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Anthocyanidins inhibit cyclooxygenase-2 expression in LPS-evoked macrophages: Structure–activity relationship and molecular mechanisms involved

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Abstract

The effects of anthocyanidins, the aglycon nucleuses of anthocyanins widely occurring in reddish fruits and vegetables, on the expression of cyclooxygenase-2 (COX-2) were investigated in lipopolysaccharide (LPS)-activated murine macrophage RAW264 cells. Of five anthocyanidins, delphinidin and cyanidin inhibited LPS-induced COX-2 expression, but pelargonidin, peonidin and malvidin did not. The structure–activity relationship suggest 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 IκB-α, nuclear translocation of p65 and CCAAT/enhancer-binding protein (C/EBP)δ and phosphorylation of c-Jun, but not CRE-binding protein (CREB). Moreover, delphinidin suppressed the activations of mitogen-activated protein kinase (MAPK) including c-Jun *N*-terminal kinase (JNK), extracellular signal-regulated kinase (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, our 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 nuclear factor-κB (NF-κB), activator protein-1 (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.

Keywords: Anthocyanidins; Delphinidin; Cyclooxygenase-2; Lipopolysaccharide; Transcription factors; Mitogen-activated protein kinase

1. Introduction

Cyclooxygenase (COX) is a rate-limiting enzyme for synthesis of dienoic eicosanoids such as prostaglandin (PG) E_2. 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 lipopolysaccharide (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]. Interestingly, some anti-oxidants 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 screen the cancer chemopreventive effects from 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 nuclear factor κB (NF-κB), CCAAT/enhancer-binding protein (C/EBP), activator protein 1 (AP-1) and CRE-binding

Abbreviations: AP-1, activator protein-1; C/EBP, CCAAT/enhancer-binding protein; COX-2, cyclooxygenase-2; CREB, CRE-binding protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun *N*-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB

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protein (CREB) have been identified to bind the *cis*-acting elements in the promoter of COX-2 and regulate the transcription [5,6]. NF-κB is a transcription factor involved in LPS-mediated induction by many cytokines and inflammatory products [8,9]. C/EBP is generally considered to regulate COX-2 production mainly with the two C/EBP family members, C/EBPδ and β [10–12]. Finally, AP-1 and CREB are essential for both basal and induced COX-2 transcription [13,14].

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 indicate that LPS stimulates COX-2 production through activating mitogen-activated protein kinase (MAPK) including extracellular signal-regulated kinase (ERK), c-Jun *N*-terminal kinase (JNK) and p38 kinase [12,15].

Anthocyanins are naturally occurring polyphenolic compounds that give the intense color to many fruits and vegetables such as berries, red grapes, purple sweet-potato and red cabbages [16,17]. They are present exclusively as glycosidic compounds in plants. The number and nature of the different attached sugar moieties are responsible for the high number of anthocyanins, more than 500 compounds [18]. The aglycon (named anthocyanidin) is a diphenylpropane-based polyphenolic ring structure and limited to a few structure variants including delphinidin, cyanidin, pelargonidin, peonidin and malvidin (Fig. 1A), that represent the aglycons of most anthocyanins in plants. Wang et al. [19] found that anthocyanins and their aglycon, cyanidin, from tart cherries could inhibit the enzyme activities of COX-2. Seeram et al. [20] found that cyanidin showed superior inhibition on the cyclooxygenase activity in vitro. However, the underlying mechanisms are not well understood.

In the present study, we used mouse macrophage cell line RAW264, which can be stimulated with LPS to mimic a state of infection and inflammation, to demonstrate the molecular mechanism of inhibitory actions of anthocyanidins on COX-2 expression. Our data showed that the inhibitory effects of anthocyanidins 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.

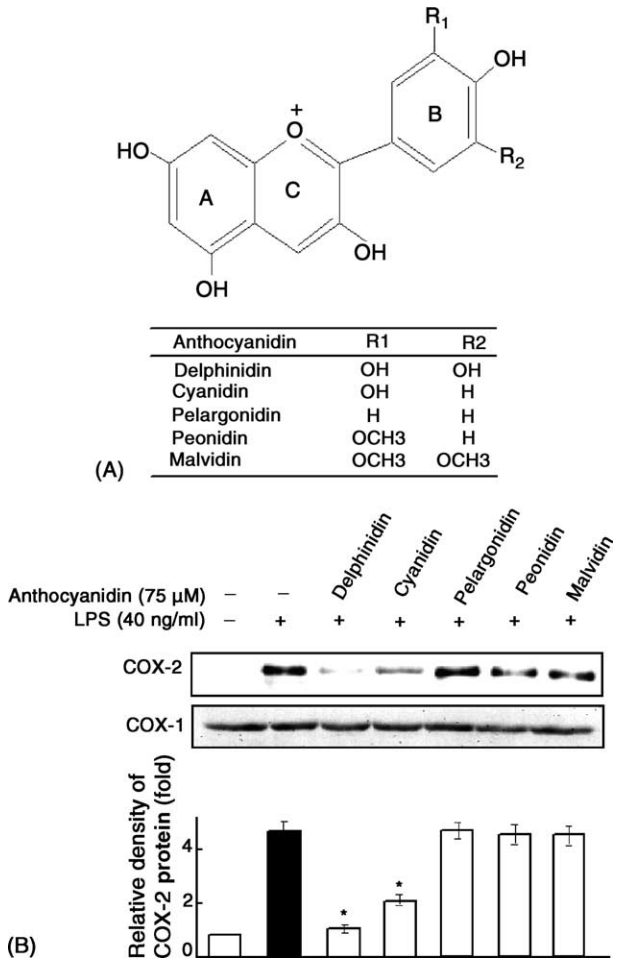

Fig. 1. (A) Chemical structures of anthocyanidins. (B) Effect of anthocyanidins on COX-2 expression in LPS-activated RAW264 cells. After RAW264 cells (1×10^6 cells) were starved in serum-free medium for 2.5 h, the cells were treated with 75 μM of delphinidin, cyanidin, peonidin, pelargonidin and malvidin for 30 min, respectively, and then exposed to 40 ng/ml LPS for 12 h. Cellular lysate was applied on 10% SDS-PAGE. The proteins of COX-2 and -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 ± S.D. of three separate experiments and the picture is a representative of those experiments each with similar results. $^*P < 0.05$ significantly different from LPS-stimulated control.

2. Materials and methods

2.1. Materials and cell culture

Anthocyanidins (delphinidin chloride, cyanidin chloride, pelargonidin chloride, peonidin chloride and malvidin chloride) purified by HPLC were obtained from Extrasynthese and dissolved in dimethyl sulfoxide (final concentration was 0.2%). Lipopolysaccharide (LPS, *Escherichia coli* Serotype 055:B5) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) were from Sigma. SB203580 was from Calbiochem and SP600125 was from Biomol Research Laboratory. U0126 and antibodies against phospho-CREB, phospho-ERK1/2, phospho-p38 kinase, phospho-c-Jun (Ser73), phospho-JNK, ERK1/2, p38 kinase, JNK and IκB-α were purchased from Cell Signaling Technology. Antibodies against COX-2, COX-1, C/EBPβ, C/EBPδ and p65 were from Santa Cruz Biotechnology.

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.

2.2. RNA extraction and RT-PCR

RNA extraction and RT-PCR were performed as described previously [21]. In brief, RAW264 (1×10^6 cells) 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 s, 55 °C for 30 s and 72 °C for 60 s 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 -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.

2.3. Nuclear protein extraction

Nuclear extracts were prepared as described previously [15]. Briefly, RAW264 cells (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 for 30 min before exposure to 40 ng/ml LPS for 4 h. Harvested cells were lysed by incubation in buffer A (10 mM 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 13,500 × *g* 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 EDTA, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride) for 15 min at 4 °C, and then centrifuged at 13,500 × *g* for 15 min at 4 °C. The supernatants containing nuclear extracts were stored at −80 °C until using.

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 Imager Gauge Software (Fuji Photo Film).

2.5. Statistical analyses

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

3. Results

3.1. Anthocyanidins suppress LPS-induced COX-2 expression with a structure–activity relationship

According to the amount of the possible intake from fruits and vegetable or their concentrated commercial extracts, we treated RAW264 cells with typical five kinds of anthocyanidins (Fig. 1A) at 25–100 μM for 30 min before exposure to 40 ng/ml LPS for 12 h, and found 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. Fig. 1B 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 cell viability as measured by MTT assay (data not

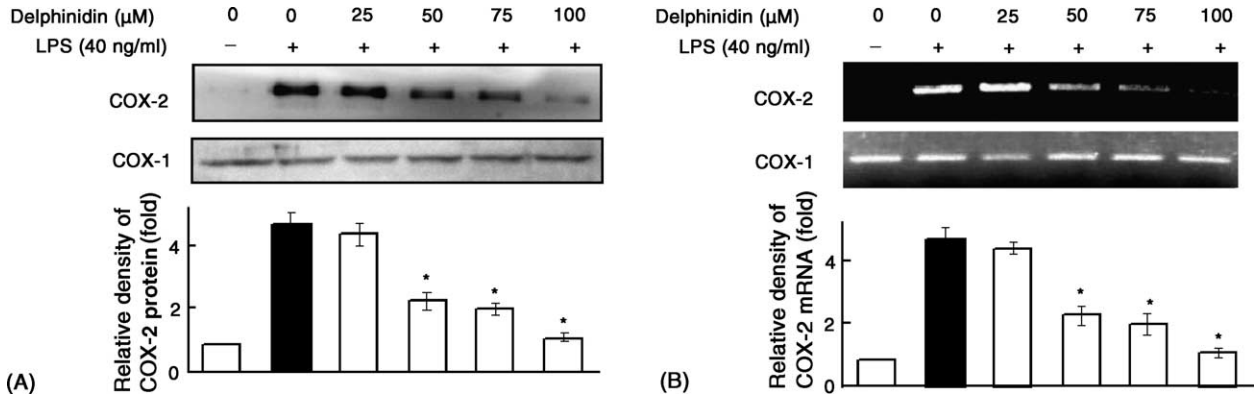

Fig. 2. Delphinidin causes a dose-dependent inhibition of COX-2 protein (A) and mRNA (B). RAW264 cells (1×10^6 cells/6 ml) were starved in serum-free medium for 2.5 h, the cells were then treated with 25–100 μM of delphinidin for 30 min, respectively. To determine the protein production, the cells were exposed to 40 ng/ml LPS for 12 h after 30 min of delphinidin treatment. The detection and quantification of COX-2 and -1 proteins were done as described in Fig. 1B. 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. Cellular RNA was extracted with ISOGEN RNA isolation kit and mRNA expressions of COX-2 and -1 were detected by RT-PCR technique as described in the Materials 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). Histograms show the densitometric analysis of COX-2 mRNA normalized to COX-1 mRNA (B). The data represent the mean ± S.D. of three separate experiments and the picture is a representative of those experiments each with similar results. $^*P < 0.05$ significantly different from LPS-stimulated control.

shown). These results indicate that delphinidin and cyanidin may be potential inhibitors for COX-2.

3.2. Delphinidin inhibits COX-2 expression at protein and mRNA levels

Because delphinidin showed the strongest inhibition on COX-2 expression among five kinds of anthocyanidins, we used delphinidin to investigate the molecular mechanism of inhibitory action in next experiments. As shown in Fig. 2A, 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 -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 (Fig. 2B). Thus, the suppression of COX-2 expression by delphinidin is due to transcription regulation.

3.3. 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 [11–13]. To identify the effects of delphinidin on these transcriptional factors, we pre-treated RAW264 cells with indicated concentrations of delphinidin for 30 min before exposure to 40 ng/ml LPS, and then examined these transcription factors using specific antibodies, respectively.

As shown in Fig. 3A, 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 (Fig. 3B), which is another important factor in LPS-mediated COX-2 expression [11,25,26].

NF-κB is another critical factor for COX-2 induction mediated by LPS or pro-inflammatory cytokines [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 [27,28]. To determine whether delphinidin can directly inhibit degradation of IκB, the level of IκB-α protein was assessed in delphinidin-treated RAW264 cells. First, we performed a time-course experiment 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 Fig. 3C). Second, we pre-treated macrophages with 25–100 μM of delphinidin for 30 min and detected IκB-α protein after exposure 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 Fig. 3C), suggesting that delphinidin may inhibit NF-κB activation by blocking LPS-induced IκB-α degradation. To confirm this, we further examined the nuclear translocation of p65, a part of p65/p50 heterodimer 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 (50–100 μM) significantly suppressed LPS-induced nuclear translocation of p65. Our data indicated that delphinidin might inhibit NF-κB 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.

3.4. 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 [12,15]. Thus, we investigated the influence of delphinidin on the activation of JNK, ERK and p38 kinase. As shown in Fig. 4, 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, we used MAPK-specific inhibitors (U0126 for MEK1/2, SB203580 for p38 kinase and SP600125 for JNK) to challenge to block LPS-mediated COX-2 induction. RAW264 cells were pre-treated with the indicated concentrations of the inhibitors for 30 min before exposure to 40 ng/ml LPS for 12 h. As shown in Fig. 5, 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.

4. Discussion

Anthocyanins have been discussed in relation to anti-inflammation activity [13–17]. However, the molecular mechanisms underlying the activity of anthocyanins are poorly defined. The present study is the first to present molecular evidence that anthocyanidins, aglycons of anthocyanins, 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 anti-oxidative [29,30], prooxidative [31,32], anti-transformation [24] and apoptotic induction [33]. For instance, delphinidin and cyanidin that possess *ortho*-dihydroxyphenyl structure on the B-ring, showed stronger scavenge activity of superoxide radicals [29], inhibitory effect on TPA-induced cell transformation [24] and apoptotic induction in human leukemia [33]. 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 [34,35]. The number of hydroxyl groups on the B-ring appear 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- or A-ring [36,37]. Anthocyanidin is subclass of flavonoids and also have similar chemical structure on the B-ring. We previously reported that delphinidin, but not peonidin, could inhibit the activation of MAPKK (SEK and MEK) and MAPK (ERK and JNK), and sequentially suppress AP-1 activation and cell transformation [24]. 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, our 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 [9,11–13]. These factors showed a redundancy in the regulating COX-2 transcription in LPS-treated macrophage/monocytic lineage [15]. In the present study, we investigated the effects of delphinidin on these transcription factors. Our data showed that delphinidin inhibited LPS-induced phosphorylation of c-Jun, a component of AP-1 complex (Fig. 3A). Delphinidin also blocked LPS-mediated nuclear translocation of C/EBPδ (Fig. 3B). Interestingly, delphinidin also had inhibitory effect on degradation of IκB-α (Fig. 3C) and nuclear translocation of p65 (Fig. 3D). These results are agreement with those from some chemopreventive compounds, such as capsaicin [38], apigenin [39] and diarylheptanoid [40], which inhibit LPS-mediated COX-2 induction by blocking the degradation of IκB-α in mouse macrophage cells. Taken

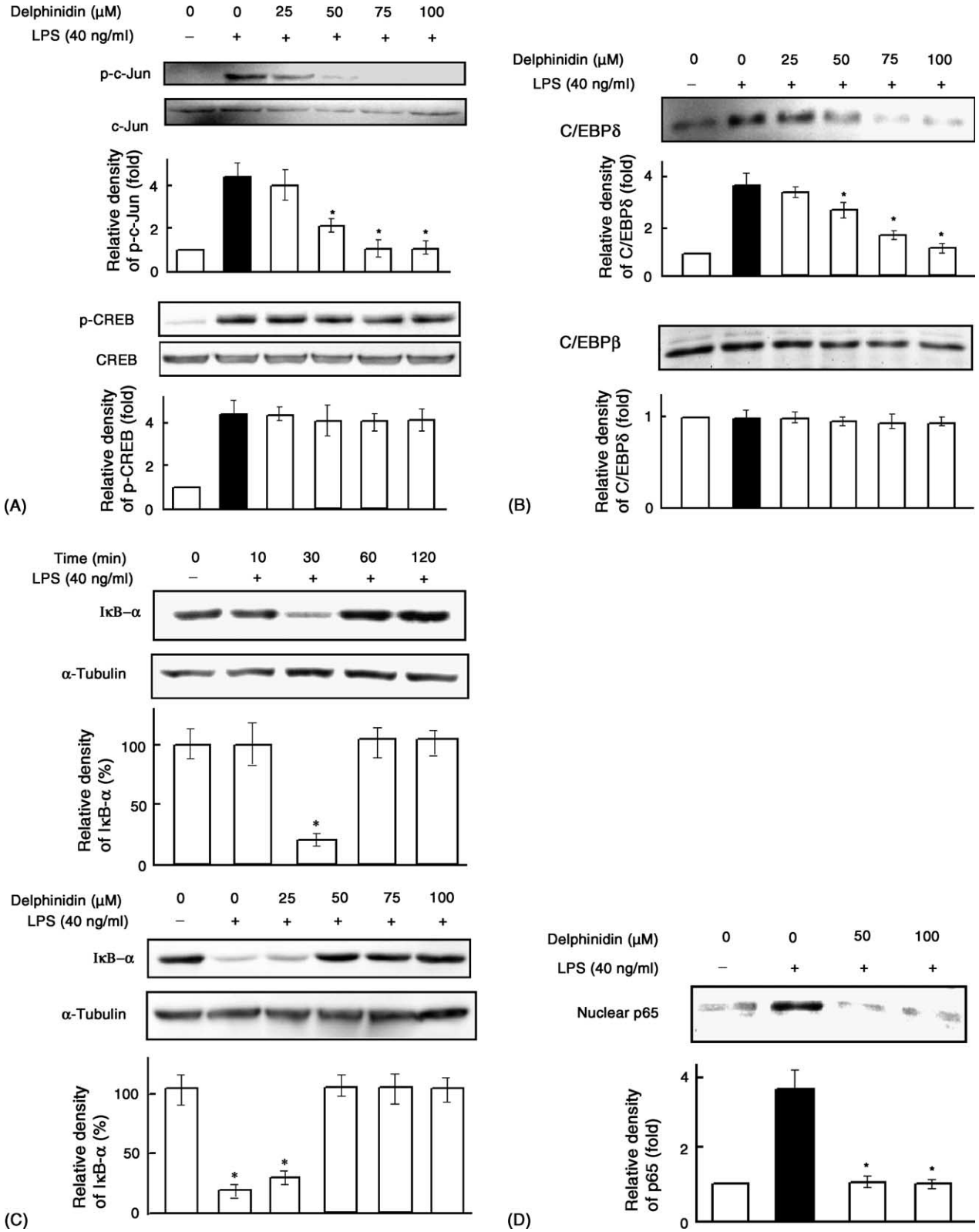

Fig. 3. Effects of delphinidin on the transcriptional factors regulating COX-2 expression. (A) Phosphorylation of c-Jun and CREB. Cell culture and Western blotting analysis were done as described in Fig. 1B. RAW264 cells were treated with 25–100 μM delphinidin for 30 min, and then exposed to 40 ng/ml 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-stimulated control. (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 C/EBPβ were detected with their antibodies. Histograms show the densitometric analysis of C/EBPδ and C/EBPβ. $^{*}P < 0.05$ significantly different from LPS-stimulated control. (C) IκB-α degradation. To identify the time of LPS-induced degradation of IκB-α protein, RAW264 cells were treated

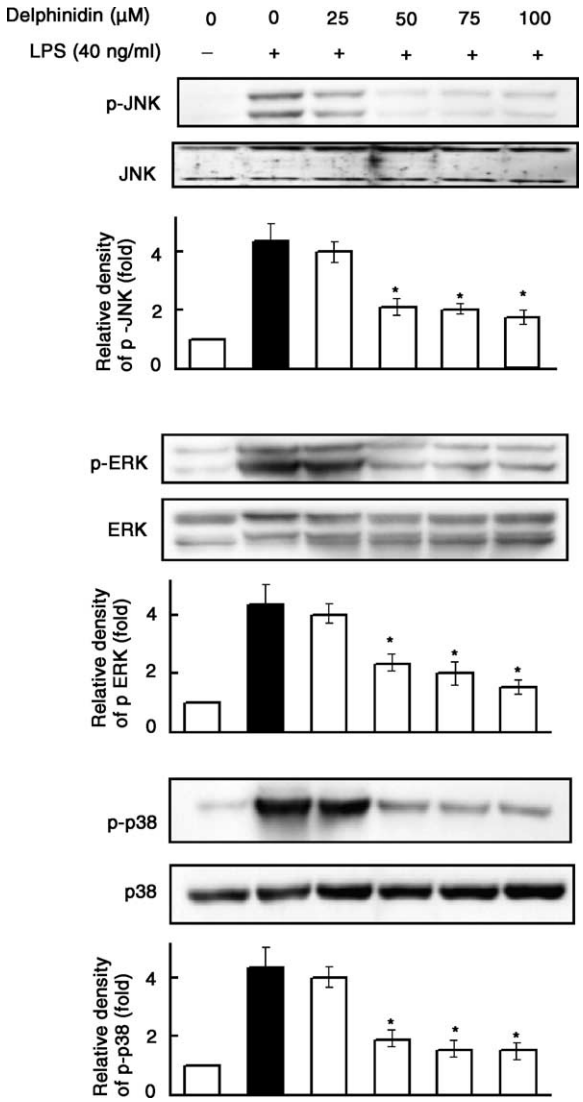

Fig. 4. Effects of delphinidin on LPS-induced phosphorylation of MAPK. Cell culture and Western blotting were done as described in Fig. 1B. RAW264 cells were treated with 25–100 μM delphinidin for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Total or phosphorylated MAPK were detected with their antibodies, respectively. Histograms show the densitometric analysis of phosphorylated MAPK normalized to total MAPK, respectively. The data represent the mean ± S.D. of three separate experiments and the picture is a representative of those experiments each with similar results. $^{*}P < 0.05$ significantly different from LPS-stimulated control.

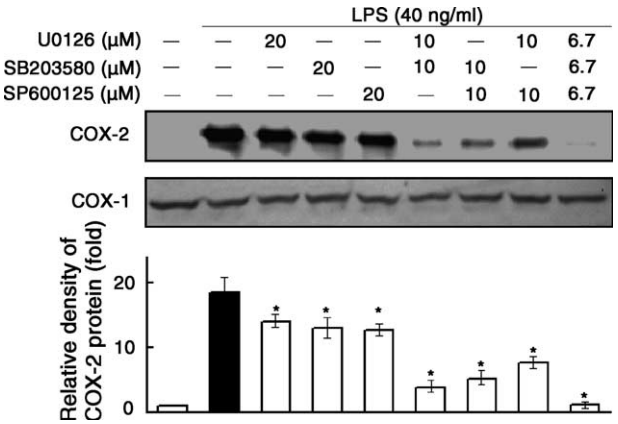

Fig. 5. Effects of MAPK inhibitors on LPS-induced COX-2 expression. Cell culture and Western blotting were done as described in Fig. 1B. RAW264 cells were treated with the indicated concentrations of U0126, SB203580 and SP600125 for 30 min, and then exposed to 40 ng/ml LPS for 12 h. COX-2 and -1 were detected with their antibodies, respectively. Histograms show the densitometric analysis of COX-2 protein normalized to COX-1. The data represent the mean ± S.D. of three separate experiments and the picture is a representative of those experiments each with similar results. $^{*}P < 0.05$ significantly different from LPS-stimulated control.

together, our 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 have been reported to play a critical role on LPS-mediated COX-2 induction [28,29]. Our 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 [40], capsaicin [41] and sesquiterpene lactones [42] that have been reported to inhibit COX-2 by targeting MAPK signaling pathways including ERK, p38 kinase and JNK.

In summary, we show the data for the first time that anthocyanidins 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. Our findings provide the first molecular basis for the anti-inflammatory properties of anthocyanidins.

with 40 ng/ml LPS from 10 to 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. (D) Nuclear translocation of p65. Cell culture and nuclear extraction were performed as Fig. 3B. Nuclear p65 were detected with p65 antibody. Histograms show the densitometric analysis of p65 in nuclear lysates. $^{*}P < 0.05$ significantly different from LPS-stimulated control. The data represent the mean ± S.D. of three separate experiments and the picture is a representative of those experiments each with similar results.

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