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Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] suppresses the expression of cyclooxygenase-2 in endotoxin-treated monocytic cells ☆

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Abstract

The anti-inflammatory activity of the predominant procyanidin dimer in cocoa, dimer B2, was investigated in this study. Pretreatment of the procyanidin dimer B2 reduced COX-2 expression induced by the endotoxin lipopolysaccharide (LPS) in differentiated human monocytic cells (THP-1) in culture. To further elucidate the underlying mechanism of COX-2 inhibition by procyanidin, we examined their effects on the activation of extracellular signal-regulated protein kinase (ERK), Jun-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK), which are upstream enzymes known to regulate COX-2 expression in many cell types. Pretreatment with procyanidin dimer B2 decreased the activation of ERK, JNK, and p38 MAPK. In addition, procyanidin dimer B2 suppressed the NF-κB activation through stabilization of IκB proteins, suggesting that these signal-transducing enzymes could be potential targets for procyanidin dimer B2. By affecting the expression rather than the activity of COX-2, these in vitro data reported herein give further evidence on the anti-inflammatory protection by procyanidins.

Keywords: Cocoa; Procyanidin; Cyclooxygenase-2 (COX-2); Mitogen-activated protein kinase (MAPK); Nuclear factor κB (NF-κB)

Cyclooxygenase (COX), the key enzyme that catalyzes the rate-limiting step in prostaglandin (PG) biosynthesis, exists in at least two isoforms, designated as COX-1 and COX-2. Although these enzymes share considerable sequence homology, these isoforms differ considerably in the tissue distribution, regulation, and function. Cyclooxygenase-1 is a housekeeping enzyme, being constitutively expressed in almost all mammalian tissues. In contrast, COX-2 is barely detectable under normal physiological conditions; however, like other early-response gene products, COX-2 can be induced rapidly and transiently by pro-inflammatory mediators and mitogenic stimuli including cytokines, endotoxins, growth factors, oncogenes, and phorbol esters. As such, COX-2 has received considerable attention for its potential role in the inflammation and disease development.

Several lines of compelling evidence from genetic and clinical studies indicate that improper upregulation of COX-2 is implicated in the progression of neurodegenerative diseases, atherosclerosis, and cancer. COX-2 expression has been shown to be increased in the frontal cortex of individuals diagnosed with Alzheimer's disease, and synthetic β-amyloid peptides induced COX-2 expression in SH-SY5Y neuroblastoma cells in vitro [1]. Furthermore, COX-2 induction occurs through a JNK/c-Jun-dependent mechanism after administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), suggesting

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that COX-2 may be playing a role in the neuropathology of Parkinson's disease. [2] Selective COX-2 inhibition has been shown to reduce early lesion formation in LDL receptor-deficient ($LDLR^{-/-}$) mice and that macrophage COX-2 expression contributes to atherogenesis in $LDLR^{-/-}$ mice [3]. The elevated expression of COX-2 is also reported in multiple malignancies, including those of esophagus, stomach, breast, pancreas, lung, colon, skin, urinary bladder, and prostate [4]. Thus, COX- 2 is recognized as a potential therapeutic target for the prevention and treatment of a number of diseases.

Epidemiologic studies have linked flavonoid-rich foods with a reduced risk of cancer and cardiovascular disease [5]. Some cocoas and chocolate can be flavonoid-rich, composed of primarily the monomeric flavanols (−)-epicatechin and (+)-catechin, and oligomeric procyanidins formed from these monomeric units. While the mechanisms underlying the suggested health benefits of flavonoid-rich foods remain to be fully elucidated, in vitro and in vivo studies have demonstrated that the flavanols and procyanidins from cocoa have a number of potential biological effects including the ability to reduce oxidative damage [6], promote endothelium-dependent relaxation [7], and decrease platelet aggregation [8]. Specifically related to inflammation, cocoa flavanols and procyanidins have been shown to modulate the production of inflammatory cytokines [9], as well as inhibit lipoxygenase activity in vitro [10]. These data together suggest that the flavanols found in cocoa may have important biological activities.

Although the potent in vitro effects of the procyanidins from cocoa have generated considerable interest in these compounds, it has been suggested that their in vivo effects may be minimal because of gastric degradation [11]. While the flavanol monomers in cocoa ((−)epicatechin and (+) catechin) are readily absorbed and metabolized in humans [12], less is known about the bioavailability and metabolism of procyanidins. Human feeding trials done with cocoa demonstrate that the procyandins can survive the acidic milieu of the stomach and as such, are not initially broken down and likely enter into the small intestine intact. In support of this, the predominant procyanidin dimer in cocoa, dimer B2 [epicatechin-(4β-8)-epicatechin] (Fig. 1), has been detected in human plasma as early as 30 min after the consumption of a flavanol-rich food such as cocoa [13]. Thus, while the metabolic fate of dimer B2 has yet to be elucidated, it is clear from this work that it can be absorbed, and as such, suggests that dimer B2 may contribute to some of the benefits observed following the consumption of flavanol/procyanidin-rich cocoa and chocolate.

For this reason, we investigated whether dimer B2 could suppress COX-2 expression induced by the endotoxin lipopolysaccharide (LPS) in a differentiated human monocytic leukemia cell line, THP-1. As the expression of COX-2 has been shown to be linked to mitogen-activated protein kinase (MAPK) signaling cascades, we also examined the cellular signaling mediators and events involved in COX-2 expression, particularly transcription factors NF-κB and upstream regulatory kinases, such as extracellular signal-regulated kinase (ERK), Jun-N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK), as potential molecular targets of dimer B2.

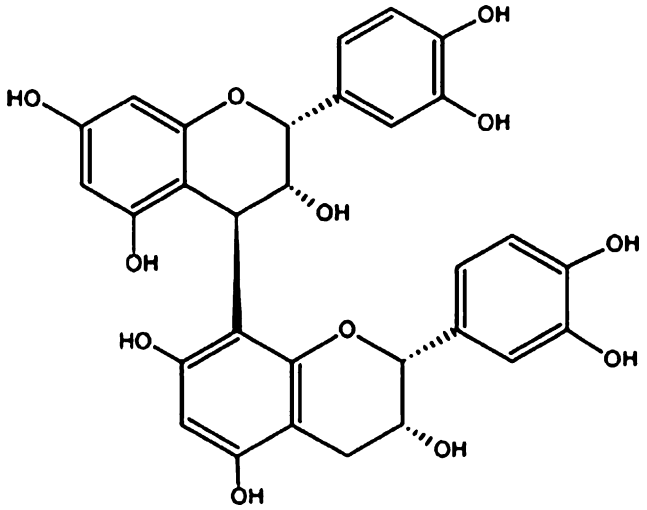

Fig. 1. Chemical structures of procyanidin dimer B2, epicatechin-(4β-8)-epicatechin (cocoa).

Materials and methods

Materials. Procyanidin dimer B2 was supplied by Mars Incorporated (purity 98.34%). Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharides (LPS, from *Escherichia coli* serotype 0111:B4), arachidonic acid, and NS398 were purchased from Sigma (St. Louis, MO). RPMI 1640, Grace's supplemented medium, L-glutamine, Hepes, 2-mercaptoethanol, fetal bovine serum, and penicillin/streptomycin were purchased from Gibco-BRL (Grand Island, NY). Anti-COX-2, anti-IκBα, and anti-NF-κB p65 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-ERK, anti-JNK, anti-p38 MAPK, and their phosphor antibodies were purchased from Cell signaling technology (Beverly, MA). Alexa Fluor 488 goat anti-mouse IgG was purchased from Molecular probes (Eugene, OR). SuperSignal West Pico chemiluminescent substrate was purchased from Pierce (Rockford, IL). All other chemicals used were in the purest form available commercially.

Cell culture. Human monocytic THP-1 cells (American Type Culture Collection, Manassas, VA) in RPMI 1640 medium, with 4.5 g/L glucose, 10 mM Hepes, 1 mM sodium pyruvate, and 50 μM 2-ME supplemented with 10% FBS, were cultured under a humidified 5% CO_2 atmosphere at 37 °C. For differentiation, THP-1 cells were plated at 1×10^6 cells/ml in the medium containing 100 nM PMA and allowed to adhere for 48 h, after which they were fed with PMA-free medium and cultured for 24 h prior to use. LPS was used at a concentration of 1 μg/ml in the medium. Insect cell line sf9 was cultured in monolayer at 28 °C in Grace's supplemented medium with 10% heat-inactivated fetal bovine serum.

Determination of COX-2 enzyme activity. The effect of procyanidin dimer B2 and selective COX-2 inhibitor NS398 on the activity of COX-2 was measured using baculovirus-expressed recombinant human COX-2 enzyme as previously described [14].

Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) to assess COX-2 mRNA expression. Total RNA was extracted from macrophages with TRIzol reagent. Real-time quantitative RT-PCR was performed using the Opticon 2 (MJ Research Inc., Waltham, MA). Sequence-specific PCR primers for COX-2 [Accession No. NM_000963; forward primer: 5′-GGGCAAAGACTGCGAAGA AG-3′; reverse primer: 5′-CCCATGTGACGAAATGACTG-3′] and

GAPDH [Accession No. NM_002046; forward primer: 5′-GTCAACGG ATTTGGTCGTATTG-3′; reverse primer: 5′-TCTCGCTCCTGGAAG ATGG-3′] were designed using the Primer Premier software version 5.00. Standard curves were run on the same plate and the relative standard curve method was used to calculate the relative gene expression.

Western blot analysis. Cells (5×10^6) were resuspended in modified RIPA lysis buffer (Tris–HCl 50 mM, pH 7.4 NaCl 150 mM, EDTA 1 mM, Na-deoxycholate 0.25%, NP-40 1%, PMSF 1 mM, Na_3VO4 1 mM, NaF 1 mM, aprotinin 10 μg/ml, leupeptin 5 μg/ml, and pepstatin 5 μg/ml) and lysed cells on ice for 45 min. The lysate was centrifuged at 14,000*g* for 15 min to sediment the particulate materials. Samples were electrophoresed in SDS–PAGE gels and separated proteins were transferred onto a PVDF membrane. The blots were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature and subsequently incubated overnight at 4 °C with appropriate primary antibody diluted in TBST [TBS, 1% (v/v) Tween 20, and 5% (w/v) BSA]. Following three washes of 10 min each with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies in blocking buffer for 1 h at room temperature. After three washes with TBST, the blots were developed with chemiluminescence reagent and exposed to X-ray film (Kodak XAR5, Eastman Kodak, Rochester, NY, USA). Semi-quantitative analysis of immunoreactivity was measured by electrophoresis image analysis system (FR-980A, Shanghai FURI Science & Technology Co., Ltd.), and the results were expressed as OD (optical density).

Preparation of nuclear extracts. The nuclear protein extracts were prepared as previously described [15] with some modifications. Briefly, 5×10^6 cells were washed twice with cold PBS. Cells lysed in 400 μl of cold buffer A (HEPES 10 mmol/L, pH 7.9, KCl 100 mmol/L, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 0.5% NP-40, and 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A) and allowed to swell on ice for 30 min, followed by vigorous vortex mixing for 1 min. The homogenate was centrifuged in a microcentrifuge at 20,800*g* for 5 min. The supernatant fluid was discarded. The nuclear pellets were washed once with cold buffer A, then resuspended in 50 μL of cold buffer C (HEPES 10 mmol/L, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, 0.5 mM PMSF, and 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A). The tube was mixed intermittently for 60 min. The nuclear extract was obtained by centrifuging at 20,800*g* for 5 min at 4 °C.

Electrophoretic mobility shift assay. Biotin end-labeled double-stranded oligonucleotides 5′-biotin-GGAGAGTGGGGACTACCCCCTCTGCT-3′ as well as non-labeled oligonucleotide containing NF-κB consensus sequence were generated using an oligonucleotide synthesizer. The binding reactions contained 5 μg of nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 1 μg of poly(dI/dC), and 20 fmol of biotin-labeled DNA. The reaction mixtures were incubated at 23 °C for 20 min. The competition reactions were performed by adding 200-fold excess unlabeled double-stranded NF-κB consensus oligonucleotide to the reaction mixture. The reaction mixtures were electrophoresed on a 6% Tris–borate–EDTA gel at 100 V for 1 h in a 0.5× Tris–borate–EDTA buffer. The reactions were transferred to a nylon membrane. The biotin-labeled DNA was detected with LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce).

Immunofluorescent staining and image capture. The cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.5% Triton X-100 for 15 min. After a 1 h incubation with blocking buffer (5% normal goat serum, 1% BSA, and 0.1% Triton X-100, 0.1% sodium azide in PBS), cells were incubated with anti-p65 Ab diluted at 1:100 in blocking buffer overnight at 4 °C, washed, and then incubated with Alexa Fluor 488 goat anti-mouse IgG, diluted at 1:1000 in blocking buffer, for 1 h. In order to identify the nuclei, the Alexa Fluor 488-labeled samples were counterstained with propidium iodide (PI) 25 μg/ml for 2 min. To acquire dual-color images, a 510 confocal laser scan confocal microscope (Leica, Germany) was used, which was equipped with a Leica inverted research biological microscope, with a 100× oil immersion objective. The samples labeled with both Alexa Fluor 488 and PI were excited at 488 nm, and the fluorescence emissions were captured through 510–550 nm (530 nm in center) and 590–620 nm (605 nm in center) bandpass with spectral grating, respectively.

Statistical analysis of data. The data are presented as means ± SD and compared with ANOVA and least significant difference test using SPSS statistical program. The level of the statistical significance was set at $P < 0.05$.

Results

Procyanidin dimer B2 inhibits LPS-induced increases in the transcription and expression of COX-2

To evaluate the effects of dimer B2 on COX-2 activity, we utilized baculovirus-expressed human recombinant COX-2 in an insect cell-based assay. Fig. 2 shows that dimer B2, even at 100 μM, had little inhibitory effect on PGE_2 synthesis, while selective COX-2 inhibitor NS398 significantly inhibited PGE_2 synthesis. We also investigated

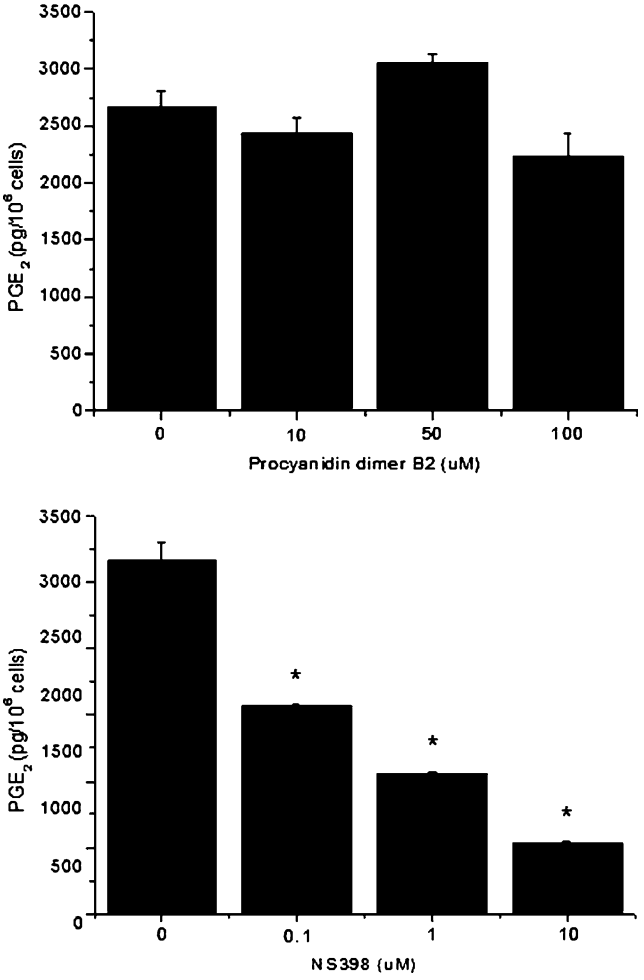

Fig. 2. Effect of procyanidin dimer B2 on COX-2 enzyme activities in differentiated THP-1 cells. Experiment was performed as described under Materials and methods. Results represent the PGE_2 production. $n = 3$. Mean ± SD. *$P < 0.01$ vs. control.

the possibility that dimer B2 suppressed the induction of COX-2. It has been reported that stimulation of human macrophage with LPS increased the levels of mRNA for COX-2 and that this change correlated well with increased prostacyclin biosynthesis [16]. Treatment of differentiated THP-1 cells with 1 μg/ml LPS for 4 h led to a dramatic increase in COX-2 transcription (Fig. 3). Pretreatment with dimer B2 inhibited the levels of mRNA for COX-2 in a concentration-dependent manner (Fig. 3). In the subsequent experiment, the inhibitory effect of dimer B2 on the LPS-induced COX-2 expression was investigated by following the same study protocol. As illustrated in Fig. 4, dimer B2 also inhibited COX-2 expression. Since only 50 μM dimer B2 has shown the significant inhibitory effects on COX-2 transcription and expression, this concentration was selected to use in the following experiments.

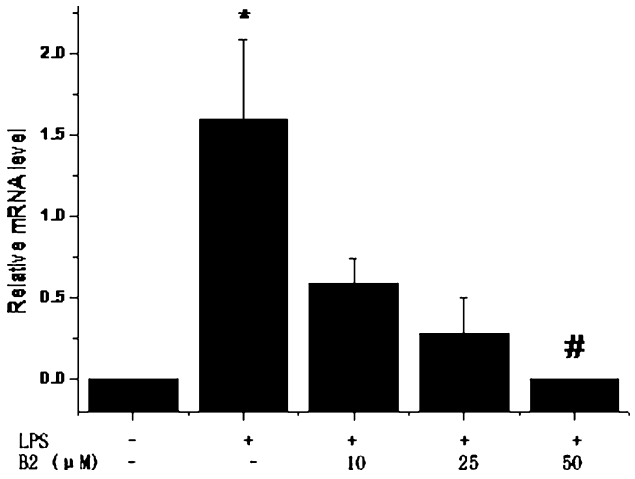

Fig. 3. Effect of procyanidin dimer B2 on COX-2 mRNA transcription in differentiated THP-1 cells. After pretreatment with procyanidin dimer B2 for 30 min, differentiated THP-1 cell was treated with LPS for 4 h. Total RNA was extracted and reverse transcribed. COX-2 mRNA expression was assessed by real-time PCR. GAPDH expression was used to normalize COX-2 and control PCR expression results. $n = 3$. Mean ± SD. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. LPS.

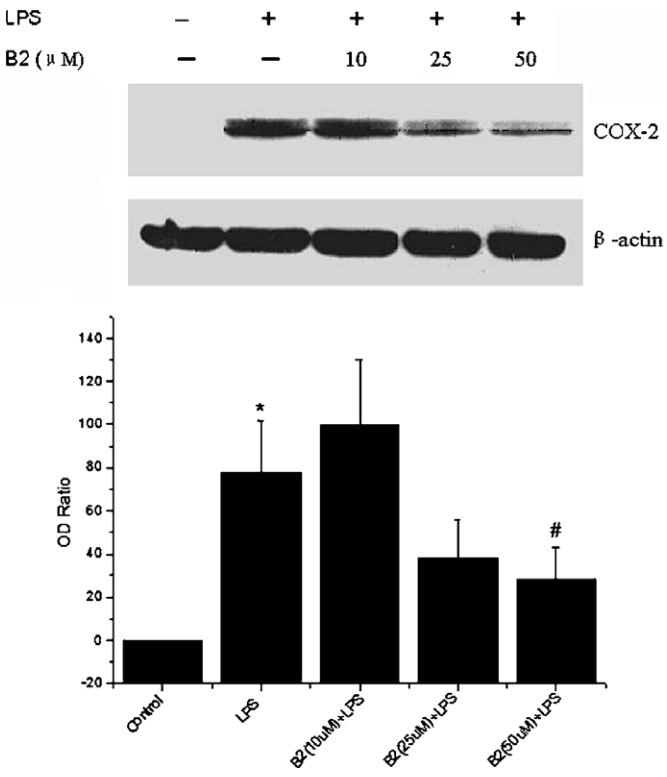

Fig. 4. Effect of procyanidin dimer B2 on LPS-induced COX-2 expression in differentiated THP-1 cells. After pretreatment with procyanidin dimer B2 for 30 min, differentiated THP-1 cell was treated with LPS for 4 h. The Western blot represents three independent experiments. $n = 3$. Mean ± SD. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. LPS.

Inhibitory effects of procyanidin dimer B2 on LPS-induced activation of MAPK in differentiated THP-1 cells

Because LPS-induced COX-2 expression is reported to be regulated, at least in part, by a series of upstream kinases collectively known as MAPKs [17], we determined the effect of the dimer B2 on LPS-induced activation of ERK, JNK, and p38 MAPK. We found that LPS significantly activated

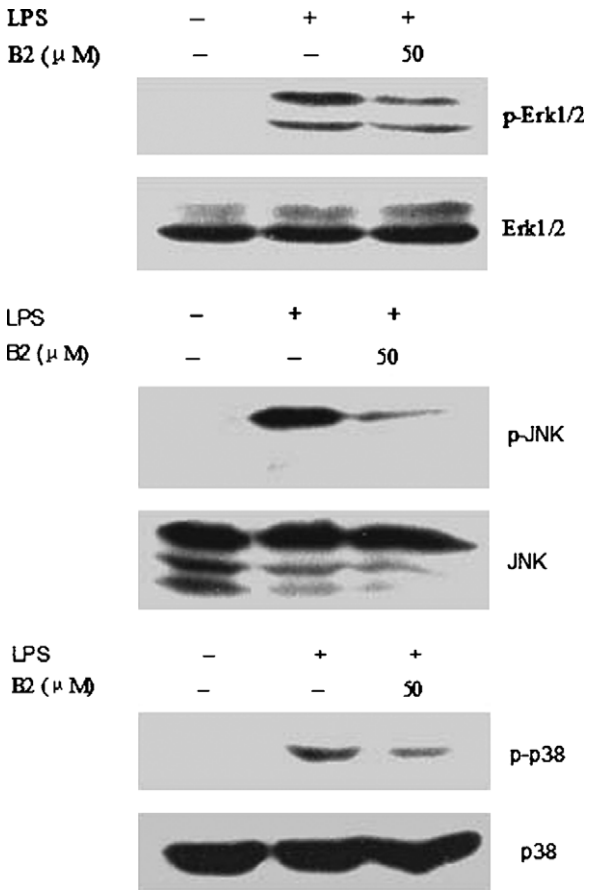

Fig. 5. Effect of procyanidin dimer B2 on LPS-induced activation of MAPKs in differentiated THP-1 cells. After pretreatment with procyanidin dimer B2 for 30 min, differentiated THP-1 cell was treated with LPS for 15 min. The Western blot represents three independent experiments.

these terminal kinases via phosphorylation of the proteins. Pretreatment of the dimer B2 at 50 μM suppressed LPS-induced activation of these protein kinases (Fig. 5).

Effects of procyanidin dimer B2 on LPS-induced DNA binding of NF-κB

Nuclear factor-κB (NF-κB) plays a central role in general inflammatory as well as immune responses. It can regulate the expression of a variety of early-response genes involved in the inflammatory and immune reactions, such as COX-2 [18]. To investigate the possible inhibitory effect of procyanidin dimer B2 on this transcription factor, the nuclear fractions from LPS-treated THP-1 cells with or without procyanidin dimer B2 pretreatment were analyzed by electromobility gel shift assay. We investigated whether procyanidin dimer B2 could suppress activation of this transcription factor in THP-1 cells stimulated with LPS. As shown in Fig. 6, DNA–protein complexes were detected using nuclear extracts from differentiated THP-1 cells and were enhanced by stimulation with LPS. The activation of NF-κB in response to LPS was reduced by pretreatment with 50 μM dimer B2.

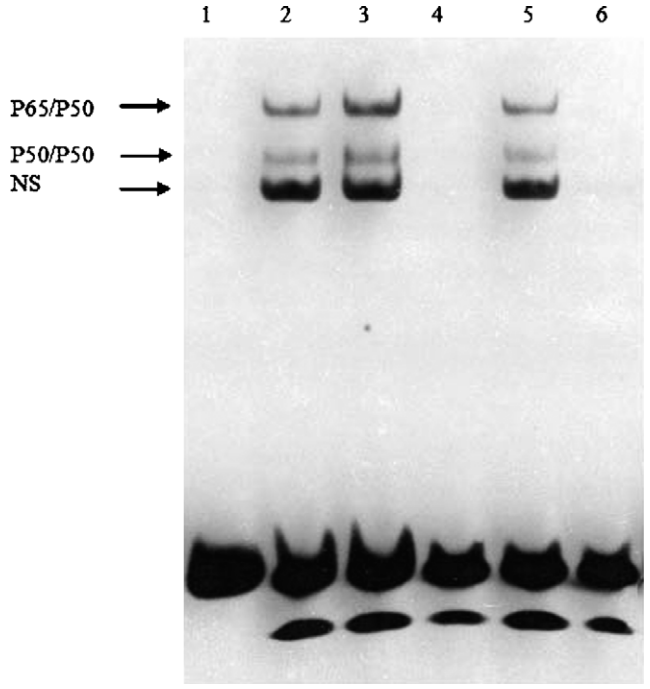

Fig. 6. Effect of procyanidin dimer B2 on the activation of NF-κB in differentiated THP-1 cells. After pretreatment with procyanidin dimer B2 for 30 min, differentiated THP-1 cell was treated with LPS for 30 min. The nuclear extracts were prepared and incubated with the biotin-labeled oligonucleotides containing NF-κB consensus sequence for analysis by electrophoretic mobility shift assay. Lane 1, free probe alone (no nuclear extract); lane 2, control; lane 3, LPS alone; lane 4, LPS alone with 200-fold unlabeled oligonucleotides; lane 5, procyanidin dimer B2 50 μM plus LPS; lane 6, procyanidin dimer B2 50 μM plus LPS with 200-fold unlabeled oligonucleotides. NS, non-specific.

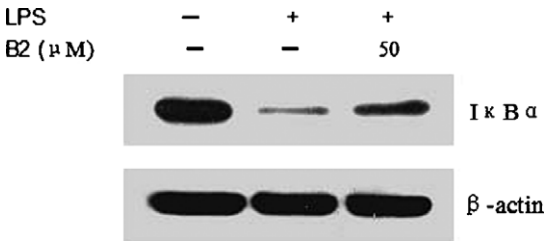

Fig. 7. Effect of procyanidin dimer B2 on IκBα degradation in differentiated THP-1 cells. After pretreatment with procyanidin dimer B2 for 30 min, differentiated THP-1 cells were treated with LPS for 30 min. The Western blot represents three independent experiments.

Effects of procyanidin dimer B2 on LPS-mediated IκB degradation and NF-κB/p65 nuclear translocation in differentiated THP-1 cells

The data from the above experiment indicate that preincubation of THP-1 cells with dimer B2 resulted in a dramatic reduction in "free" NF-κB. As the activation and translocation of NF-κB are regulated through the binding of Inhibitory kappa B protein (IκB), we evaluated IκB degradation and NF-κB translocation in LPS- and dimer B2-treated differentiated THP-1 cells. THP-1 cells pretreated with procyanidin dimer B2 for 30 min before addition of LPS 1 μg/ml for 30 min experienced a dramatic reduction in LPS-induced degradation of IκB (Fig. 7). These changes in the level of free NF-κB would be expected to result changes in the translocation of the active NF-κB into the nucleus. In a separate series of experiments, THP-1 cells treated with LPS demonstrated a dramatic increase in the translocation of NF-κB into the nucleus (Fig. 8). In the presence of procyanidin dimer B2, the LPS-induced NF-κB nuclear translocation was markedly inhibited (Fig. 8).

Discussion

While there is a substantial body of scientific literature which supports a positive role of flavonoids to health, how specific flavonoids exert these benefits is under intense investigation. Flavanols and procyanidins from cocoa have been shown to modulate inflammatory and immune responses [19,9]. While the mechanisms underlying these responses likely vary depending on the flavanol/procyanidin under investigation, the results of this study demonstrate that changes in inflammatory responses may be mediated in part through changes in the expression of COX-2. In this study, the procyanidin B2 dimer from cocoa was able to suppress LPS-mediated induction of COX-2 gene expression.

There is a growing body of compelling evidence that targeted inhibition of abnormal or improper overexpression or activity of COX-2 is valuable for not only alleviating inflammation but also preventing other diseases, such as cancer, cardiovascular disease, and neurodegenerative diseases. Drugs that interfere with the signaling mechanisms that up-regulate COX-2 should also be useful in this regard

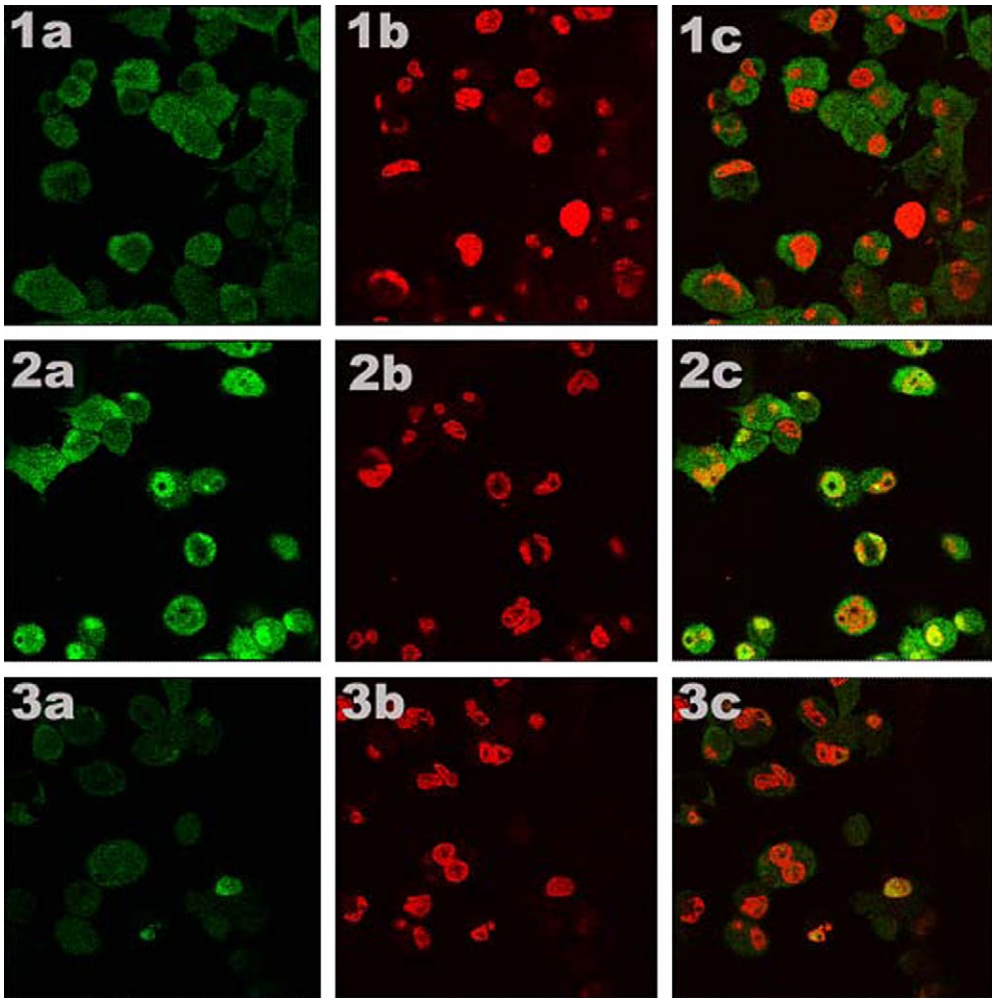

Fig. 8. Effect of procyanidin dimer B2 on LPS-induced subcellular localization of NF-κB/p65 in THP-1 cells. Indirect immunofluorescence and confocal microscopy analyses were used. (1) Control; (2) LPS 1 μg/ml for 30 min; (3) procyanidin dimer B2 50 μM for 30 min before the addition of LPS for 30 min, respectively. a, b, and c were green channel, red channel, and the combining figure. Results are representatives of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

because they also decrease total COX-2 activity. We have shown in the present studies that procyanidin dimer B2 could suppress LPS-mediated induction of COX-2 gene expression in human monocyte THP-1. The result suggests that, in addition to the inhibitory effect of B2 on proinflammatory cytokine production [19,9], the anti-inflammatory properties of procyanidins can also be attributable to its inhibitory effect on COX-2 induction. Kaul [20] reported that the procyanidins possessed inhibitory effect on the cyclooxygenase activities. However, the results of the present study clearly showed that procyanidin dimer B2 had weak inhibitory effect on COX-2 enzyme activity (Fig. 2). In contrast, procyanidin dimer B2 could inhibit the transcription and expression of COX-2 (Figs. 3 and 4). One possible explanation for these apparently contradictory results is the difference in assays used to measure COX-2 activity.

The molecular signaling mechanisms involved in the induction of COX-2 in response to various external stimuli have not been fully clarified. One of the most extensively investigated intracellular signaling cascades involved in cellular proinflammatory responses involves the MAPK pathway. The mitogen-activated protein kinase (MAPK) family of proteins belongs to distinct and evolutionarily conserved signal transduction pathways that are activated by extracellular stimuli. Accumulating evidence indicates that enzymes of the MAPK family play a role in regulating COX-2 expression. Studies using pharmacological inhibitors of MAPKs or their upstream protein kinase activators and dominant-negative mutant forms of protein kinases demonstrated the role of ERK, JNK, and p38 MAPK in transcriptional activation of COX-2 [21,22]. Moreover, MAPKs are involved in the signaling pathway for LPS-induced COX-2 expression [23,24]. Hwang et al. [25] reported that blockade of ERK and p38 MAPK activities by PD98059 and SB203580, respectively, resulted in partial suppression of LPS-induced expression of COX-2 in Raw 264.7 cells.

To determine the specific signal transduction pathway that is involved in the procyanidin dimer B2-mediated inhibition of LPS-induced COX-2 expression, we examined the

effects of procyanidin dimer B2 on the activation of MAPKs induced by LPS. Vayalil et al. [26] reported inhibitory effect of EGCG on MAPK activation in SKH-1 hairless mouse skin treated with a single or multiple exposure to UVB in which topically applied EGCG prevented depletion of antioxidant enzymes and inhibited phosphorylation of ERK1/2, JNK, and p38 MAPK [26]. Pretreatment with EGCG as well as green tea extract caused a decrease in the activation of ERK. In addition, EGCG inhibited the catalytic activity of ERK and p38 MAPK [27]. Our results demonstrated that MAPKs could be transiently activated by stimulation of LPS, and pretreatment of procyanidin dimer B2 inhibited phosphorylation of ERK1/2, JNK, and p38 MAPK (Fig. 5). Prevention of phosphorylation of MAPK proteins may thus prevent the downstream events such as inhibition of COX-2 induction. Among these three MAPKs, transient activation of ERK is responsible for proliferation and differentiation [28], and has also been shown to be involved in the tumor promotion process especially stimulated by the oxidant state [29]. Phosphorylation of JNK and p38 can mediate differentiation, inflammatory responses, and cell death [28,30,31]. Therefore, it can be suggested that treatment of procyanidin dimer B2 might be very useful in the treatment of some diseases due to its combined activities.

It has been known that NF-κB plays a central role in general inflammatory as well as immune responses. In light of its roles as a coordinating regulator in expression of a variety of early-response genes involved in the inflammatory and immune reactions, NF-κB has drawn much interest as an attractive therapeutic target for novel anti-inflammatory and immunomodulatory drugs. Included in the genes known to be regulated by NF-κB are COX-2 and iNOS. The 5′-promoter region of COX-2 contains two putative NF-κB binding sites. In line with this notion, NF-κB has been shown to be a positive regulator of COX-2 expression in murine macrophages [32] and human colon adenocarcinoma cell lines [33] exposed to LPS. Recently, more and more findings have indicated that flavan-3-ols and oligomeric procyanidins could modulate NF-κB activation. Treatment of normal human keratinocytes with EGCG resulted in an inhibition of UVB-induced IKK-α activation, phosphorylation, and degradation of IκBα, and activation and nuclear translocation of p65 [34]. EGCG and theaflavins have also been shown to inhibit TPA-induced NF-κB activity, NF-κB sequence-specific DNA binding, and IκBα phosphorylation (Ser32) in JB6 cells [35]. Pretreatment with Epicatechin, catechin, and dimeric procyanidins decreased PMA-induced NF-κB binding activity and the transactivation of the NF-κB-driven gene IL-2 [36]. In the present studies, we demonstrated that procyanidin dimer B2 inhibited activation of NF-κB induced by LPS. After THP-1 cells were treated with procyanidin dimer B2, nuclear factor binding to the NF-κB element of the COX-2 promoter was decreased (Fig. 6), the IκB protein level was increased (Fig. 7), and NF-κB nuclear translocation was inhibited (Fig. 8). The results showed that procyanidin dimer B2 suppressed the NF-κB activation through stabilization of IκB proteins.

Accumulating evidence indicates that NF-κB activation is also modulated by MAPK/ERK kinase kinase-1 (MEKK1), a kinase upstream of JNKs [37] as well as p38 MAPK [38]. MEKK1 induces site-specific phosphorylation of IκBα at Ser 32 and Ser 36 in HeLa cells and also directly activates the IκB kinase (IKK) complex [37]. Combined with aforementioned MAPK inhibition, we hypothesize that a same upstream signaling kinase of NF-κB and MAPK pathway, such as MEKK1, might be the possible molecular target of procyanidin dimer B2. The other possible explanation is that procyanidin dimer B2, as a potent antioxidant, could prevent the production of reactive oxygen species (ROS), which have been suggested to be involved in the activation of the NF-κB signaling system [39] and MAPK signal pathway [40,41]. All these conjectures are needed to be further investigated.

In conclusion, procyanidin oligomer dimer B2 showed inhibitory effect on COX-2 expression. The underlying mechanism of such inhibition may be mediated by: (i) inhibition of phosphorylation of MAPK proteins and (ii) prevention of DNA binding of NF-κB through stabilization of IκB proteins. According to the roles of COX-2 and their upstream enzymes, the present studies further provide evidence that procyanidins may represent a promising candidate for the prevention/treatment of various diseases. The consumption of flavonoid-rich foods may contribute to achieve optimal health and development as well as play an important role in reducing the risk or delaying the development of disease, such as cardiovascular disease, cancer, and other age-related diseases.

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