The procyanidin trimer C1 inhibits LPS-induced MAPK and NF-κB signaling through TLR4 in macrophages

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Natural products and dietary components rich in polyphenols have been shown to reduce inflammation; however, the molecular mechanisms underlying this anti-inflammatory activity are not completely characterized, and many features remain to be elucidated. This research was carried out to clarify the potential role of procyanidin trimer C1 in the anti-inflammatory effect of polyphenols. Procyanidin C1 inhibited inducible nitric oxide synthase-mediated nitric oxide production and the release of pro-inflammatory cytokines (interleukin-6 and tumor necrosis factor-α) in lipopolysaccharide (LPS)-induced macrophages. Treatment with procyanidin C1 resulted in a significant decrease in prostaglandin E2 and cyclooxygenase-2 levels, as well as the expression of cell surface molecules (CD80, CD86, and MHC class II), which was induced by LPS. Furthermore, our data demonstrated that the anti-inflammatory effect of procyanidin C1 occurs through inhibition of mitogen-activated protein kinase (p38 and c-Jun N-terminal kinase) and nuclear factor-κB signaling pathways. These 2 factors play a major role in controlling inflammation, through toll-like receptor 4, suggesting that procyanidin C1 plays a potent role in promoting anti-inflammatory activity in macrophages. These results represent a novel and effective therapeutic intervention for the treatment of inflammatory disease.

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1. Introduction
Polyphenolic compounds have attracted increasing attention in the fields of nutrition and medicine owing to their potential health benefits [1–4]. One of the most important of these benefits is the ability of some polyphenolic compounds to counter-regulate inflammation [5]. Polyphenolic compounds and their reaction products possess a wide range of biological activities that may contribute to their protective effects against a number of inflammation-related diseases/disorders, including cardiovascular disease, asthma, atherosclerosis, diabetes, obesity, and cancer [6,7]. The inflammatory response, which is coordinated by a large range of mediators that form complex regulatory networks, underlies a wide variety of physiological and pathological processes. Macrophages play a key role in the initiation of the inflammatory response through the production of pro-inflammatory mediators such as prostaglandin E2 (PGE2) and cytokines, e.g., interleukin (IL)-6 and tumor necrosis factor (TNF)-α [8–10]. Macrophage activation in response to bacterial products such as lipopolysaccharide (LPS) induces local inflammation and can lead to septic shock during severe infections [11]. LPS acts directly on macrophages to induce inflammatory signaling through nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK), which are major transcriptional regulators of inflammation [12–14]. NF-κB drives the expression of a number of genes that are involved in the development and progression of inflammatory processes and are predictive biomarkers of mortality, including pro-inflammatory cytokines and enzymes such as cyclooxygenase (COX)-2 [15–18]. Furthermore, previous studies have shown that COX-2 and PGE2 control the production of pro-inflammatory cytokines in LPS-induced macrophage activation [19–21]. LPS binds to and signals through a receptor complex consisting of toll-like receptors (TLRs), thereby initiating the inflammatory response. Activation of LPS-stimulated TLR4 ultimately leads to MAPK and NF-κB-mediated signaling and the induction of the inflammatory response [22,23]. Procyanidins are polyphenolic compounds...
from the flavonoid group; they are found in a variety of vegetables and fruits and have a wide range of biological activities, including antioxidant and anti-inflammatory roles [24–27]. However, the potential role of procyanidin C1 in the regulation of inflammation in macrophages has not yet been established.

For in vitro studies, the mouse macrophage cell line RAW264.7 is often used as a model system; however, because this cell line is tumorigenic, we chose to use primary bone marrow-derived macrophages (BMDMs) in the following experiments. In the present study, we investigated the ability of procyanidin C1 (Fig. 1) to modulate inflammatory signaling by BMDMs stimulated with LPS.

2. Materials and methods

2.1. Materials

Procyanidin C1, LPS from Escherichia coli O111:B4, and anti-β-actin mAb (AC-15) were purchased from Sigma (San Diego, CA, USA). Inducible nitric oxide synthase (iNOS) polyclonal (p) anti-body (Ab), COX-1/2 pAb, TLR-4 pAb, anti-phosphorylated ERK1/2 monoclonal (m) Ab, anti-ERK1/2 pAb, anti-phosphorylated p38 mAb, anti-p38 pAb, and inhibitory (I)β-α pAb were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) Ab and HRP-conjugated anti-rabbit IgG Ab were obtained from Calbiochem (San Diego, CA, USA). FITC-conjugated mAb against CD11b, and phycoerythrin (PE)-conjugated mAb against CD80, CD86, MHC class II, and cytokine detection kits for IL-6 and TNF-α were all purchased from eBioscience (San Diego, CA, USA). pGL3-NF-κB and the luciferase assay system were purchased from Promega Inc. (Madison, WI, USA). pCMV-b-gal was purchased from Clontech Laboratories Inc. (Mountain View, CA, USA).

2.2. Cell culture

RAW264.7 cells, a murine macrophage cell line, were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). RAW264.7 macrophage cells were maintained in DMEM (GIBCO, Carlsbad, CA, USA) containing 10% FBS (GIBCO), 100 U/mL penicillin and 100 U/mL streptomycin (complete medium) under humidified condition at 37 °C and 5% CO₂ in an incubator. BMDMs were isolated from C57BL/6 mice by using standard methods [28]. Briefly, bone marrow cells from the femur and tibia were cultured in DMEM containing 2 mM L-glutamine, and 25 ng/mL recombinant mouse macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN, USA) at 37 °C under condition of 5% CO₂. After 4 days, non-adherent cells were removed and allowed to differentiate into BMDMs.

2.3. Measurement of cell proliferation

Cell proliferation was examined using the EZ-Cytox cell viability kit (Daeil Laboratories, Seoul, Republic of Korea) according to the manufacturer’s instructions. BMDMs were seeded in 96-well plates at a density of 2 × 10⁴ cells/well in DMEM. After incubation at 37 °C for 4 h, the medium was replaced with DMEM containing procyanidin C1 for 24 h. EZ-Cytox kit reagent (10 μL) was added to each well, and the cells were incubated for 1 h. The optical density was measured at 450 nm in an automated micro-plate reader (Zenyth 3100; Anthos Labtec Instruments GmbH, Salzburg, Austria).

2.4. Measurement of nitric oxide (NO) production

The concentration of NO in culture supernatants was determined by measuring its oxidation product, nitrite, using the Griess method. Briefly, supernatants from experimental BMDM cultures were mixed with the Griess reagent (1:1) and incubated at room temperature for 15 min, and then, the absorbance of the solution at 517 nm was measured using a microplate reader (Zenyth 3100; Anthos Labtec Instruments GmbH). NaNO₂ freshly prepared in deionized water was used to generate a standard curve (0–100 μM) to calculate the nitrite concentration in cell culture supernatants.

2.5. ELISA

Supernatants from experimental BMDM cultures were collected and stored at −70 °C until use. The levels of IL-6 and TNF-α in the supernatants were determined using cytokine detection ELISA kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions, with detection at 450 nm using a microplate reader. The concentration of PGE₂ in cell culture supernatants was determined by using a PGE₂ detection ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA) following the manufacturer’s instructions.

2.6. Measurement of cell surface molecules by flow cytometry

Four days after differentiation, BMDMs were harvested, washed with PBS, and resuspended in washing buffer (2% FBS and 0.1% sodium azide in PBS) for fluorescence-activated cell sorting (FACS) on the FACScalibur flow cytometer (BD, Franklin Lakes, NJ, USA). The cells were pre-incubated with 0.5% BSA in PBS for 30 min and washed with PBS. The cells were then stained with PE-conjugated anti-CD11b (MHC class II), anti-CD80, and anti-CD86, along with FITC-conjugated anti-CD11b (BD PharMingen, San Diego, CA, USA) for 45 min at 4 °C. All antibodies were diluted 100-fold before use. Cells were washed 3 times and resuspended in a final volume of 500 μL of PBS. The markers of BMDM maturation were then analyzed by flow cytometry, and the data were analyzed using the Cell Quest software (BD).

2.7. Western blotting analysis

BMDMs were lysed in a lysis buffer (Cell Signaling, Danvers, MA, USA) containing phenylmethylsulfonyl fluoride (Sigma), and lysates were separated by 10% SDS-PAGE and transferred onto PVDF membranes. The PVDF membranes were blocked with 5% skim milk and incubated with each of the required primary Abs (1:1000) for 2 h, followed by incubation with HRP-conjugated secondary Abs (1:2000).
for 1 h at room temperature. Proteins were visualized using an ECL Advance kit (GE Healthcare, Little Chalfont, UK).

2.8. Luciferase reporter gene assay

RAW264.7 cells were plated in a 24-well plate. After 12 h, the cells were transiently co-transfected with the plasmids pGL3-NF-κB and pCMV-β-gal by using the Lipofectamine Plus reagent (Invitrogen Inc.), according to the manufacturer’s protocol. Briefly, a transfection mixture containing 0.5 μg pGL3-NF-κB and 0.2 μg pCMV-β-gal was mixed with the Lipofectamine Plus reagent (Invitrogen Inc.) and added to the cells. After 18 h, procyanidin C1 was added to each well and incubated. Untreated cells (designated “normal”) were used as controls. The luciferase and β-galactosidase activities were determined according to a previously described protocol[29]. The luciferase activity was normalized with respect to the β-galactosidase activity, and the results are expressed relative to the activity of the control.

2.9. Statistical analysis

All experiments were repeated ≥3 times with consistent results. Means and standard deviations for results were calculated using the SPSS software version 10.0 (2000). Differences among the mean values were examined by the Student’s two-tailed t-test. Results are expressed as the mean ± SEM. Values of p<0.05, p<0.01, and p<0.001 were considered statistically significant.

3. Results

3.1. Proliferative effect of procyanidin C1

To determine the concentration of procyanidin C1 to be used in all of the following experiments, we first investigated the proliferative effect of procyanidin C1 on BMDMs by using concentrations ranging from 25 to 200 μg/mL. In a medium supplemented with 10% FBS, procyanidin C1 significantly inhibited cell proliferation at a concentration ≥150 μg/mL compared to the control group, whereas treatment with a lower concentration did not have a cytotoxic effect (Fig. 2A). Accordingly, procyanidin C1 was used at a concentration of 100 μg/mL or less in all of the following experiments.

3.2. Effect of procyanidin C1 on iNOS-mediated NO production

Treatment of BMDMs with LPS induced the production of high levels of NO, which could be significantly inhibited by procyanidin C1 treatment (Fig. 2B). Moreover, treatment of BMDMs with procyanidin C1 also inhibited LPS-induced iNOS expression (Fig. 2C). Taken together, these data show that procyanidin C1 is capable of regulating LPS-induced macrophage activation.

3.3. Pro-inflammatory cytokines production

We next measured the ability of procyanidin C1 to modulate macrophage production of inflammatory cytokines. As shown in

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**Fig. 2** Effect of procyanidin C1 on the metabolic activity of LPS-induced bone marrow derived macrophages (BMDMs). (A) Dose-dependent effect of procyanidin C1 (25–100 μg/mL) on proliferation was analyzed using the EZ-Cytox Cell Viability Kit. (B) Dose-dependent effect of procyanidin C1 (25–100 μg/mL) on NO production was analyzed by a Griess reagent assay. The results are expressed as mean ± SEM (n = 6). Significant differences between control and procyanidin C1 groups were evaluated by unpaired Student’s t-test. N.S. denotes no significance. *p<0.05; **p<0.01; ***p<0.001 vs. LPS group. (C) Expression of iNOS induced by procyanidin C1 (50 and 100 μg/mL) treatment of BMDMs for 1 h. The relative band intensity of each protein is expressed. Data are shown as mean ± SEM (n = 3), and statistical significance (*p<0.05) is indicated for LPS-only treatment versus the LPS plus procyanidin C1 treatment.
Fig. 3A and B, treatment with procyanidin C1 (50 and 100 μg/mL) significantly decreased LPS-induced production of both TNF-α and IL-6 compared to that in BMDMs treated with LPS alone, which strongly suggests that procyanidin C1 can modulate inflammation by suppressing the production of pro-inflammatory cytokines by macrophages.

3.4. Effect of procyanidin C1 on MHC class II, CD80, and CD86 expression

To assess the ability of procyanidin C1 to modulate inflammation, we used a well-established model of inflammatory signaling: LPS-induced activation of BMDMs [28]. BMDMs showed up-regulation of MHC class II, CD80, and CD86 protein expression in response to LPS, and the expression of these molecules was significantly reduced in BMDMs treated with 50 or 100 μg/mL procyanidin C1 (Fig. 4).

3.5. Effect of procyanidin C1 on COX-2/PGE2 signaling

Previous studies have shown that the COX-2/PGE2 pathway controls the production of pro-inflammatory cytokines by macrophages in response to LPS stimulation [19], so we next measured the effect of procyanidin C1 treatment on this pathway. PGE2 levels were significantly lower in cell culture supernatants from LPS-induced BMDMs that were treated with procyanidin C1 than in BMDMs treated with LPS alone (Fig. 3C). The LPS-induced expression of COX-2 (but not COX-1) was significantly lower in procyanidin C1-treated BMDMs than in untreated controls (Fig. 5A).

3.6. Effect of procyanidin C1 on NF-κB and MAPK signaling

The activation of NF-κB signaling is a critical step in the induction of many genes involved in inflammation, and we next determined whether this pathway was influenced by procyanidin C1. The LPS-induced degradation of IκB-α was lower in procyanidin C1-treated BMDMs than in the untreated controls (Fig. 5B). Moreover, LPS-induced luciferase activity in RAW264.7 cells that were transiently transfected with an NF-κB-dependent luciferase construct and treated with procyanidin C1 was significantly lower than that in RAW264.7 cells expressing the construct and treated with LPS alone (Fig. 5D). MAPKs, including ERK-1/2 and p38, are directly involved in the activation of NF-κB. Phosphorylation of both ERK-1/2 and p38 was significantly lower in LPS-stimulated BMDMs treated with 50 or 100 μg/mL of procyanidin C1 than in BMDMs treated with LPS alone (Fig. 5C). Ultimately, the results of the present study show that procyanidin C1 inhibits LPS-stimulated TLR4 signaling, leading to MAPK and NF-κB-mediated signaling (Fig. 5A).

4. Discussion

The inflammatory response, which can be triggered by potential injurious agents such as invading bacteria, viruses, and other pathogens, underlies a wide variety of pathological processes [30,31]; however, the complex regulatory networks that coordinate inflammation have not yet been established. Procyanidins are powerful antioxidants with potent anti-inflammatory properties in both in vitro and in vivo models of inflammation [27,32]. However, the mechanisms by which procyanidins regulate inflammation are still poorly understood (Fig. 6). Primarily, to determine the maximum threshold concentration of procyanidin C1 required for all experiments, the effect of procyanidin C1 on proliferation of BMDMs was investigated in a concentration-dependent manner, which induced that procyanidin C1 (≤100 μg/mL) did not induce cell cytotoxicity and was used in all the following experiments (Fig. 2A). Macrophages play an important role in host defense against bacterial infection, and they are...
major cellular targets for LPS-induced stimulation of inflammatory mediator production [8]. In this study, LPS strongly enhanced the level of iNOS-mediated NO production in BMDMs, and this was significantly inhibited by procyanidin C1 (Fig. 2B and C). In a previous study, Chang et al. [33] reported that inflammation induced by LPS in macrophages was mediated by NO production via activation of the COX-2, PGE2, protein kinase A, p38 MAPK, and NF-κB pathways. Additionally, in the NOS family, iNOS is particularly involved in the pathological overproduction of NO, which is known to play a central role in inflammatory action [20,33].

Pro-inflammatory cytokines such as IL-6 and/or TNF-α also play an important role in the regulation of the inflammatory response, and these signaling molecules have been shown to be associated with the induction of inflammatory mediators in various cells [9,10]. This finding is consistent with our results that procyanidin C1 significantly decreased pro-inflammatory cytokine production (TNF-α and IL-6) in LPS-induced BMDMs (Fig. 3A and B). Previous studies have shown that COX-2 and PGE2 control the production of pro-inflammatory cytokines in LPS-induced macrophages [19]. In agreement with the above observations, we reported that the inhibition of LPS-induced pro-inflammatory cytokines is critically involved in the COX-2 and PGE2 pathways by demonstrating that procyanidin C1 suppressed the LPS-induced COX-2 and PGE2 expression in a time-dependent manner (Figs. 3C and 5A). Several other groups have suggested that inhibition of the NF-κB and MAPK pathways has 2 major mechanisms underlying the attenuation of LPS-induced inflammatory cytokine production [53,34]. Terra et al. [35] reported that procyanidin B1 and C1 effectively reduced the inflammation induced by LPS through the inhibition of IKKβ and ERK activation in the human monocyte cell line THP-1. Zang et al. [36] also reported that the anti-inflammatory activity of procyanidin B2 functions through down-regulation of COX-2 expression in THP-1 cells. However, to date, there has been no investigation of the anti-inflammatory action of procyanidin C1 mediated through TLR4 in primary cultured BMDMs. LPS regulates iNOS, PGE2 and COX-2 expression through a MAPK (p38 and ERK1/2) signaling pathway, leading to NF-κB activation in macrophages [12,13,18]. NF-κB is a critical regulator of many genes involved in inflammatory processes and is bound to IκB proteins, which prevent NF-κB from activating the transcriptional machinery [14,37]. Potent activators, such as TNF-α, IL-1, or LPS, induce rapid degradation of the IκBα within a few minutes [38,39], and this finding is in agreement with our result that procyanidin C1 inhibited the degradation of IκBα (Fig. 5B). Previous studies have demonstrated that activation of LPS-stimulated TLR4 ultimately leads to MAPK- and NF-κB-mediated production of pro-inflammatory cytokines (e.g., IL-6 and TNF-α), as well as an increase in surface molecule expression (e.g., CD80, CD86, and MHC class II) in macrophages [22,23,40,41]. In our study, procyanidin C1 remarkably inhibited the phosphorylation of MAPKs (ERK1/2 and p38) and the as NF-κB-dependent luciferase activity induced by LPS (Fig. 5C, and D). Procyanidin C1 markedly inhibited the expression of surface molecules (CD80, CD86, and MHC class II). Taken together, these results suggest that procyanidin C1 has a potential anti-inflammatory effect through the inhibition of the NF-κB and MAPK signaling pathways.

In conclusion, our findings show that procyanidin C1 can effectively inhibit the activation and production of a number of inflammatory mediators by macrophages in response to LPS by interfering with signal transduction directly downstream of LPS.
Thus, procyanidin C1 is a potential novel therapeutic agent for the treatment of inflammatory disease, and we recommend further in vitro and in vivo studies to elucidate the mechanism(s) underlying the anti-inflammatory activity of this compound.

**Disclosure statement**

The authors declare that there are no conflicts of interest.

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**Fig. 5.** Effect of procyanidin C1 on the LPS-induced activation of MAPKs and NF-κB signaling. Lysates from LPS-induced bone marrow-derived macrophages (BMDMs) treated with procyanidin C1 (50 and 100 μg/mL) were subjected to SDS-PAGE, and immunoblot analysis was performed using antibodies specific to phospho-p38 (p-p38), p38, phospho-ERK1/2 (p-ERK1/2), ERK1/2, COX-1/2 and IκB-α. (A) COX-1/2 and TLR4; (B) degradation of IκB-α; (C) p-p38 and p-ERK1/2. The relative band intensity of each protein is expressed. Data are shown as mean±SEM (n = 3), and statistical significance (⁎p < 0.05, ⁎⁎p < 0.01) is indicated for LPS-only treatment versus the LPS plus procyanidin C1 treatment. (D) Effect of procyanidin C1 treatment on NF-κB-dependent luciferase gene expression in RAW 264.7 macrophage cells. The luciferase activity was normalized to the β-galactosidase activity and is expressed relative to the activity of LPS alone. The results are expressed as mean±SEM.

**Fig. 6.** Proposed mechanism of procyanidin C1 on signaling pathways.