced *c-jun* expression when treated with delphinidin. As a control, the levels of *gapdh* mRNA showed no significant change under these experimental conditions. These results suggest that *c-jun* induction by delphinidin is regulated at both transcription and post-transcription levels. Comparing with the data of DNA fragmentation (Figure 4.2), delphinidin induced *c-jun* expression (Figure 4.3) in a time-subsequent and a dose-corresponding manner, suggesting *c-jun* expression may be involved in delphinidin-induced apoptosis.

4.3.3 JNK phosphorylation

c-jun expression requires the activation of JNK pathway [17, 21, 23, 27]. To test whether delphinidin-induced apoptosis involves JNK pathway activation, the effect of delphinidin on the phosphorylation of JNK, which is an essential event for activator protein-1 (AP-1) activity, was examined in the treated cells. As shown in Figure 4.4, delphinidin induced JNK phosphorylation in a time-dependent manner during 1-6 h. JNK phosphorylation increased up to 7.0-fold compared with control. On the other hand, total JNK proteins showed no change in delphinidin-treated cells. Delphinidin stimulates

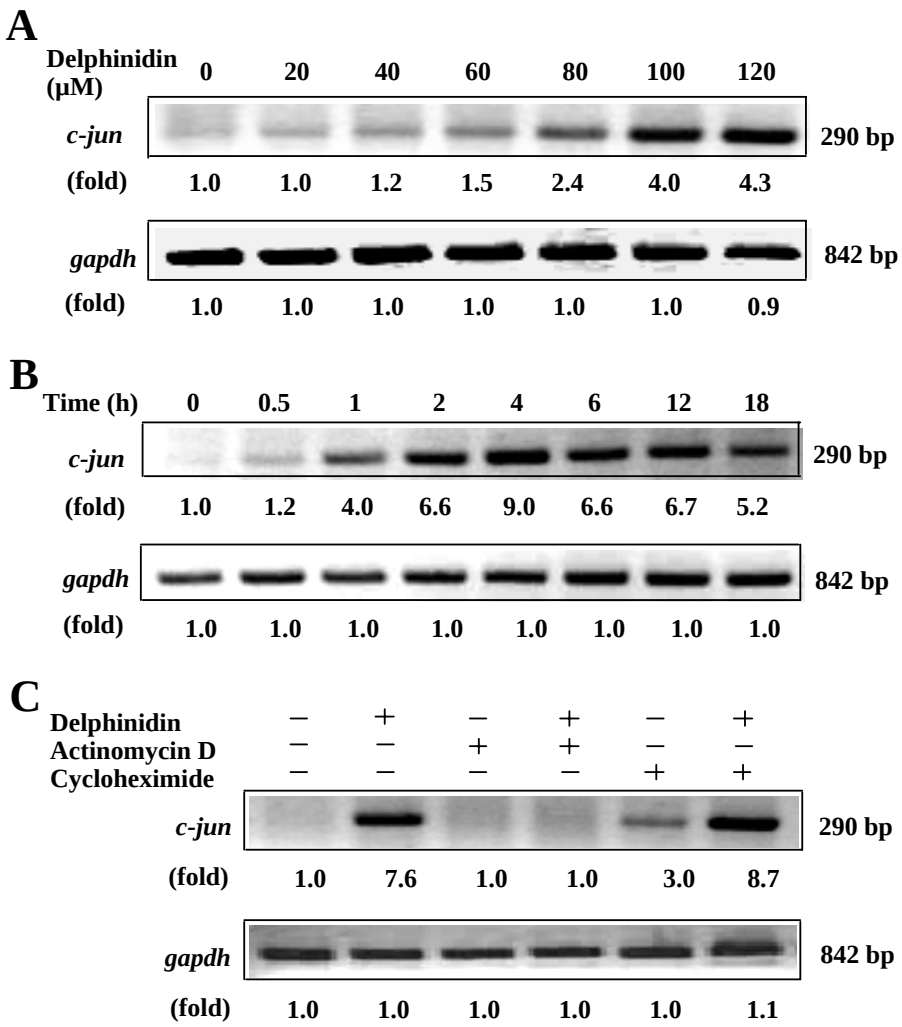


Figure 4.3. Delphinidin induces *c-jun* mRNA expression. (A) A dose-dependence of *c-jun* mRNA induction. The cells were exposed to the indicated concentrations of delphinidin for 6 h. **(B)** A time-course of *c-jun* mRNA induction. The cells were exposed to 100 μM delphinidin for the indicated times. Expression of *gapdh* gene was used as a control. **(C)** Delphinidin regulates *c-jun* induction at transcriptional and post-transcriptional levels. HL-60 cells were pretreated with 5 μg/ml actinomycin D or 3 μg/ml cycloheximide for 1 h and then exposed to 100 μM delphinidin for 3 h. Expression of *gapdh* gene was used as a control. RNA was extracted with ISOGEN RNA isolation kit, and RT-PCR was performed as described in Material and methods. The RT-PCR products were separated on 2% agarose gel, and digitally imaged after staining with ethidium bromide. Quantification of the bands was performed by Imager Gauge Software (Fuji Photo Film). The results are expressed as the ratios of *c-jun* mRNA in the treated cells to those of control.

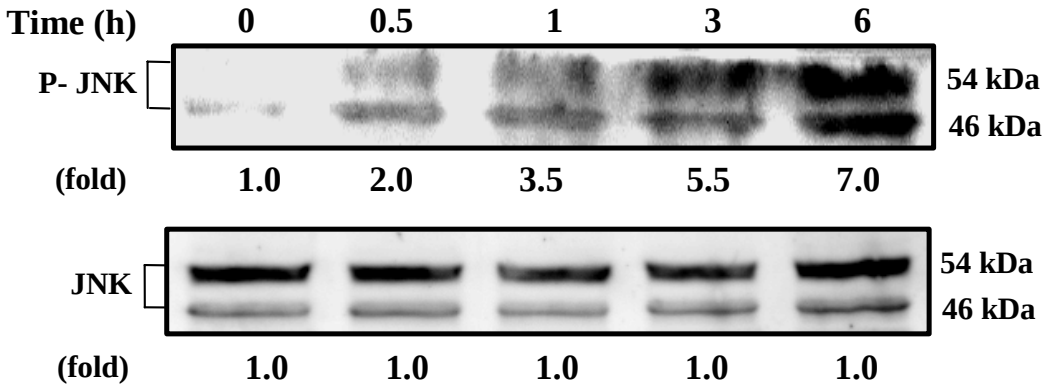


Figure 4.4. Delphinidin stimulates JNK phosphorylation. HL-60 cells were exposed to 100 μM delphinidin for the indicated times. Lysate protein of 50 μg was applied on 10% SDS-PAGE. The proteins of total and phosphorylated JNK were detected with corresponding specific antibodies, and visualized by chemiluminescence ECL kit. The amounts of the proteins were quantified, using Imager Gauge Software (Fuji Photo Film) and expressed as a fold of specific protein in the treated cells to those of control (0 min).

JNK phosphorylation and *c-jun* expression in a time-subsequent manner. The appearance of JNK phosphorylation and *c-jun* gene expression by delphinidin is earlier than that of DNA fragmentation. These data suggest that JNK pathway activation may be involved in triggering the apoptosis by delphinidin.

4.3.4 Intracellular ROS generation

Based on *c-jun* expression and JNK activation, an oxidative stress such as ROS generation may be induced in delphinidin-treated cells. For this, a fluorescent probe H_2DCF-DA was used to monitor the intracellular ROS levels [48]. As shown in Figure 4.5A, a dose-dependent increase of intracellular ROS levels was detected when the cells were treated at 30 min with the indicated concentrations of delphinidin. An increase of intracellular ROS levels by 100 μM delphinidin was detected from 30 min. After 6 h, the

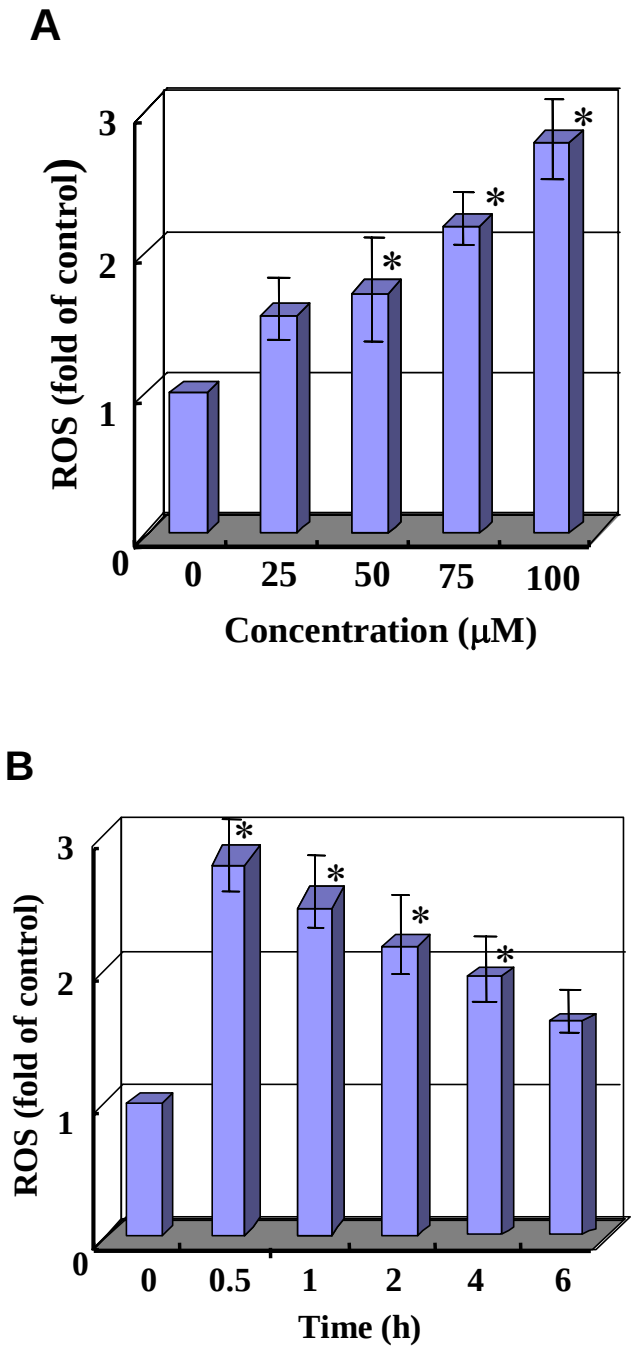


Figure 4.5. Anthocyanidins elevate intracellular ROS. (A) A dose-dependence of the production of ROS by delphinidin. Cells were exposed to the indicated concentrations of delphinidin for 30 min. **(B)** A time-course of the production of ROS by delphinidin. Cells were exposed to 100 μM delphinidin for the indicated times. **(C)** The production of ROS in anthocyanidin-treated cells. Cells were exposed to 100 μM of different anthocyanidins for 30 min, respectively.

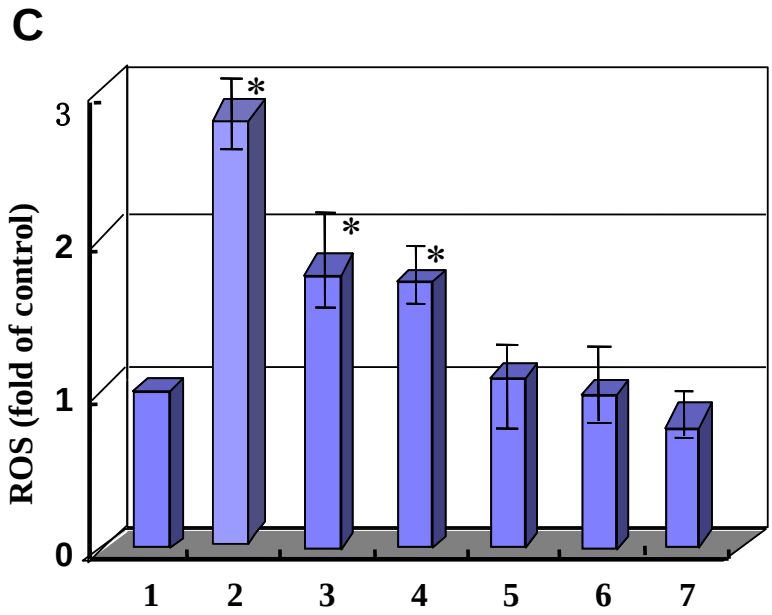


Figure 4.5 ***(continued)***

1, 0.1% DMSO; 2, delphinidin; 3, cyanidin; 4, petunidin; 5, pelargonidin; 6, peonidin; 7, malvidin. To determining intracellular ROS, the cells were incubated with 20 μM of 2',7'-dichlorofluorescin diacetate for 30 min, and the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission of 530 nm using a fluorescent Mutilabel Counter (Perkin Elmer). The relative amount of intracellular ROS production by anthocyanidins was expressed as the fluorescence ratio of the treated to control. Data represent the mean ± SD of 3-4 separate experiments. * $P<0.05$, significantly different from non-treatment control.

level was reduced to the control level (Figure 4.5A). A maximum amount of ROS production was observed with the treatment at 100 μM for 30 min. Moreover, the ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins appears to be essential for intracellular ROS production because pelargonidin, peonidin and malvidin, having no such ortho-dihydroxyphenyl structure, failed to show an increased level (Figure 4.5C). These data indicated that the increment of ROS might play a role as an early mediator in anthocyanidin-induced apoptosis.

4.3.5 Mitochondrial cytochrome *c* release, Bid truncation and ΔΨm loss

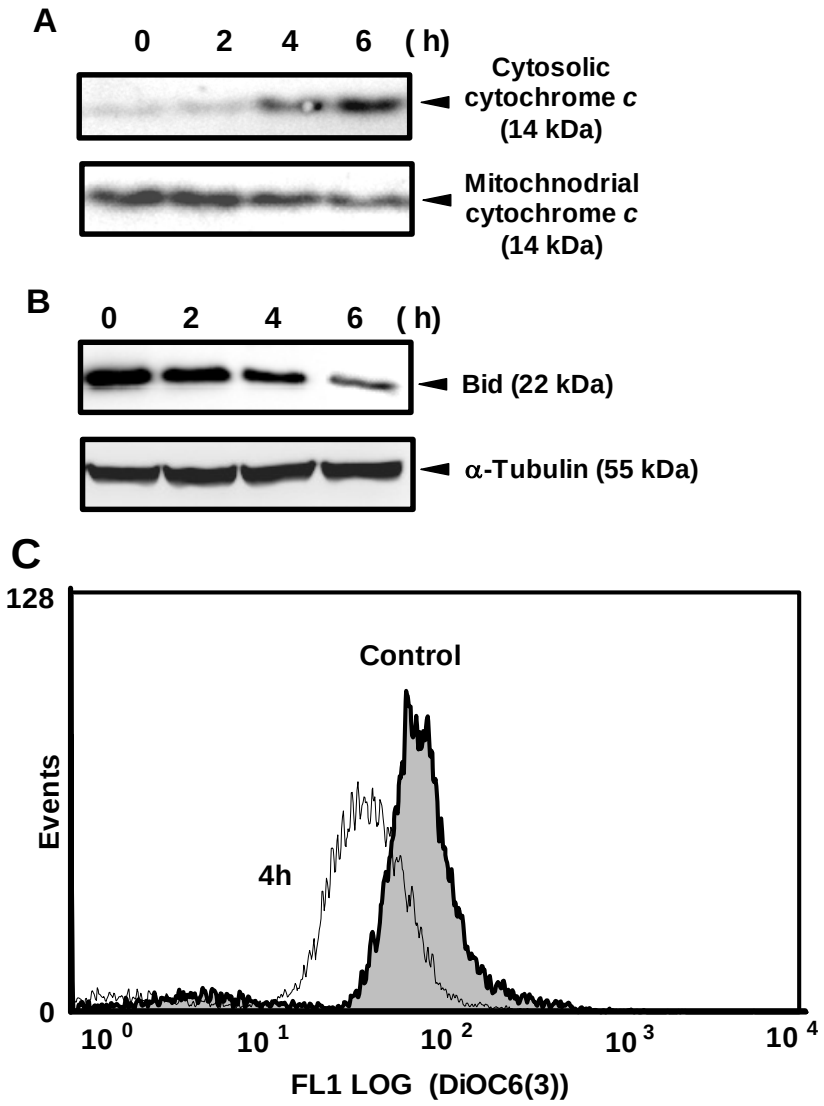


Figure 4.6. Dp3-Sam-induced apoptosis involves mitochondrial dysfunction pathway. **(A)** Dp3-Sam induced cytochrome *c* release. HL-60 cells (1×10^6 cells/ml) were treated with 100 μM of Dp3-Sam for 2-6 h. Mitochondrial cytosolic fractions were isolated as described in Materials and methods. Cytochrome *c* was detected by Western blotting analysis with cytochrome *c* antibody. **(B)** Dp3-Sam induced Bid truncation. HL-60 cells (1×10^6 cells/ml) were treated with 100 μM of Dp3-Sam for 2-6 h, and whole-cell lysate was used for Western blotting analysis with Bid antibody. **(C)** Dp3-Sam caused ΔΨm loss. HL-60 cells (1×10^6 cells/ml) were treated with 100 μM of Dp3-Sam for 4 h. The harvested cells were then incubated with 100 nM of $DiOC_6$ (3) for 30 min, followed by flow cytometric analysis as described in Materials and methods.

Dp3-Sam is a glycoside with delphinidin aglycon and is shown to have same biological activities with delphinidin. For instance, it also produced ROS and induced apoptosis in HL-60 cells with the same fashion with delphinidin (data not shown). Dp3-Sam was used for the next experiments because it was easily extracted from *Hibiscus sabdariffa L.* (Malvaceae) (42).

Several lines of evidence indicate ROS can cause ΔΨm loss by activating mitochondrial permeability transition, and induce apoptosis by releasing apoptogenic protein such as cytochrome *c* to cytosol [31, 32]. To elucidate whether mitochondrial cytochrome *c* releases into cytosol in Dp3-Sam-treated apoptosis, Cytochrome *c* in mitochondrial and cytosolic fractions was detected by Western blotting analysis. As shown in Figure 4.6A, a time-dependent accumulation of cytochrome *c* in the cytosol was detected, and the efflux in mitochondrion was observed simultaneously in Dp3-Sam-treated cells. Current evidence suggests that truncated Bid may play an important role in the release of cytochrome *c*. Thus, cell lysates were also subjected to Western blotting analysis. Likewise, a decreased Bid protein level was also observed from 4 h treatment (Figure 4.6B) while α-tubulin showed no change. Due to cytochrome *c* release links to the loss of ΔΨm [19, 20], the effect of Dp3-Sam on the ΔΨm was further examined with a mitochondria-specific dye, $DiOC_6$ (3) (48). A marked loss of ΔΨm was observed at 4 h exposure to 100 μM of Dp3-Sam (Figure 4.6C). These data indicate that apoptosis induced by Dp3-Sam involved a mitochondrial dysfunction pathway.

4.3.6 Activation of caspase-8, -9 and-3

Accumulated results indicate cytosolic cytochrome *c* induces caspase-9-dependent activation of caspase-3, which further cause cleavage of the DNA reparatory protein, PARP [51]. Therefore, caspases play pivotal roles in the terminal, execution phase of apoptosis induced by diverse stimuli [32]. Of which, caspase-3 was classically divided into

executioner caspases, and caspase-8 and -9 were into initiator caspases according to their function and their sequences of activation [32]. To investigate whether caspases were involved in the apoptotic response induced by Dp3-Sam, HL-60 cells were treated with 100 μM Dp3-Sam in the indicated time. As shown in Figure 4.7A, Dp3-Sam caused a time-dependent activation of caspase-8, -9 and -3. The appearances of active forms of the caspases were observed 4 h after the addition. Moreover, pretreatment with the inhibitors of caspase-8 (IETD), -9 (LEHD) and -3 (DEVD), the activity of caspase-3 induced by Dp3-Sam was significantly inhibited ($P< 0.05$) (Figure 4.7B) and the survival rate of HL-60 cells was augmented significantly ($P< 0.05$) (Figure 4.7C). These results indicate that a caspase-3-dependent pathway might be involved in Dp3-Sam-induced apoptosis in HL-60 cells.

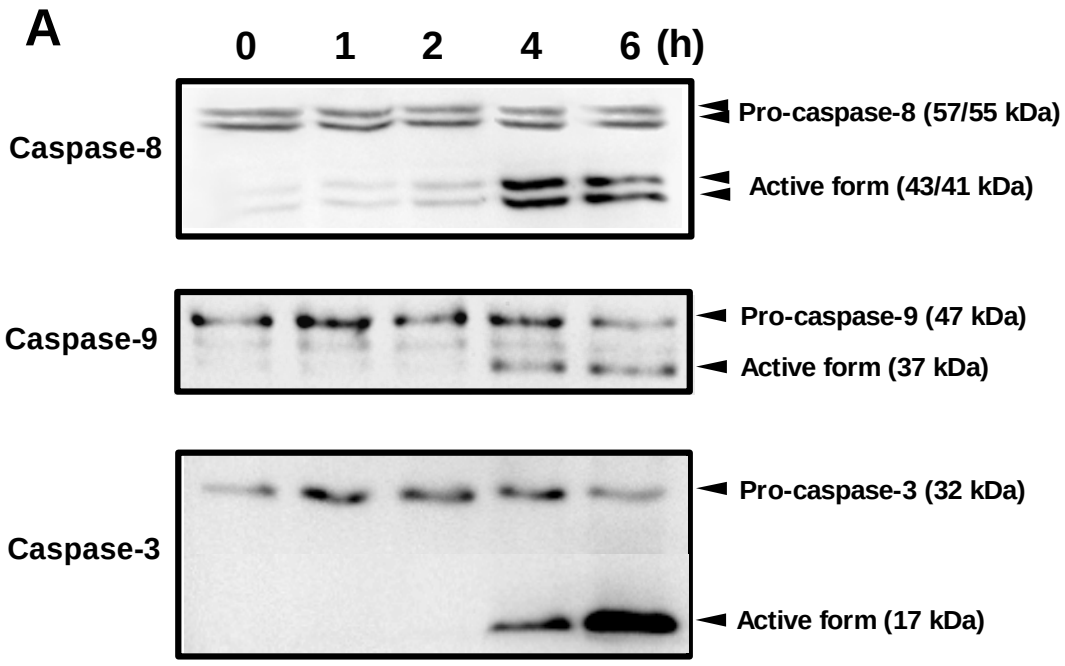


Figure 4.7. Dp3-Sam-induced apoptosis involves the activation of caspase-3, -8 and -9. (A) Dp3-Sam induced the activation of caspase-3, -8 and -9. HL-60 cells (1×10^6 cells/ml) were treated with 100 μM of Dp3-Sam for the indicated times, whole-cell lysate was used for Western blotting analysis with the indicated specific antibodies, respectively.

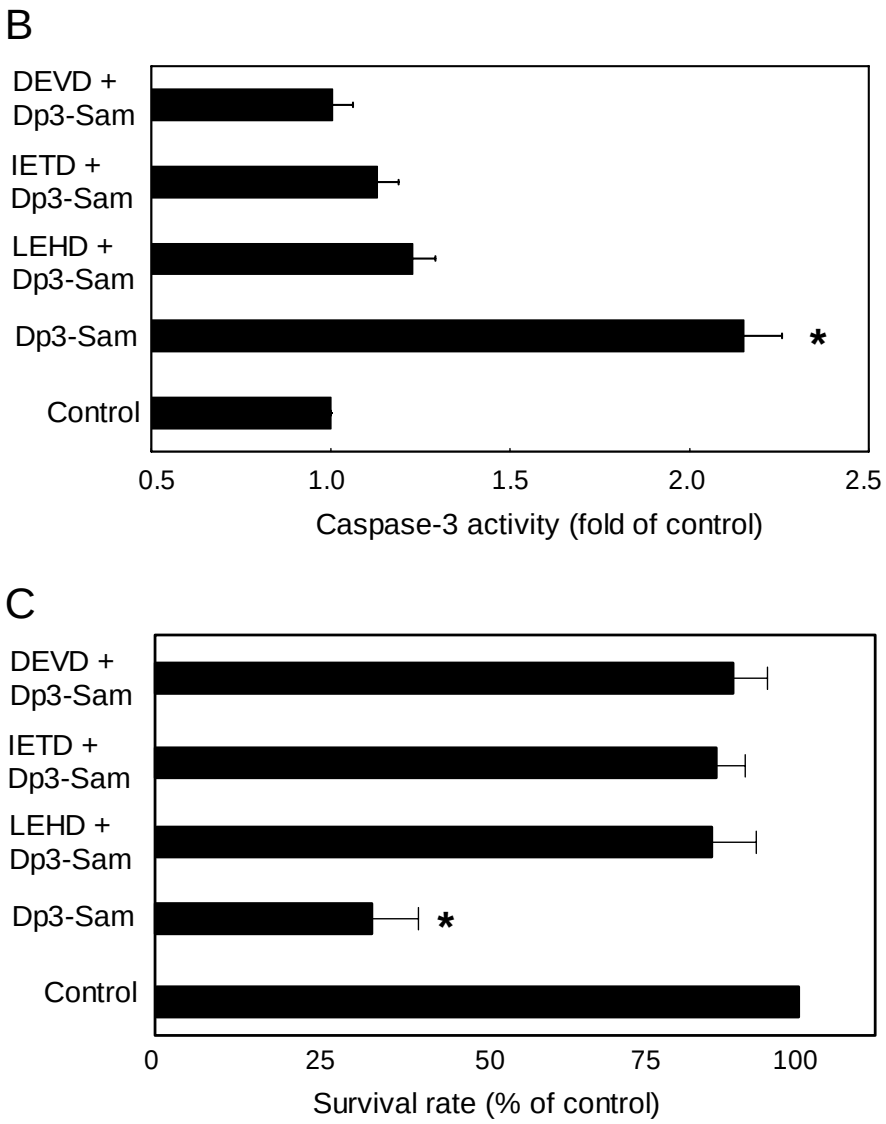


Figure 4.7 ***(continued)***

(B) Caspase inhibitors suppress the activity of caspase-3 induced by Dp3-Sam. HL-60 cells were pretreated with the 100 μM of caspase-8 inhibitor (IETD) or caspsae-9 inhibitor (LEHD) or caspase-3 inhibitor (DEVD) for 1 h, and then exposed 100 μM of Dp3-Sam for 6 h. The caspase-3 activity was measured as described in Materials and methods. **(C)** Caspase inhibitors augmented survival rate of HL-60 cells. Cell were pretreated with caspase inhibitors as (B), and then exposed to 100 μM of Dp3-Sam for 24 h. Cell survival was determined by a MTT assay. The results are shown as means ± SD of three independent experiments. * $P<0.05$, significantly different from non-treatment control.

4.3.7 Antioxidants protect against apoptosis by blocking ROS generation, JNK phosphorylation and caspase activation

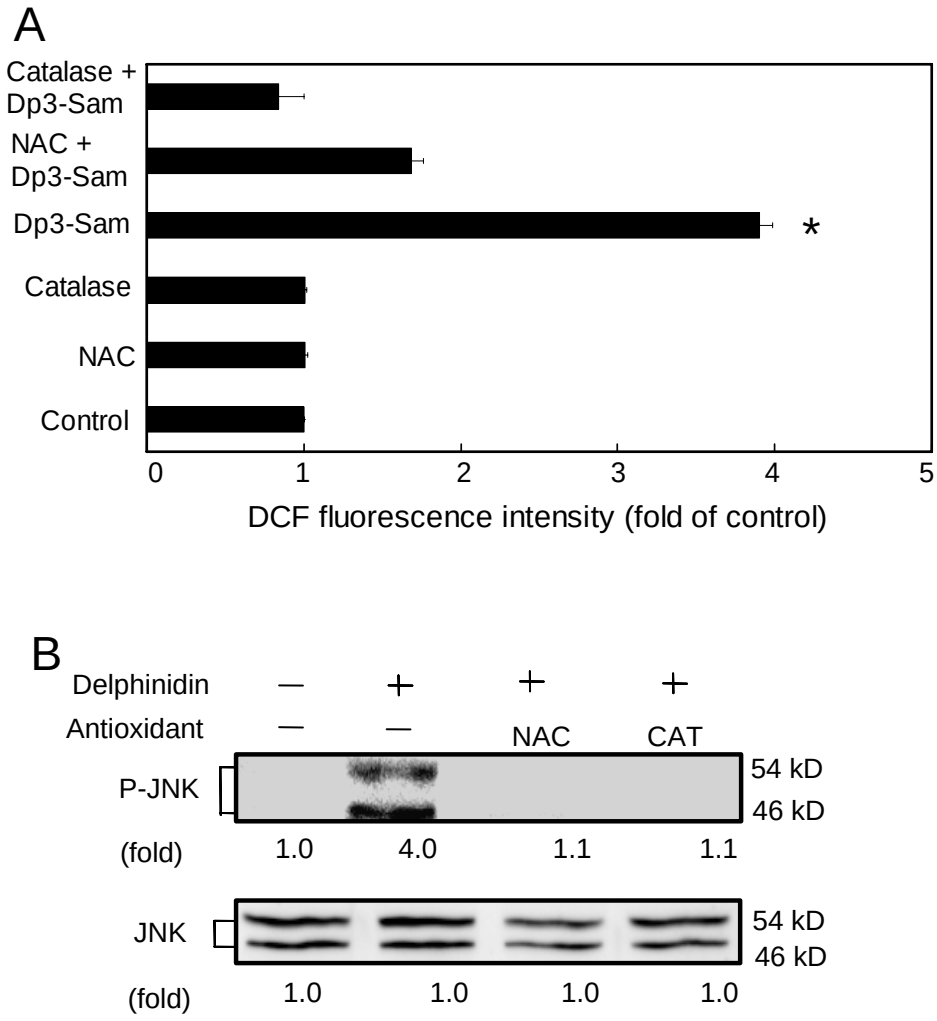
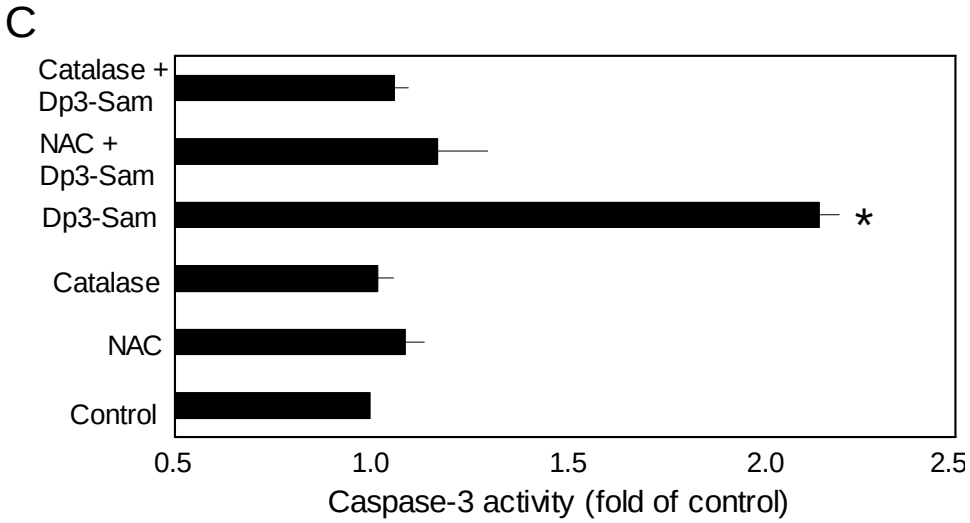


Figure 4.8. Antioxidants block ROS generation, JNK activation, caspase-3 activity and DNA fragmentation induced by Dp3-Sam or delphinidin. (A) Catalase and NAC block Dp3-Sam-induced ROS. HL-60 cells (1×10^6 cells/ml) were pretreated with 5 mM of NAC or 100 U/ml of catalase for 1 h, and then challenged to 100 μM of Dp3-Sam for 15 min. ROS assay was performed as described in Figure 4.5. Data represent the mean ± SD of three independent experiments. * $P<0.05$, significantly different from non-treatment control. **(B)** The cells were exposed to 100 μM delphinidin for 3 h. Total and phosphorylated JNK were detected with specific antibodies as described in Figure 4.4.



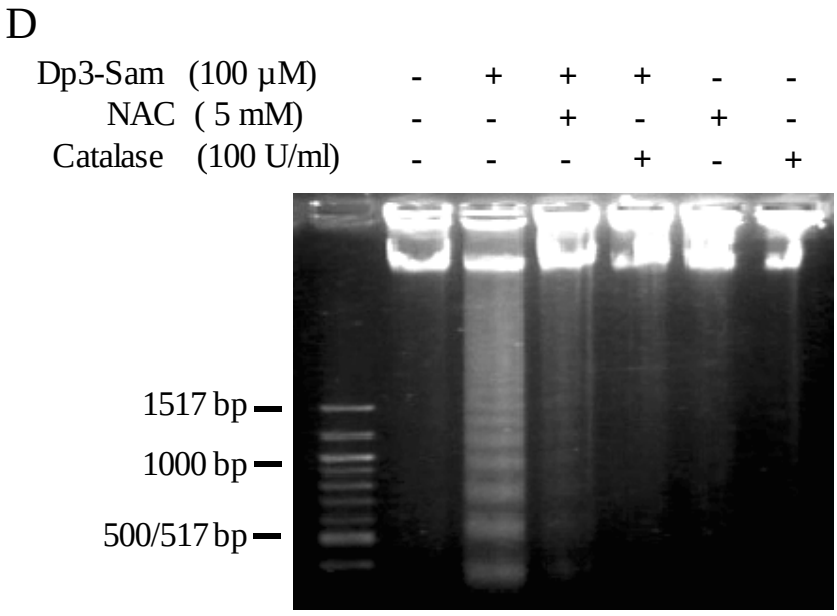


Figure 4.8 ***(continued)***

(C) Catalase and NAC inhibit Dp3-Sam-induced the activity of caspase-3. HL-60 cells (1×10^6 cells/ml) were pretreated with 5 mM NAC or 100 U/ml catalase for 1 h, and then challenged to 100 µM of Dp3-Sam for 6 h. Caspase-3 activity was measured as described in Figure 4.7B. Data represent the mean ± SD of three independent experiments. * $P<0.05$, significantly different from non-treatment control. **(D)** Catalase and NAC inhibited Dp3-Sam-induced DNA fragmentation. HL-60 cells (1×10^6 cells/ml) were pretreated with 5 mM NAC or 100 U/ml catalase for 1 h, and then challenged to 100 µM of Dp3-Sam for 6 h. DNA fragmentation was detected as described in Figure 4.1A.

To determine whether the intracellular ROS is essential to apoptosis induction, antioxidants including NAC and catalase were used to challenge to protect against Dp3-Sam-induced ROS generation, JNK phosphorylation, caspase-3 activity and DNA fragmentation. The cells were pretreated for 1 h with NAC and catalase at 5 mM and 100 U/ml, at which cell viability was not affected (data not shown), and then exposed to 100 μM Dp3-sam for the indicated times. Both NAC and catalase completely inhibited Dp3-sam-induced ROS generation (Figure 4.8A), JNK phosphorylation (Figure 4.8B), Caspase-3 activity (Figure 4.8C) and DNA fragmentation (Figure 4.8D). It is known that NAC is a scavenger of reactive oxidative species, and catalase is an enzyme that hydrolyzes H_2O_2 to H_2O [52, 53]. Both two antioxidants can protect against delphinidin-induced molecular events, providing another piece of evidence for an essential role of ROS-mediated JNK activation and mitochondrial dysfunction in Dp3-Sam-induced apoptosis.

4.4 Discussion

Anthocyans have been discussed in relation to antitumor activity [2-6]. However, the molecular mechanisms underlying the antitumor activity of anthocyans are poorly defined. The present study is the first time to present molecular evidence that active anthocyanins and anthocyanidins induces apoptosis in HL-60 cells through ROS-mediated JNK activation and mitochondrial dysfunction pathways.

Six kinds of the representative anthocyanidins were used to investigate the potency of apoptosis induction in the present study. Special attention was paid to the number of hydroxyl groups on the B-ring of the structure. It is noteworthy the data from the DNA fragmentation and ROS generation assay indicate that the number of hydroxyl groups on the B-ring might be associated with their actions. Anthocyanidins containing single hydroxyl group at B-ring such as pelargonidin, peonidin and malvidin provided no activity.

Cyanidin and petunidin containing two hydroxyl groups at the B-ring showed higher activities. Delphinidin, a compound with three hydroxyl groups on the B-ring, exhibited the highest activity in both ROS generation and DNA fragmentation. These data indicate that ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins is, at least, essential for ROS generation and apoptosis induction. Recent studies show that the number of hydroxyl groups on the B-ring of flavonoids is associated with both antioxidant and prooxidant activities [54-56]. For instance, kaempherol-3,7,4'-trimethylether containing single hydroxyl group at B-ring diminished the activity. Quercetin and luteolin containing two hydroxyl groups showed higher activity, and myricetin with three hydroxyl groups showed the highest activity. Thus, these findings together with other reports indicate that the biological activity of flavonoids including anthocyanidins appears to be associated with the structure of B-ring.

A ROS-mediated JNK activation pathway may be involved in delphinidin-induced apoptosis. Delphinidin treatment resulted in rapid and persistent phosphorylation of JNK (Figure 4.4). *c-jun* expression and apoptosis induction in a time-subsequentant and dose-corresponding manner were also observed after JNK phosphorylation. Moreover, antioxidants such as NAC and catalase could protect against the delphinidin-induced JNK phosphorylation, caspase-3 activation and DNA fragmentation apoptosis. These data suggest that ROS may be present in delphinidin-treated cells. In fact, the production of ROS in anthocyanidins-treated cells was directly measured, using H_2DCF-DA. These data strongly suggest that anthocyanidin might generate a toxic oxidant signal to stimulate JNK pathway activation and then to induce apoptosis. Considering some important antioxidant enzymes such as SOD or catalase might be interfered by flavonoids, and this interference may subsequently result in an elevation of ROS with target cells. However, the exact mechanism by which anthocyanidins induce ROS is unknown and remains to be investigated. In agreement with this view, a similar longer stimulation of JNK activation

and apoptosis induction was also observed in several cancer chemopreventive agents such as cisplatin [11, 12], paclitaxel [13], phenethyl isothiocyanate [15, 16], adriamycin [17] and dopamine [57].

Mitochondrion has been reported to play a key role in the regulation of apoptosis [58]. Mitochondrial dysfunction including the loss of mitochondrial membrane potential ($\Delta\Psi m$), permeability transition, and release of cytochrome *c* from the mitochondrion into the cytosol are associated with apoptosis [59]. In this study, a time-dependent release of mitochondrial cytochrome *c* and loss of $\Delta\Psi m$ were also observed in Dp3-Sam-treated HL-60 cells. Moreover, a decreased Bid protein level was also observed in Dp3-Sam-treated cells. According to the present understanding, caspase-8 can be activated either by cytokines or chemicals that in turn induces Bid truncation. The truncated Bid causes the cytochrome *c* efflux from mitochondrion, caspase-3 activation and PARP inactivation, and finally results in apoptosis [60, 61]. Thus, Dp3-Sam may firstly activate caspase-8, which leads to Bid truncation and then activates mitochondrion-mediated downstream events, including cytochrome *c* release and sequential activation of caspase-9 and caspase-3. Thus, a mitochondrial damage-dependent pathway might be involved in Dp3-Sam-induced apoptosis in HL-60 cells.

Accumulating data indicate caspases play critical roles in the initiation of apoptosis. Caspases can be grouped into “apoptotic initiator”, such as caspase-8, and “apoptotic effector”, such as caspase-3, according to their substrate specificities and target proteins [62]. In the present study, the induction of apoptosis by Dp3-Sam occurred within several hours, suggesting that Dp3-Sam may activate the pre-existing apoptosis machinery. In fact, we detected the DNA fragmentation, PARP inactivation and activation of caspase-3, -8, and -9 in Dp3-Sam-treated cells. These actions showed a time-subsequent and a dose-corresponding manner. Furthermore, the caspase-3 inhibitor, DEVD-CHO, inhibited Dp3-Sam-induced caspase-3 activity, and the inhibitors of caspase-8 (IETD-CHO), and -9

(LEHD-CHO), also blocked the caspase-3 activities, respectively (Figure. 4B). These results demonstrated that Dp3-Sam-induced apoptosis might involve a caspase-3-mediated mechanism, and the activation of caspase-8 and -9 might act as the upstream of caspase-3 activation.

In summary, this study showed that anthocyanidins could induce apoptosis in HL-60 cells with structure-activity relationship. The ortho-dihydroxyphenyl structure of anthocyanidins might generate an oxidant signal to stimulate JNK pathway activation, caspase-3 activation and DNA fragmentation. Antioxidants including NAC and catalase can block delphinidin-induced those actions. Our result is the first evidence that delphinidin may trigger an apoptotic death program through an oxidative stress-involved JNK signaling pathway. These findings suggest anthocyans may represent a promising class of compounds, which might be interesting in the view of chemoprevention.

4.5 Abstract

To investigate their anticancer effects of anthocyans, induction of apoptosis was tested in human leukemia cells (HL-60), which is a valid model for testing antileukemic or general antitumoral compounds. Of six anthocyanidins representing the aglycons of most of anthocyanins, only those with an ortho-dihydroxyphenyl structure on the B-ring induce apoptosis, suggesting that the ortho-dihydroxyphenyl structure of anthocyanidins may contribute to the induction of apoptosis. Delphinidin, the most potent inducer, causes apoptosis in a time- and dose-dependent manner. Concomitant with the apoptosis, delphinidin or Dp3-Sam stimulated ROS generation, JNK phosphorylation, *c-jun* gene expression, Bid truncation, mitochondrial membrane potential ($\Delta\Psi m$) loss, and cytochrome *c* release and activation of caspases. Antioxidants including *N*-acetyl-L-cysteine (NAC) and catalase effectively block ROS generation, JNK phosphorylation,

caspase-3 activation, and DNA fragmentation. Thus, anthocyans trigger an apoptotic death program in HL-60 through an oxidative stress-involved JNK signaling pathway.

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Chapter 5 Discussion and conclusion

5.1 Discussion

5.1.1. Multiple roles and molecular targets of anthocyans

In the present study, the cancer chemopreventive effects of anthocyans were characterized at molecular levels using different cell lines. The results showed that anthocyans could block cell transformation and inflammation, and could induce apoptosis of cancer cells, which are important steps for cancer chemoprevention (Figure 5.1).

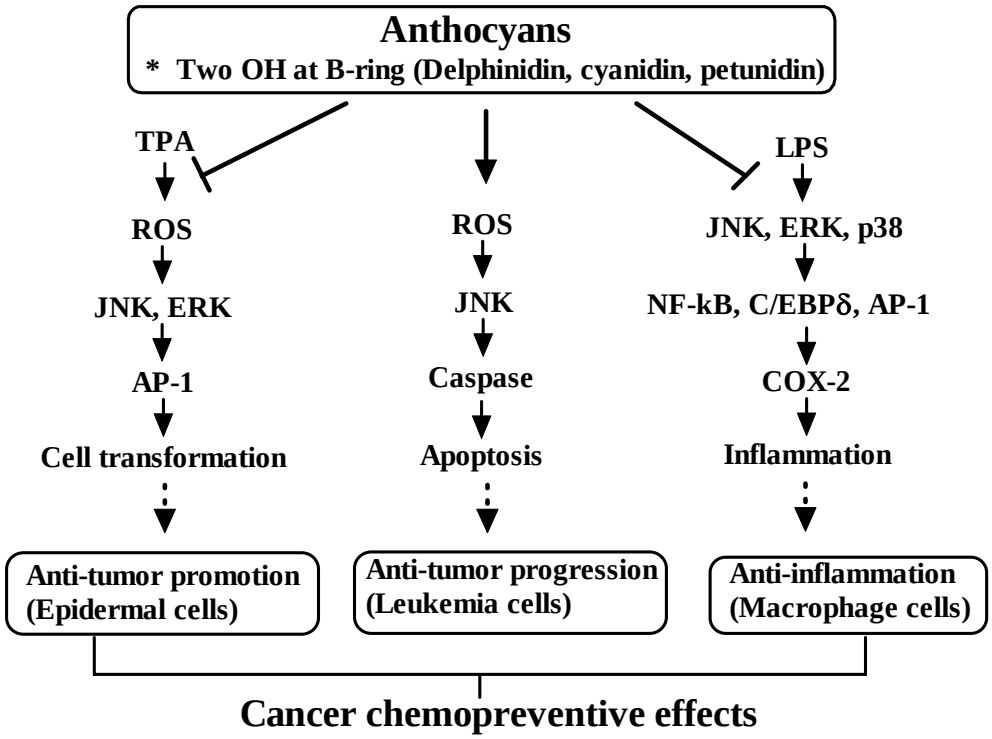

Figure 5.1. A schematic molecular view of cancer chemoprevention by anthocyans. Anthocyans at least contribute to cancer chemoprevention through targeting three different signal transduction pathways and downstream genes. AP-1: activator protein-1; ERK: extracellular signal-regulated kinase; JNK: c-Jun NH_2-terminal kinase; LPS: lipopolysaccharide; NF-κB: nuclear factor κB; ROS: reactive oxygen species; TPA: 12-*O*-tetradecanoylphorbol-13-acetate.

They modulated a variety of molecules including signaling factors (such as MAPK), transcription factors (such as AP-1, NF-κB and C/EBPδ) and genes (such as COX-2). These data suggest that anthocyans might play multiple roles in cancer chemoprevention by targeting a number of molecules.

One of the potential mechanisms for the multiple roles of anthocyans is that anthocyans can act as antioxidants or pro-oxidants to initiate the cell signaling. Due to their polyphenolic nature, the anthocyans are efficient antioxidants in *in vitro* systems [1-4], and in animal models [5-10]. It was noticed that anthocyans showed higher antioxidant capacity in vitamin E-depleted rats than that in control rats [5] and also in oxidative stress condition [6, 7], suggesting the antioxidant potency of anthocyans might depend on the redox status of animal or cells. On the other hand, phenolic antioxidants also can act as pro-oxidants in 1) *in vitro* system containing redox-active metals, and 2) cellular system containing peroxidase. In *in vitro* system, phenolic antioxidants with the pyrogallo or catechol B-ring have been showed to autoxidize in the presence of transition metals, such as copper and iron, to produce ROS and phenoxyl radicals [11, 12]. Peroxidase has been reported to catalyze oxidation of phenolic B ring-containing flavonoids into pro-oxidant phenoxyl radicals, such quinine-type metabolites, and then result in ROS through redox cycling [13-15]. In the present study, the mouse epidermal cells and macrophage cells were treated with TPA and LPS to mimic cell transformation and inflammation. It was known that TPA and LPS caused the cells to generate ROS [16-19], and then to initiate cellular signals. Thus, anthocyans might act as antioxidants to scavenge ROS and then attenuate ROS-initiated signals. On the other hands, ROS production and apoptosis induction were observed in HL-60 cells treated with B ring-containing anthocyans, suggesting anthocyans might act as pro-oxidant in this case. HL-60 cell line is a bone marrow-derived leukemia

cell line and contains myeloperoxidase. Incubation of HL-60 cells with polyphenols caused cells to produce ROS, which was prevented by myeloperoxidase inhibitors [20, 21]. In the present study, it can be also speculated that anthocyans might be oxidized into ROS by cellular myeloperoxidase in HL-60 cells. Generated ROS then initiated the cell signaling to induce a ROS-mediated apoptosis. As controls, ROS generation and apoptosis induction were not observed in JB6 cells (a mouse normal epidermal-drived cell line) treated with the same dose and times (data not shown). Thus, anthocyans may exert effects in cancer prevention in two opposite forms; 1) they can scavenge free radical and ROS to become the reduced form, and act as antioxidant to protect against carcinogenesis in normal cells; 2) they may also can be oxidized into ROS by cellular peroxidase, and generated ROS sequentially induces apoptosis in cancer cells (Figure 5.2). Both antioxidant and prooxidant action of anthocyans may be important mechanisms for their cancer chemopreventive effects. The beneficial effects of flavonoids in cancer therapy have often been linked to their ability to act as antioxidants, which includes their reducing

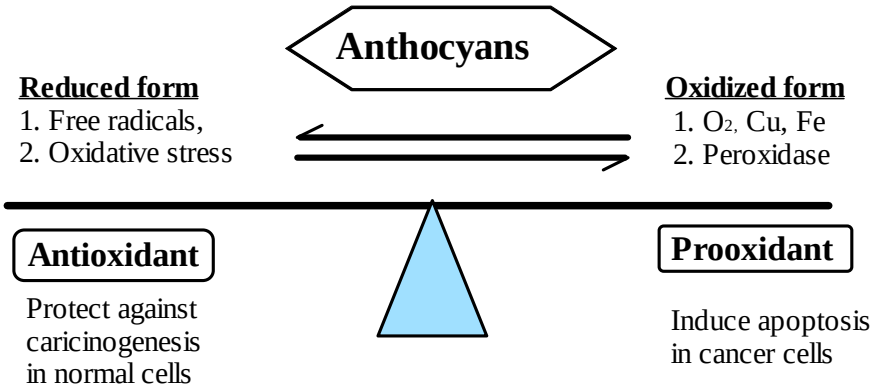

Figure 5.2. Diagram representing the balance between antioxidant and prooxidant characteristics of anthocyans. The forms reduced by free radicals or oxidative stress act as antioxidant; however, the forms oxidized by peroxidase or oxygen under copper and iron can have prooxidant activities.

capacities and ROS-scavenging capabilities. However, recent reports suggest that the prooxidant action of flavonoids may be another important mechanism for their anti-cancer properties by apoptosis induction [22-24].

Another possible mechanism is that anthocyans might exert modulatory effects in cells through selective actions at some protein kinase signaling cascades, which may be independent of classical antioxidant capacity. Several lines of studies have indicated that flavonoids targeted different components of a number of protein kinase and lipid kinase signaling cascades such as phosphoinositide 3-kinase (PI 3-kinase), Akt/PKB, tyrosine kinases, protein kinase C (PKC), and MAP kinases [25-31]. Inhibitory or stimulatory actions at these pathways are likely to profoundly affect cellular function by altering the phosphorylation state of target molecules and/or by modulating gene expression. Recent studies have indicated that flavonoids have the potential to bind to the ATP binding sites of a large number of proteins [32], including mitochondrial ATPase [33], calcium plasma membrane ATPase [34], protein kinase A [35], protein kinase [30,36-39] and topoisomerase [40]. The binding of flavonoids to the ATP binding site presumably causes three-dimensional structural changes in the kinase leading to its inactivity. Flavonoids may also interact with mitochondria; interfere with pathways of intermediary metabolism, and/or downregulate the expression of adhesion molecules [41-43]. The structures of flavonoids have been proven to determine whether or not they act as potent inhibitors of protein kinase C, tyrosine kinase, and PI 3-kinase [29, 44]. It appears that the number and substitution of hydroxyl groups on the B-ring and the degree of unsaturation of the C2-C3 bond are important determinants of this particular bioactivity. Anthocyans are flavanols, a subclass of flavonoids, and also have similar chemical structure on the B-ring. Moreover, a synergistic, not additive, inhibition for AP-1 activity (Chapter 2) was observed between

SOD and delphinidin treatment. These findings together with other reports suggest that the inhibitory effects of anthocyans on AP-1 activation might be due, in part, to their potent antioxidant activity and, in part, to blocking protein kinases such as MAPKs. Both targets are important for their cancer chemopreventive actions. Thus, anthocyans may have cancer chemopreventive effects by scavenging ROS and by targeting selective protein kinases such as MAPKs with the attendant activation of downstream factors including AP-1, NF-κB and C/EBPδ.

Taken together, the potency of anthocyans to block cell transformation and inflammation, and to induce apoptosis of cancer cells were demonstrated at cellular and molecular levels. According to the dose-response, the inhibitory effects on cell transformation are able to obtain from the daily intake of anthocyans dietary. On the other hand, the effects on anti-inflammation and apoptosis induction are possible to obtain only from the dosing of high concentrated extracts of anthocyans. Thus, anthocyans mainly have chemopreventive effect rather than chemotherapeutic effect from intake of daily dietary.

5.1.2 Structure-activity relationship

The results indicated that the potency of cancer chemoprevention of anthocyans including anti-cell transformation, anti-inflammation and apoptosis induction is associated with the number of hydroxyl groups on the B-ring, more than the sugar units. The ortho-dihydroxyphenyl structure on the B-ring appears essential for these actions. Extensive structure-activity studies of anthocyans by many investigators have shown that the numbers of sugar units and hydroxyl groups on aglycons, especially on the B-ring are associated with biological activities including antioxidation, antimutagenicity and COX

inhibition. Yoshimoto *et al.* [45] showed that antimutagenic activity of cyanidin appears stronger than that of peonidin. This might be related to the antioxidative activity of anthocyans, which depends on the number of hydroxyl groups on the B-ring [5]. Wang *et al.* [46] found that anthocyanins and their aglycon, cyanidin, from tart cherries could inhibit the activities of the human prostaglandin endoperoxide H synthase-1 and -2 isozymes (PGHS-1, and –2), and cyanidin showed highest inhibitory activity. Seeram *et al.* [47] reported that both antioxidant activity and cyclooxygenase inhibitory activity of cyanidin glycosides increased with a decreasing number of sugar units. Cyanidin-rutinose showed higher activity than cyanidin-glucosylrutinose, and the aglycon cyanidin showed the highest activity. Kamei *et al* [48] has investigated the effect of the chemical structure of flavonoids on the growth inhibition of human tumor cell (HCT-15) *in vitro*, and found that type of sugar combined with the A ring and hydroxyl groups bounded to the B ring

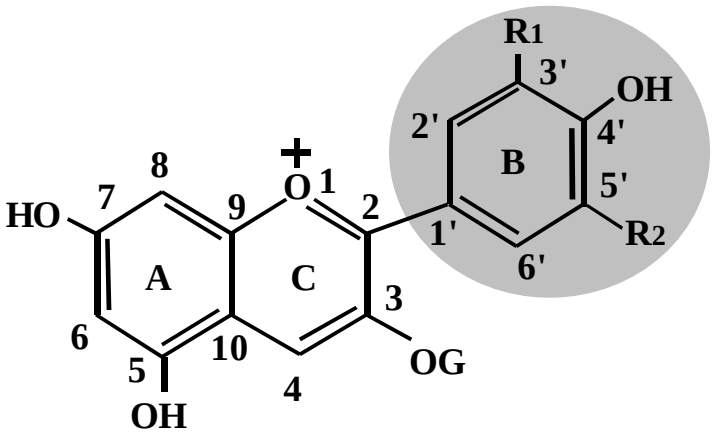

Figure 5.3. Chemical structure and properties. The aglycon of anthocyans consists of three rings (A, C and B). In general, the B-ring contributes to the biological function such as antioxidant or prooxidant activity. The potency depends on the catechol group or ortho-dihydroxyphenyl structure at 3', 4' and 5'. On the other hand, the A- or C- ring contributes to the stability or color intensities of anthocyans in pH-dependent manner at solution. At pH<3, the ring C harbors a flavylium cation with intense color, however, at neutral pH anthocyans occur as chalcone (yellow) with an open C ring.

played an important role on the tumor suppression. The glucose attachment at A ring caused suppression of tumor cell growth, but other sugars such as rhamnose and lutinoside at that position did not suppress the growth. Flavonoids with hydroxyl groups conjugated to the 3', 4', and 5' of B ring were stronger in antitumor effect than those with hydroxyl groups attached at the 3' and 4' or 4' only. Several lines of studies reported that flavonoids with ortho-dihydroxy on the B-ring such as quercetin, rhamnetin, fisetin and luteolin showed stronger inhibition on COX-2 expression [49, 50]. The number of hydroxyl groups on the B-ring appeared to link to a molecular conformation that influences the interactions between flavonoids and enzymes such as tyrosine kinase and protein kinase C. Indeed, the flavonoids, which inhibit tyrosine kinase and protein kinase C, have an ortho-dihydroxy on the B-ring or A-ring [29, 44]. Anthocyans are a subclass of flavonoids. The chemical structure on the B-ring is very similar to those required for the inhibition of tyrosine kinase and protein kinase C in flavonoids. Therefore, the ortho-dihydroxy structure on the B-ring of anthocyans might be associated with 1) the antioxidant and/or pro-oxidant potency, and with 2) the molecular conformation that influences the interactions between anthocyans and enzymes such as tyrosine kinase and protein kinase C, which are involved in carcinogenesis.

5.1.3 Stability

Anthocyans are extremely water-soluble and occur in different pH-dependent conformations with varying colors or color intensities [51-53] (Figure 5.1). In strong acid (pH <3), ring C acquires aromaticity involving a favylium cation, which imparts intense red color on the molecule (i). Under mildly acidic conditions the ring is closed to form a carbinol pseudo-base with colorless (ii). At neutral pH anthocyans occur as chalcones with

an open C ring, and shows light yellow (iii). In alkali, oxidation of ring A generates a quinoid structure with elimination of the positive charge; this species shows purple color (iv). The ring-opened chalcone can be reformed at neutral pH. Although the stability of the favylium cation is compromised by increasing pH [52, 53], the implications of the different conformational manifestations of anthocyans for pharmacological activity are unclear. In the present study, the pH at culture medium is closed to neutral. The color change was also observed from red to light yellow. This condition is equivalent to the situation of (iii), suggesting that the form of anthocyans in culture medium should be as chalcones with an

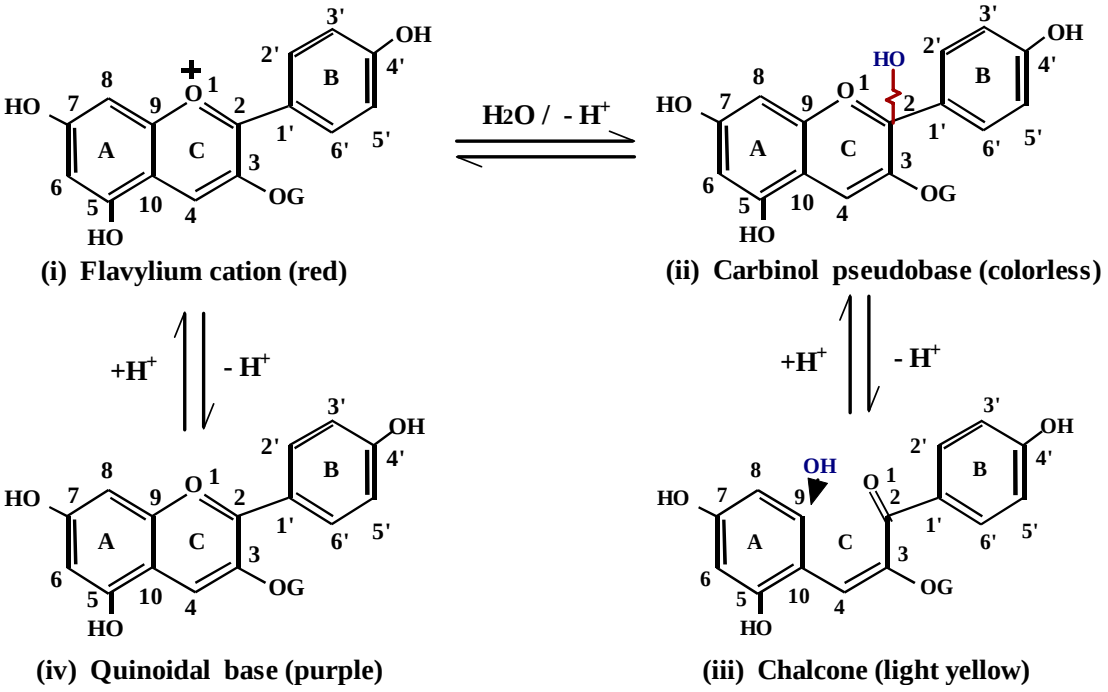

Figure 5.4. Stability of anthocyans, shown here for anthocyanins bearing a sugar (G) on C3. At neutral pH, anthocyans occur as chalcones with an open C ring (iii). Under mildly acidic conditions the ring is closed to form a carbinol pseudo base (ii). In strong acid (pH 2), ring C acquires aromaticity involving a flavylium cation, which imparts intense color on the molecule (i). In alkali, oxidation of ring A generates a quinoid structure with elimination of the positive charge, this species is also colored (iv). The ring-opened chalcone can be reformed at neutral pH [51].

open C ring. It is noteworthy that that the structure on the B-ring, which is demonstrated to be an important active ring in the present study, is not changed in all pH range from strong acid to alkali. The biological activity of anthocyans at different color intensities was also observed without significant change (data not shown). Thus, this finding indicates that biological activity of anthocyans is independent on color intensities. In other words, the neutral culture medium in this study did not influence the biological activity of anthocyans although the neutral medium changed the red color into light yellow.

5.1.4 Bioavailability

The evidence described in the present study and in the results from many other groups intimates that anthocyans are more potent chemopreventive agents because they regulated neoplastic promotion/progression *in vitro* and *in vivo*. The emergence of renewed interest is regarding the bioavailability and efficacy of anthocyans in mammalian organism. Several lines of studies indicated that anthocyans were very rapidly absorbed to serum with lower concentrations, and started to eliminate 30 min after intake [54]. Thus, they seen absorbed with poor bioavailability. However, most of these estimations were only based on the measurement of unchanged glycosides of anthocyans *in vivo*, some important metabolites were ignored. Recently, glucuronides and sulfates of anthocyans were identified in human urine with HPLC-mass spectrometry/mass spectrometry analysis [55-59]. The monoglucuronides of anthocyans accounted for 80% of the total metabolites when analyses were performed immediately after urine collection [56]. Other underestimated reasons are due to 1) most analyses were performed with UV-visible light detection on the basis of complete conversion of all of the chemical forms of anthocyans into a colored flavylium cation with acidification. However, it is possible that some forms existing at

neutral pH would not be converted into the flavylium form, because of putative binding to, or chemical reactions with, other components of the plasma or urine. 2) The metabolites of anthocyans were very unstable and were extensively degraded when acidified urine samples were frozen for storage. This probably explains why such metabolites were not observed in previous studies. Therefore, it seems crucial to reconsider anthocyanin bioavailability with available methods that allow, e.g. 1) measurement at *in vivo* pH (near neutral), 2) preservation of all of the metabolites in frozen samples. 3) identification of all of anthocyans and their metabolites by labeling.

Another is noteworthy that the plasma concentrations of anthocyans, which are much lower than those at *in vitro* studies, in animal experiments and human volunteers also showed biological activities, such as antioxidant property [60, 61] and vision improvement [62]. A positive correlation between postprandial serum anthocyanin content and antioxidant status was observed in human volunteers [62] although a maximum amount of 13 ng/ml of total anthocyans was only detected in the serum after 4 h. Those results suggest a possibility, at least, that their metabolites of anthocyans may also have modulatory effects although there is little information. Thus, further studies on chemopreventive effects of both intact compounds and their metabolites of anthocyans are necessary.

5.2 Conclusion

This study characterized the effects of cancer chemoprevention of anthocyans at cellular and molecular levels through targeting the key steps involved in carcinogenesis. The conclusions include that

1. Anthocyans inhibited cell transformation induced by TPA in mouse epidermal cells through targeting ERK- and JNK-mediated AP-1 pathway.
2. Anthocyans suppressed COX-2 overexpression in LPS-evoked mouse macrophage RAW264 cells through blocking ERK-, JNK-, and p38 kinase-mediated activation of transcriptional factors such as NF-κB, AP-1 and C/EBPδ.
3. Anthocyans induced apoptosis in human leukemia cell via ROS-dependent mitochondrial death pathway.
4. Structure-activity study indicated that the ortho-dihydroxyphenyl structure on the B-ring of anthocyans appears essential for these actions.

These findings provided the first molecular basis for the inhibitory effects of anthocyans on carcinogenesis including promotion, inflammation and progression, suggesting that anthocyans have potency for cancer chemoprevention.

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