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RESEARCH PAPER

Potent anti-inflammatory effect of a novel furan-2,5-dione derivative, BPD, mediated by dual suppression of COX-2 activity and LPS-induced inflammatory gene expression via NF-κB inactivation

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BACKGROUND AND PURPOSE

We previously reported that 3-(benzo[*d*]-1,3-dioxol-5-yl)-4-phenylfuran-2,5-dione (BPD) showed strong inhibitory effects on PGE_2 production. However, the exact mechanism for the anti-inflammatory effect of BPD is not completely understood. In this study, we investigated the molecular mechanism involved in the effects of BPD on inflammatory mediators in LPS-stimulated macrophages and animal models of inflammation.

EXPERIMENTAL APPROACH

The expressions of COX-2, inducible NOS (iNOS), TNF-α, IL-6 and IL-1β, in LPS-stimulated RAW 264.7 cells and murine peritoneal macrophages, were determined by Western blot and/or qRT-PCR, respectively. NF-κB activation was investigated by EMSA, reporter gene assay and Western blotting. Anti-inflammatory effects of BPD were evaluated *in vivo* in carrageenan-induced paw oedema in rats and LPS-induced septic shock in mice.

KEY RESULTS

BPD not only inhibited COX-2 activity but also reduced the expression of COX-2. In addition, BPD inhibited the expression of iNOS, TNF-α, IL-6 and IL-1β at the transcriptional level. BPD attenuated LPS-induced DNA-binding activity and the transcription activity of NF-κB; this was associated with a decrease in the phosphorylation level of inhibitory κB-α (IκB-α) and reduced nuclear translocation of NF-κB. Furthermore, BPD suppressed the formation of TGF-β-activated kinase-1 (TAK1)/TAK-binding protein1 (TAB1), which was accompanied by a parallel reduction of phosphorylation of TAK1 and IκB kinase (IKK). Pretreatment with BPD inhibited carrageenan-induced paw oedema and LPS-induced septic death.

CONCLUSION AND IMPLICATIONS

Taken together, our data indicate that BPD is involved in the dual inhibition of COX-2 activity and TAK1-NF-κB pathway, providing a molecular basis for the anti-inflammatory properties of BPD.

Abbreviations

AP-1, activator protein-1; IKK, inhibitor of κB kinase; iNOS, inducible NOS; IκB, inhibitor of κB; PGN, peptidoglycan; TAB, TAK-binding protein; TAK1, transforming growth factor β activated kinase-1; TLR, toll-like receptor

Introduction

Prolonged inflammation contributes to the pathogenesis of many inflammatory diseases, such as, bronchitis (Vernooy *et al.*, 2002), inflammatory bowel disease (Fichtner-Feigl *et al.*, 2005) and rheumatoid arthritis (Akaogi *et al.*, 2006). Thus, massive resources have been dedicated to the development of anti-inflammatory drugs. Furthermore, the inhibition of PG synthesis is at the centre of current anti-inflammatory therapies, and COX is the key enzyme for prostaglandin biosynthesis. There is accumulating evidnece indicate that COX-2 is involved in many inflammatory processes and induced in various carcinomas, which suggests that COX-2 plays a key role in inflammation and tumourigenesis (Mutoh *et al.*, 2006; Fujimura *et al.*, 2007). Thus, direct inhibition of the enzyme activities of COX-2 has also been actively pursued as another efficient pharmacological approach to treat inflammation, as exemplified by the active development of COX-2 inhibitors such as celecoxib (Deeks *et al.*, 2002).

New anti-inflammatory drugs are being discovered on the basis of their effects on signal transduction and their efficacies as anti-cytokine agents. NF-κB is one of the pivotal regulators of pro-inflammatory gene expression, and the aberrant regulation of NF-κB activity has been implicated in the pathogenesis of immune deficiencies and disorders, neurodegenerative disorders, autoimmune and inflammatory diseases, ischaemia–reperfusion injury and cancer (Liu and Malik, 2006). NF-κB proteins in cytoplasm are associated with inhibitory proteins known as IκBs, whereas the main active form of NF-κB is a heterodimer composed of p65 and p50 subunits (Li and Verma, 2002). NF-κB induction in response to pro-inflammatory stimuli involves the phosphorylation of IκBs at two critical serine residues (Ser^{32}, Ser^{36}) by the inhibitor of κB kinase (IKK) signalosome complex. After IκB has been phosphorylated, it is ubiquitinated and degraded by 26S proteosome (Basak and Hoffmann, 2008), and the resulting free NF-κB translocates to the nucleus, where it binds to κB binding sites in the promoter regions of target genes and induces the transcriptions of pro-inflammatory mediators, such as iNOS, COX-2, TNF-α and IL-1β, IL-6 and IL-8 (Lappas *et al.*, 2002).

Recent studies indicate that LPS binds to toll-like receptor 4 (TLR4) in the cellular membrane and activates a TLR4-mediated NF-κB pathway (Doyle and O'Neill, 2006; Gloire *et al.*, 2006). Although the molecular events are not fully understood in LPS signalling, TAK1 and IKKs are thought to be the two important factors. In unstimulated conditions, TAK1 binds to TNF receptor-associated factor 6 (TRAF6) together with TAB, forming a complex of TRAF6/TAK1/TAB1/TAB2 in the membrane (Doyle and O'Neill, 2006; Wang *et al.*, 2001). Phosphorylation of TAK1 initiates the release of the complex from the membrane, and then phosphorylates downstream targets such as the IKKs and MAPKs (Wang *et al.*, 2001; Shim *et al.*, 2005). Once activated, IKKs subsequently induce phosphorylation and degradation of IκBs (Hacker and Karin, 2006).

Diarylheterocycles and other central ring pharmacophore templates have attracted much attention as potential selective COX-2 inhibitors. Accordingly, we designed a novel 1H furan-2,5-dione derivative, 3-(benzo[*d*]-1,3-dioxol-5-yl)-4-phenylfuran-2,5-dione (BPD), from the vast library of synthetic analogues, and we previously demonstrated that BPD selectively inhibits the production of PGE_2 (Moon *et al.*, 2010). The chemical structure of BPD is shown in Figure 1A. As a part of our ongoing screening programme to evaluate the anti-inflammatory potentials of new compounds, we investigated the molecular mechanism by which BPD produces its anti-inflammatory properties in activated macrophages and in murine animal models.

Methods

Material

The BPD used for this study was synthesized as previously described (Moon *et al.*, 2010) and was >98% pure by LC-MS. Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, USA). Antibodies against iNOS, COX-2, p65, p50, PARP, p-IκB-α, IκB-α and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p-IKK, IKK, p-TAK1, TAK1 and TAB1 monoclonal antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). HRP-conjugated anti-mouse, anti-rabbit and anti-goat Ig were purchased from Jackson Immunoresearch (West Grove, PA, USA). The enzyme immunoassay (EIA) kits for PGE_2, TNF-α, IL-1β and IL-6 were obtained from R&D Systems (Minneapolis, MN, USA). Random oligonucleotide primers and M-MLV reverse transcriptase were purchased from Promega (Madison, WI, USA). SYBR green ex Taq was obtained from TaKaRa (Shiga, Japan). iNOS, COX-2, TNF-α, IL-6, IL-1β and β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sulphanilamide, aprotinin, leupeptin, PMSF, dithiothreitol (DTT), $_L$-N6-(1-iminoethyl)lysine ($_L$-NIL), NS-398, LPS (*Escherichia coli*, serotype 0111:B4), poly(I:C),

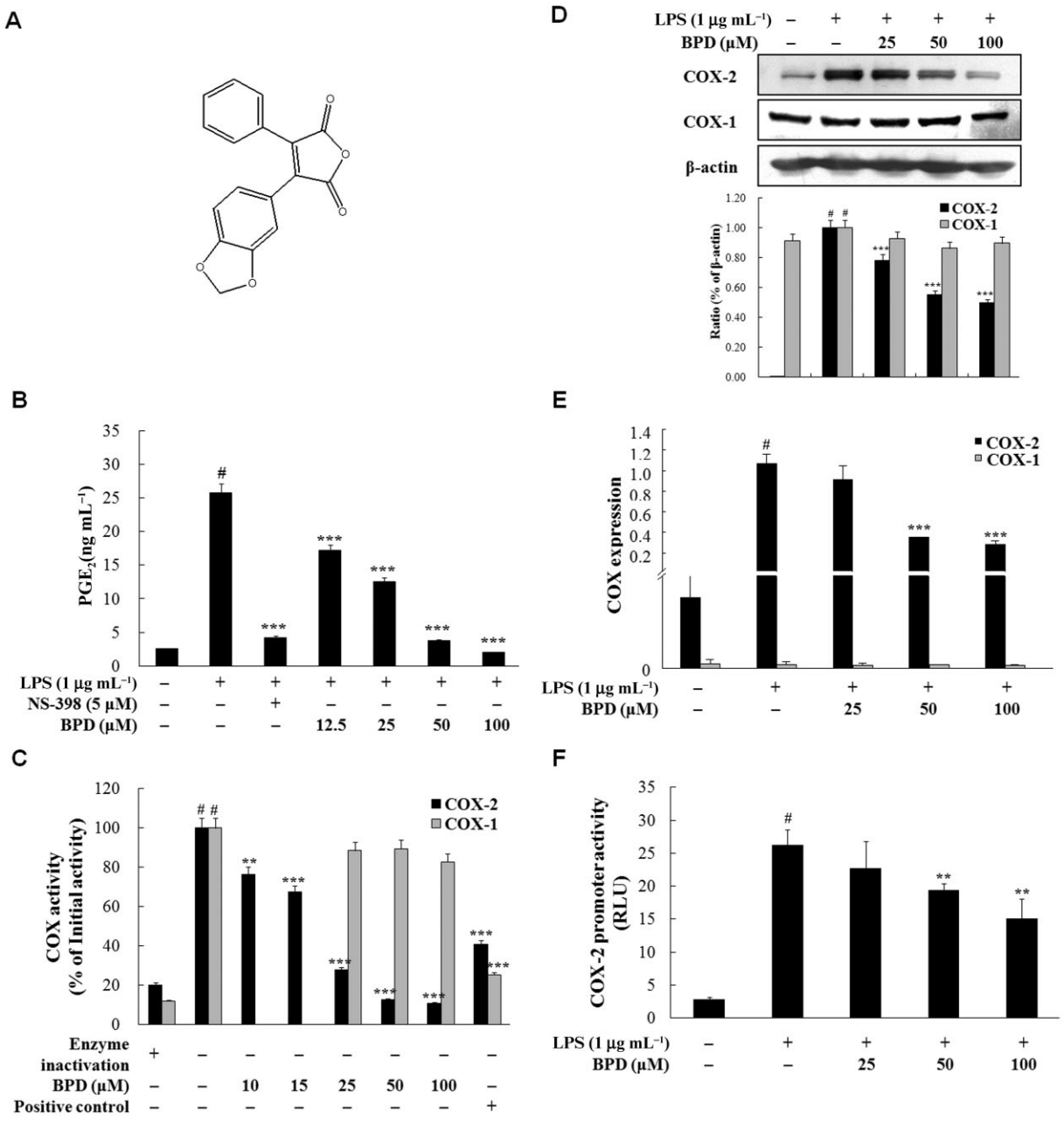

Figure 1

Effects of BPD on the PGE_2 production, COX-2 enzyme activity and COX-2 expression in RAW 264.7 cells. (A) The chemical structure of BPD. (B) Cells were pretreated with/without the indicated concentrations of BPD for 1 h and then stimulated with LPS (1 μg·mL) for 24 h. Detection of PGE_2 were measured by an EIA as described in Methods. NS-398 (5 μM) was used as a positive control to inhibit PGE_2 production. Controls were not treated with LPS or BPD. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{***}P < 0.001$ versus the LPS-induced group; significant differences between groups were determined by ANOVA and Dunnett's *post hoc* test. (C) Recombinant COX-1 or COX-2 enzyme was treated *in vitro* with the indicated concentrations of BPD for 10 min. As a negative control, these enzymes were inactivated by boiling for 3 min. SC-560 (1 μM) and Dup-697 (100 nM) were used as positive COX-1 and COX-2 inhibitor controls, respectively. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the negative control group; $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-induced group; significant differences between groups were determined by ANOVA and Dunnett's *post hoc* test. (D, E) Lysates or total RNA were prepared from cells pretreated with/without the indicated concentrations of BPD for 1 h and then with LPS (1 μg·mL^{-1}) for 24 h or 4 h. The protein and mRNA levels of COX-1 and COX-2 were determined by Western blot and qRT-PCR, respectively, as described in Methods. Controls were not treated with LPS or BPD. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{***}P < 0.001$ versus the LPS-induced group; significant differences between groups were determined by ANOVA and Dunnett's *post hoc* test. (F) Cells were transfected with a pGL3-COX-2 promoter vector and the phRL-TK vector as an internal control. Luciferase activity levels were determined as described in Methods. Controls were not treated with LPS or BPD. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{**}P < 0.01$ versus the LPS-induced group; significant differences between groups were determined by ANOVA and Dunnett's *post hoc* test.

peptidoglycan (PGN), carrageenan lambda, ibuprofen, Triton X-100 and all other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture and sample treatment

The RAW 264.7 macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown at 37°C in DMEM medium supplemented with 10% FBS, penicillin (100 U·mL^{-1}) and streptomycin sulphate (100 μg·mL^{-1}) in a humidified 5% CO_2 atmosphere. Murine peritoneal macrophages were elicited by i.p. injection of 2 mL of 5% thioglycollate into the peritoneal cavity of 6–10 weeks old C57BL/6 mice. After 4 days, cells in the peritoneal exudates were obtained by lavage with ice-cold DMEM. The cells were washed twice, re-suspended in HEPES-buffered DMEM (supplemented with $NaHCO_3$, 10% FBS and antibiotics, namely 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin sulphate) and were seeded in sterile disposable culture plates. Cells were pre-incubated with the tested samples at increasing concentrations (25, 50, 100 μM) or reference drugs for 1 h and then stimulated with LPS (1 μg·mL^{-1}) for the indicated times.

MTT assay for cell viability

RAW 264.7 cells were plated at a density of 10^5 cells·per well in 96-well plates. To determine the appropriate concentration not toxic to cells, cytotoxicity studies were performed 24 h after treating cells with various concentrations of BPD. Viabilities were determined using colorimetric MTT assays, as described previously (Won *et al.*, 2006).

PGE_2, TNF-α, IL-6 and IL-1β assays

RAW 264.7 cells were pretreated with BPD for 1 h and then stimulated with LPS (1 μg·mL^{-1}) for 24 h. Levels of PGE_2, TNF-α, IL-6 and IL-1β in the culture media were quantified using EIA kits (R&D Systems).

COX activity assay

BPD was evaluated for its potency and selectivity of inhibition *in vitro* using COX Inhibitor Screening Assay (Cayman, MI, USA). Recombinant COX-1 (ovine) or COX-2 (human) proteins were pre-incubated with BPD for 10 min at 37°C. The reaction was started by the addition of 100 μM arachidonic acid and allowed to proceed for 2 min. The reaction was terminated by addition of HCl solution containing $SnCl_2$. The COX activity assay directly measures $PGF_{2\alpha}$ produced by $SnCl_2$ reduction of COX-derived PGH_2. The prostanoid product is quantified via EIA. As control inhibitors for COX-1 or COX-2, SC-560 (1 μM) or Dup-697 (100 nM) were used.

Western blot analysis

Control and BPD-treated RAW 264.7 cells were collected by centrifugation and washed twice with PBS. Washed cell pellets were re-suspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM PMSF, 0.5 mM DTT, 5 mM NaF and 0.5 mM sodium orthovanadate) containing 5 μg·mL^{-1} of leupeptin and aprotinin, and incubated for 20 min at 4°C. Cell debris was removed by microcentrifugation, and supernatants were rapidly frozen. Protein concentrations were determined using Bio-Rad protein assay reagent, according to the manufacturer's instruction. Forty micrograms of cellular proteins from treated or untreated cell extracts was separated on 10% or 7% SDS-PAGE onto a PVDF, which was incubated for 1 h with blocking solution at 4°C, and then with primary antibody overnight. Blots were then washed four times with Tween 20 / Tris-buffered saline (TBS/T), incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, rewashed three times with TBS/T and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, USA).

Quantitative real-time RT-PCR (qRT-PCR)

Total cellular RNA was isolated by Easy Blue® kits (Intron Biotechnology, Seoul, Korea). From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM deoxyribonucleotide triphosphate (dNTP) and oligo ($dT_{12\text{-}18}$) 0.5 μg·μL^{-1}. PCR amplification was performed using the incorporation of SYBR green. The oligonucleotide primers for iNOS, designed from mouse (NM_010927), were CATGCTACTGGAGGTGGGTG (forward) and CATTGATCTCCGTGACAGCC (reverse); for TNF-α, designed from mouse (NM_013693.2), were AGCACA GAAAGCATGATCCG (forward) and CTGATGAGAGG GAGGCCATT (reverse); for IL-6 (NM_031168) were GAGGATACCACTCCCAACAGACC (forward) and AAGTG CATCATCGTTGTTCAT ACA (reverse); for COX-2, designed from mouse (NM_011198), were TGCTGTACAAG CAGTG GCAA (forward) and GCAGCCATTTCCTTCTCTCC (reverse); for IL-1β, designed from mouse (NM_008361), were ACCT GCTGGTGTGTGACGTT (forward) and TCGTTGCTTGGT TCTCCTTG (reverse); and the suitable size of synthesized cDNA were 209, 220, 142, 149 and 187 bp respectively. The oligonucleotide primers for β-actin used as a housekeeping gene designed from mouse (NM_007393) were ATCACTAT TGGCAACGAGCG (forward) and TCAGCAATGCCTGGGTA CAT (reverse), and the suitable size of synthesized cDNA was 200 bp. Steady-state mRNA levels of iNOS, TNF-α, IL-6, COX-2, IL-1β and β-actin were determined by real-time qPCR using the Takara thermal cycler dice® (Takara Bio Inc., Shiga, Japan). The results are expressed as the ratio of optimal density to β-actin.

Transient transfection and luciferase assay

The mouse COX-2 promoter plasmid (pGL3-COX-2;-965/+39) and iNOS promoter plasmid (pGL3-iNOS; –1592/+185) were prepared as described previously (Lowenstein *et al.*, 1993; Kraemer *et al.*, 1996). RAW 264.7 cells were co-transfected with pGL3-COX-2, pGL3-iNOS, NF-κB-Luc or AP-1-Luc reporter plasmid vector plus the phRL-TK plasmid (Promega) using Lipofectamine LTX™ (Invitrogen, Carlsbad, CA, USA) as instructed by the manufacturers. After 4 h of transfection, cells were pretreated with BPD for 1 h and then stimulated with LPS (1 μg·mL^{-1}) for 18 h. Each well was washed with cold-PBS, and cells were lysed and the luciferase activity was determined using the Promega luciferase assay system (Promega).

Nuclear extraction and EMSA

RAW 264.7 cells were plated in 100 mm dishes (1×10^6 cells·mL^{-1}). The cells were treated with various BPD

concentrations (25, 50, 100 μM), stimulated with LPS for 1 h, washed once with PBS, scraped into 1 mL of cold PBS and pelleted by centrifugation. Nuclear extracts were prepared as described previously, with slight modification (Won *et al.*, 2006). Nuclear extract 5 μg was mixed with the double-stranded NF-κB and AP-1 oligonucleotide, 5′-AGTTGAGG GGACTTTCCCAGGC-3′, and 5′-CGCTTGATGACT-CAGCC GGAA-3′, respectively, and end-labelled by [γ-^{32}P]-dATP. The specificity of binding was examined by competition with the 80-fold unlabeled oligonucleotide. DNA–protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5 × TBE buffer. The gels were vacuum-dried for 1 h at 80°C and exposed to X-ray film at –70°C for 24 h.

Immunocytochemistry

RAW 264.7 cells were pretreated without or with BPD (100 μM) for 1 h and then treated with LPS (1 μg·mL^{-1}) in the presence or absence of BPD, and then fixed with 100% methanol for 30 min. The cells were incubated with 0.1% Triton X-100 for 30 min then blocked with 1% NGS for 1 h. Cells were probed with mouse anti-p65 antibody (Santa Cruz Biotechnology; diluted 1:100) overnight at 4°C, followed by goat anti-rabbit 594® Alexa-conjugated secondary antibody (Invitrogen, Eugene, OR; diluted 1:100) 1 h at 37°C, washed with PBS three times and then mounting with mounting medium containing. NF-κB p65 subunit was observed with a microscope.

Immunoprecipitation

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl, 50 mM NaCl, 0.5% NP-40, 0.25% sodium deoxycholate, 0.05% SDS, 0.5 mM EDTA, pH 7.4) containing 1 mM PMSF and 1 × protease inhibitor cocktail by sonication. After removal of debris, 2 μg of anti-TAK1 antibodies and 20 μL of PureProteome™ protein A magnetic beads (Millipore, Billerica, MA, USA) were added to extracts and incubated overnight at 4°C. After centrifugation, beads were collected using a magnetic separation rack and then washed four times with RIPA buffer. Bound proteins were subjected to SDS-PAGE and then analysed by Western blotting.

Nitrite determination

RAW 264.7 cells were plated at 5 × 10^5 cells per well in 24-well plates and then incubated with or without LPS (1 μg·mL^{-1}) in the absence or presence of various concentrations (25, 50, 100 μM) of BPD for 24 h. Nitrite levels in culture media were determined using the Griess reaction and presumed to reflect NO levels (Won *et al.*, 2006). Absorbance was then measured at 540 nm using a microplate reader. Fresh culture media were used as blanks in all experiments. Nitrite levels in samples were read off a standard sodium nitrite curve.

Animals

All animal care and experimental procedures complied with the Guidelines of the Committee for Animal Care and Use of laboratory animals, College of Pharmacy, Kyung Hee University according to an animal protocol (Approval number # KHP-2009–10-06 and KHP-2010–11-04). C57BL/6 male mice weighing 20–25 g and Sprague–Dawley male rats weighing 180–200 g were purchased from the Orient Bio Inc. (Seongnam-si, Korea) and maintained under constant conditions (temperature: 20 ± 2°C, humidity: 40–60%, light/dark cycle: 12 h) for 2 weeks or more; 12 h before the experiment, only water was provided.

Carrageenan-induced oedema in rats

The initial hind paw volume of the Sprague–Dawley strain of rats was determined volumetrically, a 1% solution of carrageenan in saline (0.1 mL per rat) was injected s.c. into the right hind paws 1 h after the test sample (25 or 50 mg·kg^{-1}) had been administered orally. The test samples were first dissolved in vehicle solution (5% EtOH and 5% Tween 20 and diluted with saline). Paw volumes were measured for up to 5 h after injections, and oedema volumes were measured using a plethysmometer. All animals were killed with CO_2 at 5 h after carrageenan injection, the paw tissues were then removed and immediately frozen (–70°C) for the determination of myeloperoxidase (MPO) activity. Ibuprofen (100 mg·kg^{-1}), an anti-inflammatory drug, was used as a positive control. MPO activity was measured in soft tissue from carrageenan-injected paws, as a marker of neutrophil influx into the tissue. The tissue was thawed and homogenized. The homogenate was then centrifuged at 1500× *g* for 15 min, and the resulting supernatant was assayed for MPO assay using EIA kits (Hycult Biotechnology, The Netherlands). And PGE_2 level in tissue was measured from homogenate using EIA kits (R&D Systems).

Septic shock in mice

Sepsis was induced in C57BL/6 mice (male, 6–8 weeks old) by i.p. injection of LPS (*Salmonella enterica*, 25 mg·kg^{-1}). The mice were administered BPD (25 or 50 mg·kg^{-1}, p.o.) 1 h before the LPS injection. Survival of the mice was monitored over the next 22 h.

Statistical analysis

Results are expressed as the mean ± SD of triplicate experiments. Statistically significant values were compared by ANOVA and Dunnett's *post hoc* test, and *P*-values of less than 0.05 were considered statistically significant.

Results

BPD inhibits the enzyme activity and the expression of COX-2, leading to suppression of LPS-induced PGE_2 production in RAW 264.7 cells

As previously demonstrated (Moon *et al.*, 2010), we observed that BPD acts as an inhibitor of PGE_2 production through COX-2 activity. BPD was found to inhibit LPS-induced PGE_2 production (Figure 1B) and selectively inhibited the COX-2 activity (IC_{50} value = 18.5 μM) with no effect on the COX-1 activity in a concentration-dependent manner (Figure 1C). We further investigated whether the inhibitory effects of BPD on PGE_2 production are related to COX-2 expression via Western blot and qRT-PCR. COX-2 protein and mRNA levels were found to be markedly up-regulated in response to LPS,

and BPD inhibited these responses in a concentration-dependent manner (Figure 1D and E). However, it had no effect on the expressions of COX-1 protein and mRNA in RAW 264.7 cells (Figure 1D and E). Next, we analysed the transcriptional activity of COX-2 gene promoter. As shown in Figure 1F, LPS significantly enhanced the COX-2 promoter activity, and BPD inhibited this increase in a concentration-dependent manner. The inhibitory action of BPD was not due to a cytotoxic effect, because BPD did not affect cell viability, as measured by the MTT assay, at the concentrations (25–100 μM) that suppressed COX-2 protein and mRNA (Moon *et al.*, 2010).

Effects of BPD on the LPS-induced activations of NF-κB and AP-1 in RAW 264.7 cells

Since the activations of NF-κB and AP-1 are critically required for the LPS-induced activation of COX-2 (Hatziieremia *et al.*, 2006; Lin *et al.*, 2007), we examined the effect of BPD on the LPS-induced NF-κB and AP-1-dependent reporter gene assay. Analysis of reporter gene expression using pNF-κB-luc or pAP-1-luc demonstrated that BPD concentration-dependently inhibited NF-κB-dependent luciferase activity, but had no effect on AP-1-dependent luciferase activity (Figure 2A). LPS stimulation resulted in a significant increase in the DNA-binding activities of NF-κB, as determined by EMSA, and BPD attenuated this LPS-induced NF-κB-binding in a concentration-dependent manner (Figure 2B), but not AP-1 (data not shown). In general, the translocation of transcriptional factors into the nucleus is believed to be essentially required for the activations of target genes. Accordingly, we investigated whether BPD prevents the translocations of the subunits of NF-κB (p65 and p50) to the nucleus. We found that pre-treatment with BPD before LPS stimulation concentration-dependently attenuated p65 and p50 translocations from the cytosol to the nucleus (Figure 2C). Immunofluorescence staining analysis confirmed that LPS-induced translocation of p65 into nucleus were reduced by BPD treatment (Figure 2D).

Effects of BPD on the LPS-induced degradation of IκB-α and activation of IKK and TAK in RAW 264.7 cells

In its inactive state, NF-κB binds with its inhibitor protein, IκB-α, in the cytoplasm. However, after cellular stimulation, IκB-α is phosphorylated at specific serine residues and undergoes poly-ubiquitination and proteasomal degradation, which frees NF-κB and allows it to be translocated to the nucleus (O'Connell *et al.*, 1998; Basak and Hoffmann, 2008). Thus, we explored whether BPD inhibits the LPS-induced degradation of IκB-α in RAW 264.7 cells. Figure 3A shows that LPS-induced degradation and phosphorylation of IκB-α were significantly inhibited by BPD pretreatment. Since IKK-α and β are upstream kinases of IκB in the NF-κB signal pathway, we examined the effects of BPD on LPS-induced IKK-α and -β activation in RAW 264.7 cells by Western blot. It was found that BPD markedly reduced LPS-induced IKK-α/β phosphorylation, whereas BPD did not affect the total amounts of IKK-α and -β (Figure 3B).

Furthermore, since protein kinase TAK1 has been implicated in the regulation of IKK-α/β phosphorylation by LPS (Wang *et al.*, 2001), we further investigated the effect of BPD on the LPS-induced phosphorylation of TAK1. RAW 264.7 cells were pretreated with BPD (25, 50 or 100 μM) for 1 h and then stimulated with LPS (1 $\mu g \cdot mL^{-1}$), and as shown in Figure 3B, LPS-induced TAK1 phosphorylation was significantly inhibited by BPD in a concentration-dependent manner. To determine whether BPD affects TAK1 phosphorylation by inhibiting the formation of the TAK1/TABs complex, we performed immnoprecipitation with the anti-TAK1 antibody. Binding of TAK1 to TAB1 was observed after LPS treatment, but binding was significantly reduced after treatment with BPD (Figure 3C), and the level of immunoprecipitated TAK1 remained unchanged by LPS. These findings suggest that BPD suppresses LPS-induced NF-κB activation by down-regulation of the TAB1/TAK1-mediated NF-κB pathway.

Effects of BPD on LPS-induced PGE_2 production and COX-2 protein expression via NF-κB signalling in mouse peritoneal macrophages

To determine whether these anti-inflammatory effects of BPD occur in primary cells as well, we further examined the effects of BPD on LPS-induced PGE_2 production and COX-2 expression in peritoneal macrophages isolated from C57BL/6 mice. Culture media were harvested, and PGE_2 levels were measured. Although the productions of PGE_2 and other cytokines were almost 10 times lower than that seen in the Raw 264.7 cell line, BPD was found to significantly inhibit LPS-induced PGE_2 production (Figure 4A). Furthermore, in these cells, LPS markedly up-regulated the expression of COX-2, and BPD inhibited this in a concentration-dependent manner (Figure 4B). These inhibitory effects of BPD were not caused by a toxic effect as determined by MTT reduction assay (cell viability under these conditions used was >97%). Consistent with our findings in RAW 264.7 cells, BPD suppressed the phosphorylation of TAK1 and consequently translocation and transcriptional activity of NF-κB (Figure 4C and D). These results confirm that BPD also inhibits NF-κB activation by suppressing the TAK1/IKKs/IκBα pathway in LPS-induced primary mouse peritoneal macrophages.

Effects of BPD on TLR-2 and TLR-3 ligands-induced PGE_2 production and COX-2 protein expression in RAW 264.7 cells

To evaluate whether the inhibitory action of BPD is specific to LPS-TLR4 signalling, we exposed cells to other TLRs activators; PGN (a cell wall component of the Gram-positive bacteria), activates TLR2, and synthetic double-stranded RNA, a polyriboinosinic polyribocytidylic acid [poly(I:C)], activates TLR3 (Akira and Takeda, 2004). These TLRs ligands increased PGE_2 production in RAW 264.7 cells, and BPD reduced this PGE_2 production-induced by these ligands in a concentration-dependent manner. NS-398 (5 μM) was used as a positive COX-2 inhibitor (Figure 5A and B). Furthermore, COX-2 protein levels were markedly up-regulated in response to PGN or poly(I:C), and BPD (100 μM) strongly inhibited this up-regulation (Figure 5C). These findings suggest the anti-inflammatory effects of BPD might not be restricted to the TLR4 signal pathway, but it also inhibits the TLR2- and TLR3- pathways in macrophages.

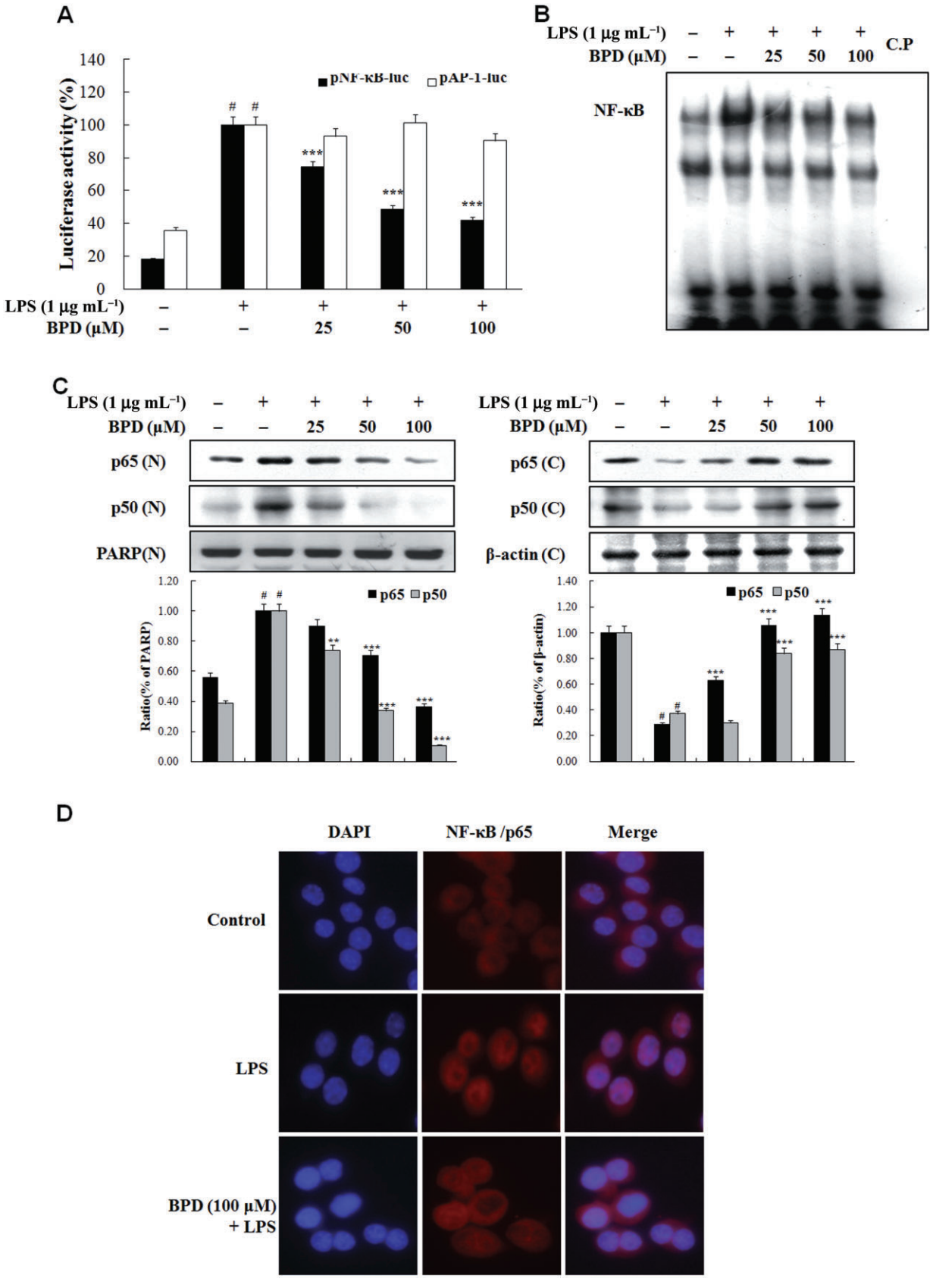
A
Luciferase activity (%)
pNF-κB-luc
pAP-1-luc
LPS (1 μg mL⁻¹)
BPD (μM)
B
LPS (1 μg mL⁻¹)
BPD (μM)
C.P
NF-κB
C
LPS (1 μg mL⁻¹)
BPD (μM)
p65 (N)
p50 (N)
PARP(N)
p65 (C)
p50 (C)
β-actin (C)
Ratio(% of PARP)
Ratio(% of β-actin)
p65
p50
D
DAPI
NF-κB /p65
Merge
Control
LPS
BPD (100 μM) + LPS

Figure 2

Effects of BPD on the LPS-induced activation of NF-κB and AP-1 in RAW 264.7 cells. (A) Cells were co-transfected with pNF-κB-luc or pAP-1-luc reporter with phRL-TK vector. Cells were treated with/without the indicated concentrations of BPD for 1 h and then stimulated with LPS (1 μg·mL^{-1}) for 18 h. Cells were then harvested and luciferase activity levels were determined as described in Methods. Controls were not treated with LPS or BPD. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{***}P < 0.001$ versus the LPS-induced group; significant differences between groups were determined by ANOVA and Dunnett's *post hoc* test. (B) Nuclear extracts were prepared from cells pretreated with/without the indicated concentrations of BPD for 1 h and then with LPS (1 μg·mL^{-1}) for 1 h, and analysed for NF-κB binding by EMSA. The specificity of binding was examined by competition with the 80-fold unlabelled NF-κB oligonucleotide (C.P). The experiment was repeated three times, and similar results were obtained. (C) Cells were pretreated with the indicated concentrations of BPD for 1 h and then stimulated with LPS (1 μg·mL^{-1}) for 1 h. Nuclear (N) and cytosolic (C) extracts were isolated and the levels of p65 and p50 in each fraction were determined by Western blot. PARP and β-actin were used as internal controls. The experiment was repeated three times, and similar results were obtained. (D) p65 localization was assessed under a fluorescence microscope as described in Methods. Cells were pretreated with 100 μM BPD for 1 h and then with LPS (1 μg·mL^{-1}) for 1 h. The data shown are representative of three independent experiments.

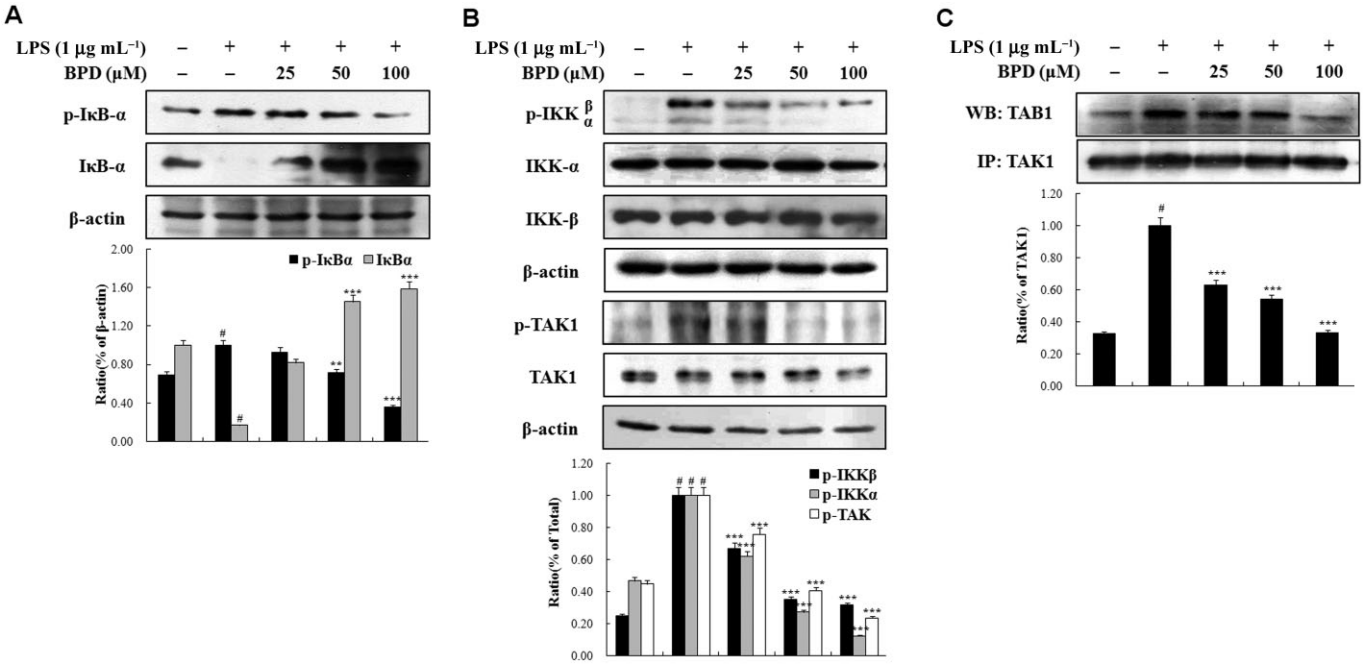

Figure 3

Effects of BPD on the LPS-induced degradation of IκB-α and activation of IKK and TAK in RAW 264.7 cells (A) Cells were pretreated with/without the indicated concentrations of BPD for 1 h and then stimulated with LPS (1 μg·mL^{-1}) for 15 min. Lysates were analysed by Western blot using antibodies against p-IκB-α and IκB-α. The experiment was repeated three times, and similar results were obtained. (B) Cells were pretreated with/without the indicated concentrations of BPD for 1 h and then stimulated with LPS (1 μg·mL^{-1}) for 10 min. Lysates were analysed by Western blot using antibodies against p-IKKα/β, IKKα, IKKβ, p-TAK1 and TAK1. The experiment was repeated three times, and similar results were obtained. (C) Total proteins (500 μg) in whole extracts were prepared from cells pretreated with/without the indicated concentrations of BPD for 1 h and then with LPS (1 μg·mL^{-1}) for 10 min, and analysed by immunoprecipitation. TAK1was immunoprecipitated using anti-TAK1 antibody and then precipitated with protein A-Sepharose beads as described in Methods. The experiment was repeated three times, and similar results were obtained.

BPD suppresses LPS-induced iNOS, TNF-α, IL-6 and IL-1β expression

There is accumulating data indicating that NF-κB activation plays a pivotal role in the LPS-induced production of inflammatory mediators such as NO, TNF-α, IL-6 and IL-1β (Guha and Mackman, 2001; Tian and Brasier, 2003). LPS (1 μg·mL^{-1} for 24 h) markedly increased NO production, but BPD decreased this effect of LPS through inhibition of iNOS at the protein and mRNA expression levels, as determined by Western blotting and qRT-PCR, respectively (Figure 6A, B and D). Moreover, BPD inhibited LPS-induced promoter activity of iNOS in a concentration-dependent manner (Figure 6C). We further examined the effects of BPD on the LPS-induced pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β by EIAs and RT-PCR, respectively, in RAW 264.7 cells. It was found that BPD pretreatment of these cells considerably reduced the LPS-induced productions of TNF-α, IL-6, and IL-1β and their mRNA expressions (Figure 6A and D). We further examined the production of NO and cytokines in LPS-induced primary macrophages pretreated with BPD. BPD considerably reduced LPS-induced productions of NO and TNF-α (Supplementary Figure S1).

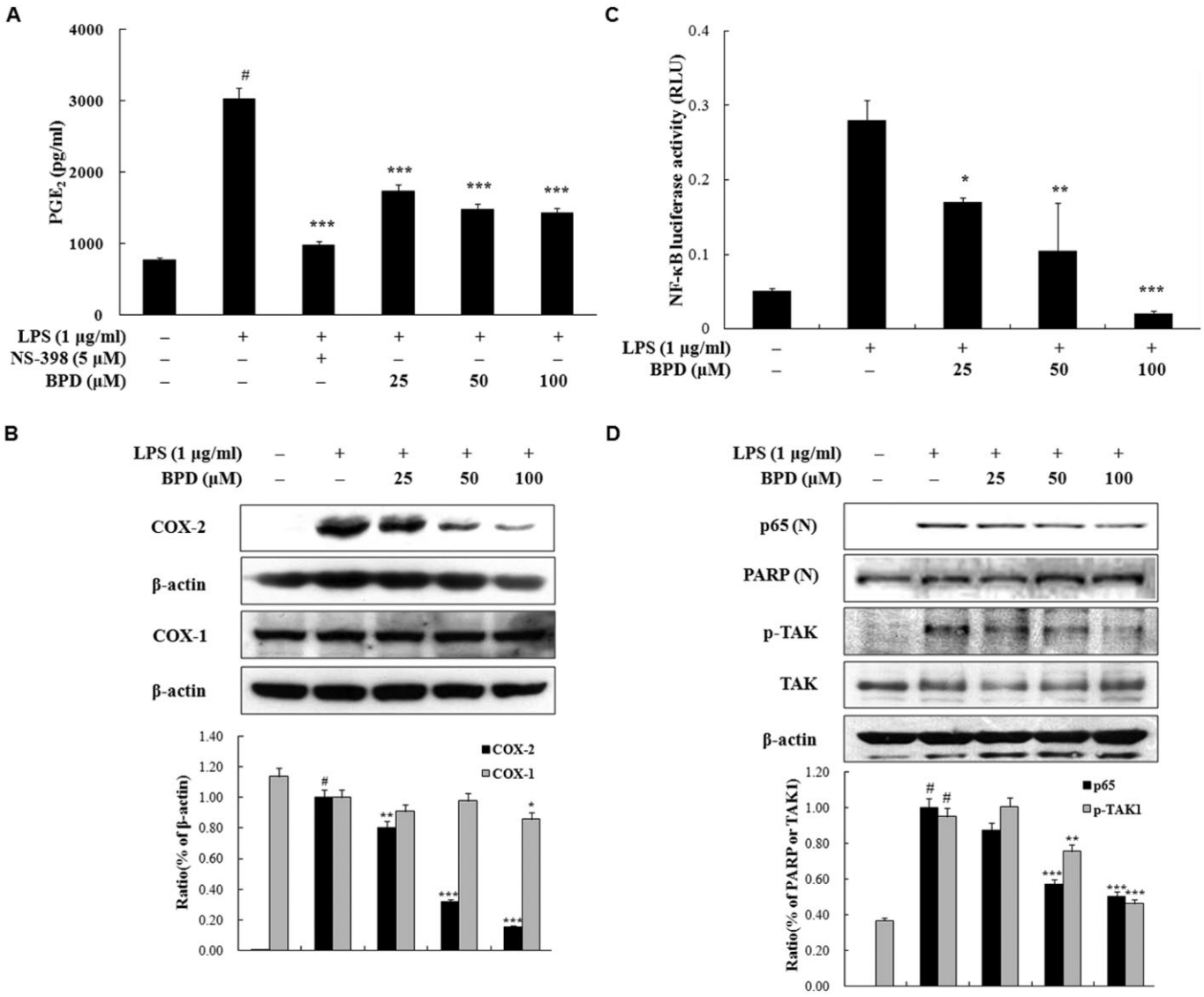

Figure 4

Effects of BPD on LPS-induced PGE_2 production and COX-2 protein expression via NF-κB signalling pathway in mouse peritoneal macrophages (A) Macrophages from 5% thioglycollate-primed C57BL/6 mice were pretreated with/without the indicated concentrations of BPD for 1 h and then stimulated with LPS (1 μg·mL^{-1}) for 24 h. Culture supernatants were collected, and amounts of PGE_2 were determined by an EIA as described in Methods. Controls were not treated with LPS or BPD. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{***}P < 0.001$ versus the LPS-induced group; significant differences between groups were determined by ANOVA and Dunnett's *post hoc* test. (B) Lysates were prepared from primary mouse peritoneal macrophages pretreated with/without the indicated concentrations of BPD for 1 h and then with LPS (1 μg·mL^{-1}) for 24 h. The protein levels of COX-1 and COX-2 were determined by Western blot as described in Methods. The experiment was repeated three times, and similar results were obtained. (C) Cells were transfected with a pNF-κB-luc reporter vector and the phRL-TK vector as an internal control. After 4 h of transfection, cells were pretreated with/without BPD and then stimulated with LPS (1 μg·mL^{-1}) for 18 h. Luciferase activity levels were determined as described in Methods. Controls were not treated with LPS or BPD. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-induced group; significant differences between groups were determined by ANOVA and Dunnett's *post hoc* test. (D) Nuclear (N) or whole extracts were prepared from cells pretreated with/without the indicated concentrations of BPD for 1 h and then with LPS (1 μg·mL^{-1}) for 1 h or 10 min, respectively. Nuclear (N) and whole extracts were isolated and levels of p65 and TAK-1/p-TAK1 in each fraction were determined by Western blot. PARP and β-actin were used as internal controls. The experiment was repeated three times, and similar results were obtained.

The anti-inflammatory effects of BPD in carrageenan-induced paw oedema and LPS-induced septic shock models

The anti-inflammatory effects of BPD were examined in a well-established carrageenan-induced paw oedema animal model. The inhibitory effects of ibuprofen and BPD were observed at 1 h and sustained for 5 h. At 3 h, the inhibitory effects of BPD were 24.7% (25 mg·kg^{-1}) and 38.6% (50 mg·kg^{-1}) compared with the vehicle group (Figure 7A). Neutrophil migration into carrageenan-stimulated rat paws was indirectly determined by means of MPO activity. Injec-

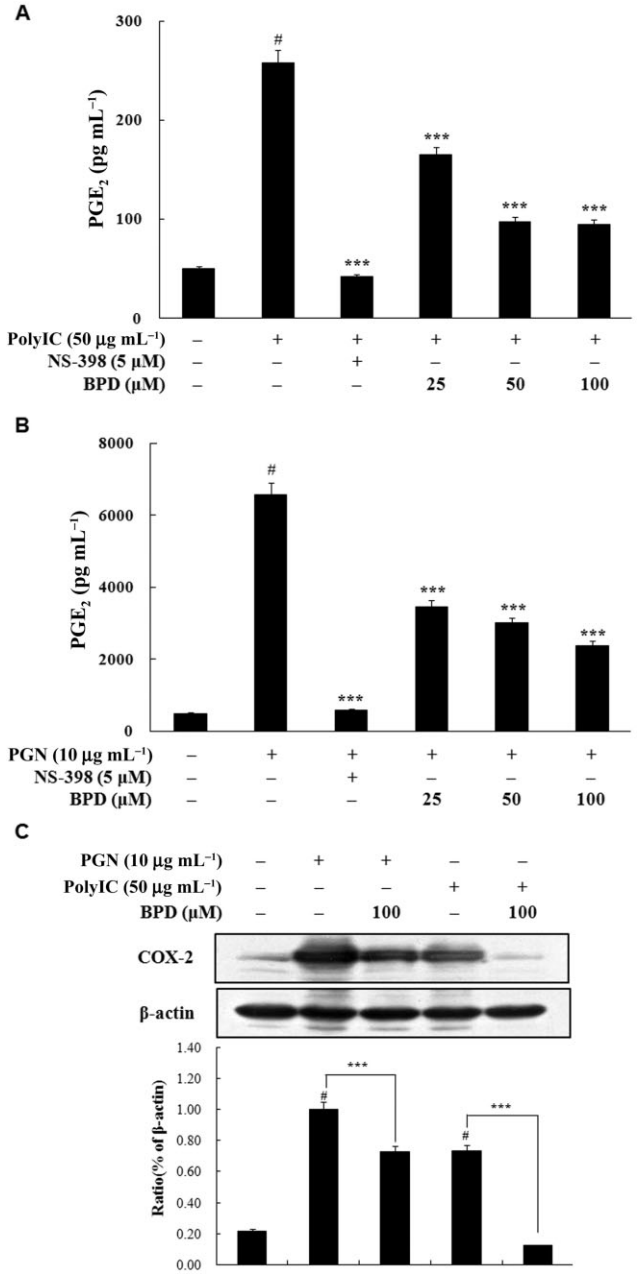

Figure 5

Effects of BPD on the PGE_2 production and COX-2 protein expression by TLR ligands in RAW 264.7 cells. (A, B) Cells were treated with/without the indicated concentrations of BPD for 1 h and then stimulated with PGN (10 μg·mL^{-1}) (A) or poly(I:C) (50 μg·mL^{-1}) (B) for 24 h, and then PGE_2 level was determined by an EIA as described in Methods. NS-398 (5 μM) was used as a positive control to inhibit PGE_2 production. Controls were not treated with PGN, poly(I:C) or BPD. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{***}P < 0.001$ versus the PGN or poly(I:C)-induced group; significant differences between groups were determined by ANOVA and Dunnett's *post hoc* test. (C) Lysates were prepared from cells pretreated with/without the indicated concentrations of BPD for 1 h and then with LPS for 24 h. The protein levels of COX-2 were determined by Western blot as described in Methods. The experiment was repeated three times, and similar results were obtained.

tion of carrageenan into rat paws resulted in an up-regulation of MPO activity in tissue, but treatment with BPD (25 or 50 mg·kg^{-1}, p.o.) prevented the increase in carrageenan-induced MPO activity (11.8% and 56.3%, respectively). Ibuprofen (100 mg·kg^{-1}) also inhibited the MPO activity (66.1%) (Figure 7B). Moreover, changes in PGE_2 production were similar to the observed reductions in MPO activity (Figure 7C). To examine the inhibitory effects of BPD on endotoxin shock induced by LPS, we examined the survival rate of LPS-induced endotoxin shock in mice. In the absence of BPD, all of mice died within 16 h of injection of the LPS (25 mg·kg^{-1}, i.p.). When 50 mg·kg^{-1} BPD was administered 1 h before the injection of LPS, the rate of survival was improved by 60% (Figure 7D).

Discussion

Development of new generations of anti-inflammatory drugs is aimed at enhancing the anti-inflammatory and analgesic activities of classic nonsteroidal anti-inflammatory drugs (NSAIDs) and at reducing the adverse effects of these agents, such as gastrointestinal toxicity, renal dysfunction. Selective COX-2 inhibitors are enthusiastically viewed because they are similar to traditional NSAIDs in terms of efficacy but spare constitutively active COX-1 and are comparatively free of stomach-associated complications. The recent withdrawal of the selective COX-2 inhibitors rofecoxib and valdecoxib because of their adverse cardiovascular side effects demonstrates the necessity for identifying new inhibitors of PGE_2 without the side effects of known agents (Herrero *et al.*, 2003). Despite the undesirable effects of these drugs, they are among the most widely used therapeutic classes of compounds, and COXs and PGE_2 are still attractive drug targets for the relief of pain and inflammation (Tegeder *et al.*, 2001). Thus, the development of drugs that preferentially inhibit NF-κB-activation would appear to provide a more promising route to the amelioration of inflammatory responses via the synergic suppression of COX-dependent PGE_2 production and NF-κB-induced pro-inflammatory mediators, and to the minimization of the adverse effects caused by excessive inhibition of PG production. Furthermore, it was interesting to find that BPD simultaneously suppressed COX-2 enzymic activity and protein synthesis, whereas most known COX-2 inhibitors only target this enzyme's activity.

In a previous study, we constructed a library of novel small 1H furan-2,5-dione-derived analogues and through our screening process identified a novel derivative, BPD, that exhibits strong inhibitory effects on PGE_2 production (Moon *et al.*, 2010). The lack of an observed effect of BPD on COX-1 activity shows that BPD directly and selectively inhibits COX-2 activity (Figure 1C). In addition, we found that the concentration-dependent inhibition of COX-2 protein by BPD was consistent with the suppression of its mRNA and promoter activity (Figure 1D, E and F). Thus, we suggest that the inhibition of PGE_2 production by BPD is due to its inhibition of the enzyme activity and expression of COX-2. However, NS-398, selective COX-2 inhibitor, did not affect COX-2 protein expression, which contrasts with BPD results (Supplementary Figure S2). Thus, the inhibitory effect of BPD

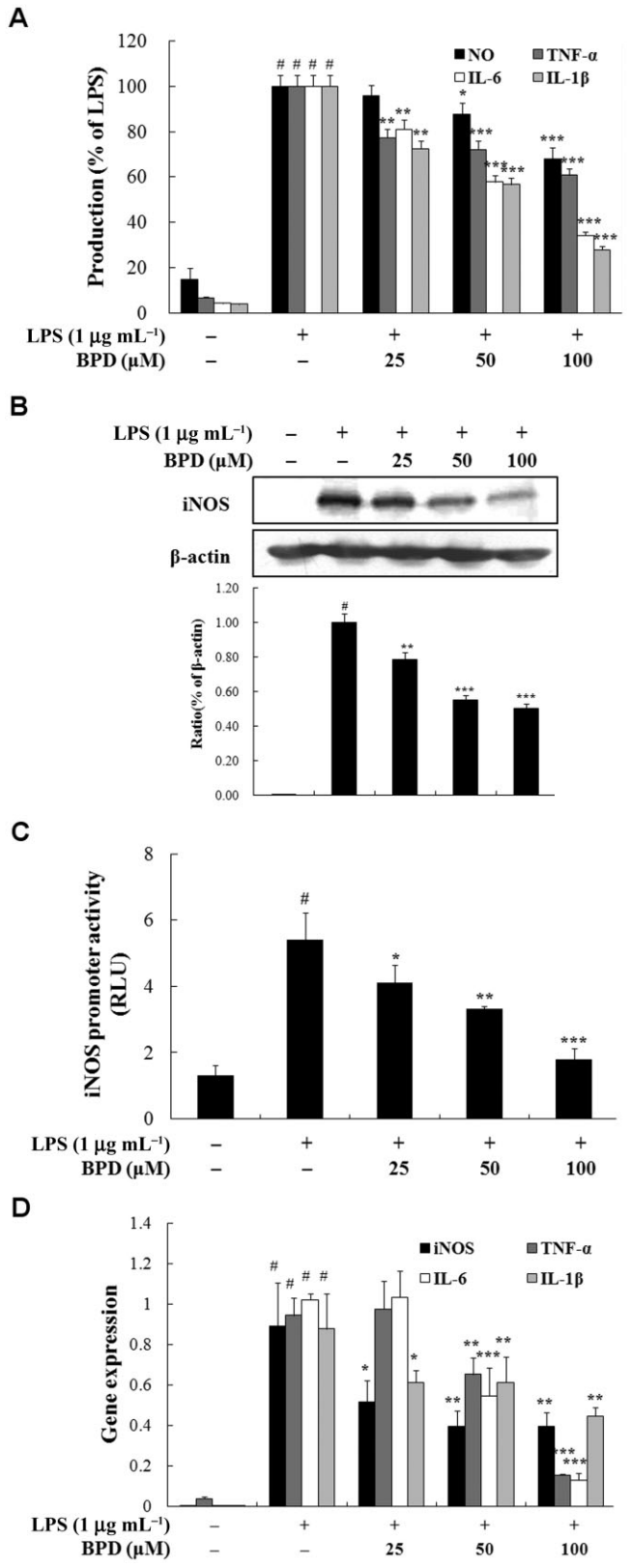

Figure 6

Effects of BPD on LPS-induced NO and cytokine production and their protein and mRNA expressions in RAW 264.7 cells. (A) Cells were pretreated with/without the indicated concentrations of BPD for 1 h and then stimulated with LPS (1 μg·mL^{-1}) for 24 h. Amount of NO was determined using the Griess reaction. The production of TNF-α, IL-6 and IL-1β were determined using EIA kits respectively. Controls were not treated with LPS or BPD. (B) Lysates were prepared from cells pretreated with/without the indicated concentrations of BPD for 1 h and then with LPS for 24 h. The protein levels of iNOS were determined by Western blot as described in Methods. The experiment was repeated three times, and similar results were obtained. (C) Cells were transfected with a pGL3-iNOS promoter vector and the phRL-TK vector as an internal control. Luciferase activity levels were determined as described in Methods. Controls were not treated with LPS or BPD. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-induced group; significant differences between groups were determined by ANOVA and Dunnett's *post hoc* test. (D) Total RNA was prepared from cells pretreated with/without the indicated concentrations of BPD for 1 h and then with LPS for 4 h. The mRNA levels of iNOS, TNF-α, IL-6 and IL-1β were determined by qRT-PCR as described in Methods. Controls were not treated with LPS or BPD. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-induced group.

on COX-2 expression led to the hypothesis that BPD may exhibit COX-2 activity-independent anti-inflammatory effects.

LPS-induced COX-2 transcription is regulated through multiple redundant mechanisms involving several central response elements, such as NF-IL-6, AP-1, NF-κB, CRE and C/EBP, present in the COX-2 promoter (Murakami and Kudo, 2004). EMSA and NF-κB-dependent reporter gene data revealed that BPD inhibited the binding complex of NF-κB–DNA and transcriptional activity in pNF-κB-luc-transfected RAW 264.7 cells but did not affect AP-1 (Figure 2A and B). Western blot results revealed that BPD inhibited LPS-induced phosphorylation and degradation of IκB-α (Figure 3A), and subsequently reduced p65 and p50, subunits of NF-κB, in the nucleus (Figure 2C and D), in a concentration-dependent manner. These data suggest that the inhibition of NF-κB activation by BPD might be the result of the inhibition of IκB-α phosphorylation and degradation, and then reduction of p65 and p50 nuclear translocation in LPS-induced RAW 264.7 cells. However, the activity of AP-1 is controlled by different MAPK cascades. In particular, JNK phosphorylates and thereby activates the transcriptional potential of c-Jun, a critical component of AP-1 (Chang and Karin, 2001). It was found that BPD inhibited the LPS-induced phosphorylations of ERK and p38 but had no effect on the phosphorylation of JNK. These results are consistent with the minimal effects of BPD on AP-1 activation (Supplementary Figure S3).

The activation of TLR4s can eventually lead to the activation of a complex consisting of TAK1 and the adaptor proteins TAB1 (TAK1 activator) and TAB2/3 (ubiquitin-binding protein) and, subsequently, to the activation TAK1 by autophosphorylation (Sakurai *et al.*, 2000; Prickett *et al.*, 2008; Brown *et al.*, 2011). Phosphorylation of TAK1 initiates the release of the complex from the membrane, and the active TAK1 phosphorylates the IKK complex, which in turn phosphorylates IκB for degradation (Adhikari *et al.*, 2007). Our data show that BPD suppresses TAK1-TAB1 complex formation, TAK1 phosphorylation and subsequent downstream events, such as phosphorylation of IKK-α/β and IκB-α

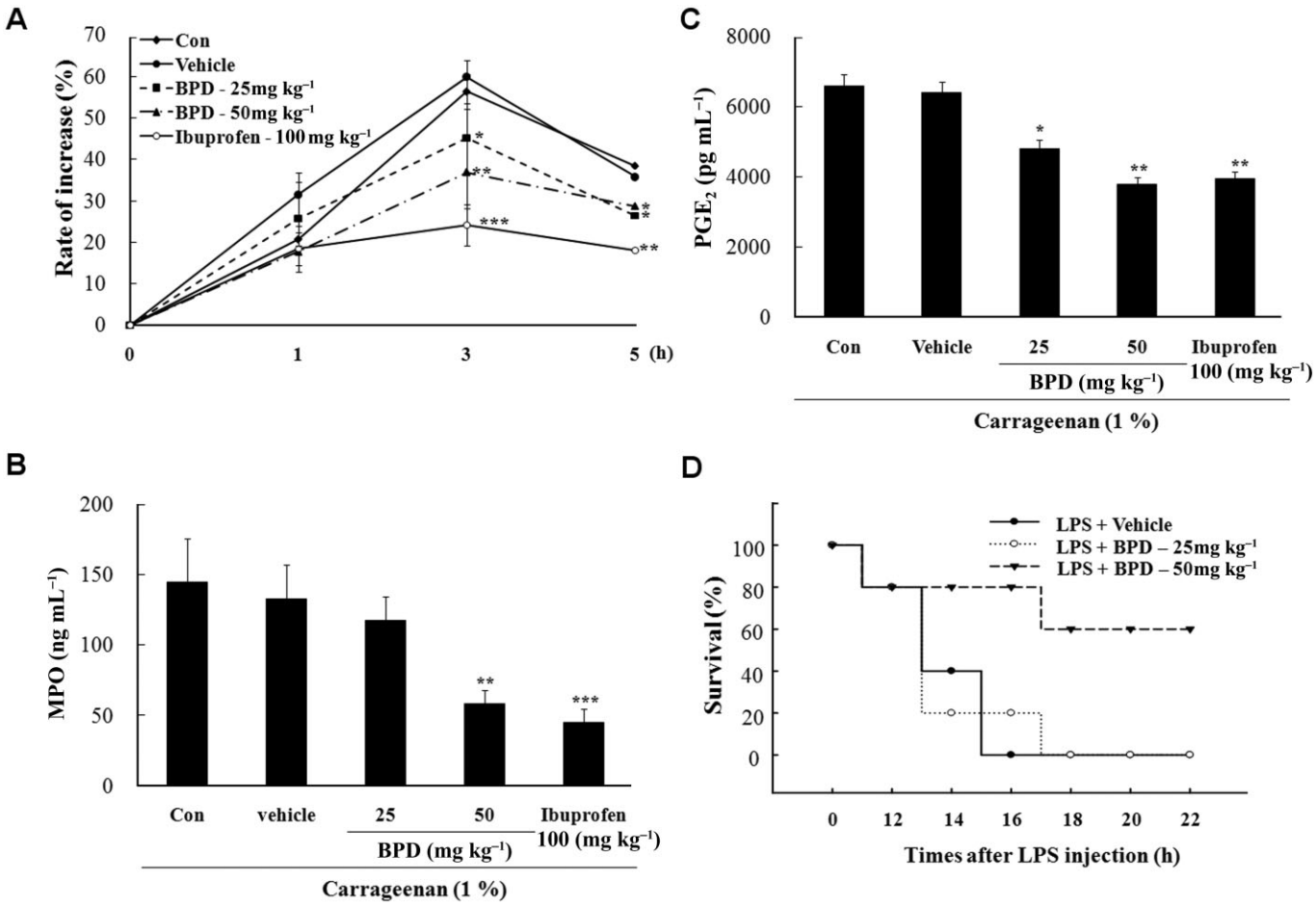

Figure 7

Inhibitory effects of BPD pretreatment on (A) carrageenan-induced paw oedema in rats and (D) LPS-induced septic shock in mice. (A) Paw oedema was induced by carrageenan injection, and paw volumes were measured at 1, 3 and 5 h as described in Methods. Ibuprofen (100 mg·kg^{-1}) was used as positive control. Values shown are mean ± SD (n = 5–6). *P < 0.05, **P < 0.01 versus the carrageenan-injected group; significant differences between groups were determined by ANOVA and Dunnett's *post hoc* test. (B, C) Soft tissues from carrageenan-injected paws were collected at 5 h after carrageenan injection and homogenized. Amounts of MPO and PGE_2 in homogenates were measured using EIA kits as described in Methods. Values shown are mean ± SD (n = 5–6). *P < 0.05, **P < 0.01, ***P < 0.001 versus the carrageenan-injected group; significant differences between groups were determined by ANOVA and Dunnett's *post hoc* test. (D) Five mice per group were treated with vehicle only or BPD and after 1 h were injected with LPS (25 mg·kg^{-1}, i.p.). Survival rates of these mice were observed over the next 22 h.

(Figure 3). These findings indicate that BPD inhibits the LPS-induced TAK1 phosphorylation, which is the pivotal upstream signal for NF-κB activation, by attenuating TAK1/TAB1 complex formation in LPS-induced RAW 264.7 cells.

To evaluate whether the inhibitory action of BPD is specific to LPS-TLR4 signalling, experiments using other TLRs activators were performed. It is conceivable that the activation of TLR4 elicits two distinct downstream intracellular signalling pathways, a myeloid differentiation primary-response protein 88 (MyD88)-dependent or independent pathway, whereas TLR2 ligand activates transcription factor via a MyD88-dependent pathway (Akira and Takeda, 2004) and TLR3 appears to transduce its signals through a MyD88-independent pathway (Alexopoulou *et al.*, 2001; Doyle and O'Neill, 2006). In the present study, BPD inhibited PGE_2 release and COX-2 protein expression induced by two TLRs ligands, PGN (for TLR2) and double-stranded RNA (for TLR3) (Figure 5). Thus, it is possible that BPD does not exclusively require MyD88 protein to inhibit NF-κB activation and suppress the diverse molecules that are required to regulate TLRs signalling. We also checked the mRNA expression of adaptor proteins involved in TLR signalling processes after LPS stimulation. It was found that BPD suppressed the LPS-induced mRNA expressions of MyD88, IL-1 receptor-associated kinase 1 (IRAK-1) and TRIF in a concentration-dependent manner (Supplementary Figure S4). Interestingly, BPD potently inhibited PGN-induced NF-κB-dependent luciferase activity, whereas it only mildly reduced poly(I:C)-induced NF-κB-dependent luciferase activity (Supplementary Figure S5). However, BPD significantly reduce poly(I:C)-induced COX-2 expression, indicating that it might affect other intracellular

signalling proteins, such as TBK1 and IRF3, that are mediators of NF-κB-independent TRIF-downstream signalling. Further investigation is needed about the effect of BPD on the TLR3 pathway.

To confirm the effect of BPD on NF-κB-regulated gene transcription, we have shown that BPD inhibited LPS-induced expressions of inflammatory mediators, such as iNOS, TNF-α, IL-6 and IL-1β. In this study, it was clearly shown that BPD could suppress induction of iNOS expression through down-regulation of their promoter activities and subsequent production of NO. Our findings indicate that BPD inhibits the expressions of TNF-α, IL-6 and IL-1β at the transcription level in a concentration-dependent manner, with the associated reduction of TNF-α, IL-6 and IL-1β. These results indicated that the suppression of NF-κB activation by BPD might inhibit pro-inflammatory gene expression at the transcriptional level. In addition, BPD also inhibited the production of NO and TNF-α in PGN- or double-stranded RNA-induced RAW 264.7 cells and LPS-induced primary macrophages (Supplementary. Figures S1 and S6).

To confirm whether the suppressive effect of BPD on LPS-induced inflammatory mediators *in vitro* could have an anti-inflammatory effect *in vivo*, we examined for the first time the effects of BPD in animal models. Activation of innate immunity by carrageenan is dependent on TLR4, in which the activated inflammatory cascade can lead to systemic inflammatory syndrome like oedema and sepsis in the clinical setting (Morris, 2003; Bhattacharyya *et al.*, 2008). Recent studies have shown that carrageenan induces the peripheral release of NO and PGE_2, and TNF-α, which subsequently promotes IL-1 and IL-6 production in tissues (Cunha *et al.*, 1992; Omote *et al.*, 2001). In our study, BPD inhibited the carrageenan-induced paw oedema and the tissue levels of MPO and PGE_2, which indicates BPD could alleviate acute inflammation by inhibiting the infiltration of inflammatory cells and production of pro-inflammatory mediators. To confirm the effect of BPD in systemic inflammatory responses, we also examined the protective effect of BPD in a LPS-induced sepsis model. Death caused by septic shock is crucially dependent on the inflammatory responses and pro-inflammatory cytokine production (Chaudhry *et al.*, 2008). Pretreatment with BPD improved survival during sepsis in a dose-dependent manner.

In summary, our results show that BPD exhibited its anti-inflammatory effect through dual inhibitory effect on the COX-2 enzyme activity and NF-κB-dependent pathways via TAK1 inactivation. These mechanisms of action are thought to contribute to the ability of BPD to reduce carrageenan-induced paw oedema and increase the survival rate of mice in the LPS-induced septic shock model. Accordingly, we conclude that BPD has the potential to ameliorate the inflammation and pain associated with various pathological conditions.

Acknowledgements

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Conflicts of Interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effects of BPD on LPS-induced NO (A) and TNF-α (B) production in mouse peritoneal macrophages. Cells isolated from 5% thioglycollate-primed C57BL/6 mice were treated with BPD (25, 50 or 100 μM) for 1 h and then stimulated with LPS (1 μg·mL^{-1}) for 24 h. Controls were not treated with LPS or BPD. $_{L}$-NIL (10 μM) was used as a positive control. Amounts of NO and TNF-α were determined using the Griess reaction and an EIA kit respectively. The experiment was repeated three times, and similar results were obtained. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$, $^{***}P < 0.001$ versus the LPS-induced group.

Figure S2 Effects of NS-398 on the expressions of COX-2 in TLR ligand-induced RAW 264.7 cells. Lysates were prepared from cells pre-treated with/without the NS-398 (5 μM) for 1 h and then with LPS (1 μg·mL^{-1}), PGN (10 μg·mL^{-1}) or poly(I:C) (50 μg·mL^{-1}) for 24 h. The protein levels of COX-2 were determined by Western blot as described in Methods. The experiment was repeated two times, and similar results were obtained. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group.

Figure S3 Effects of BPD on the LPS-induced phosphorylations of MAPKs in RAW 264.7 cells. Lysates were prepared from cells pre-treated with/without different concentrations (25, 50 or 100 μM) of BPD for 1 h and stimulated with LPS (1 μg·mL^{-1}) for 15 min. The protein levels of MAPKs were detected by Western blotting. The experiment was repeated three times, and similar results were obtained. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{***}P < 0.001$ versus the LPS-induced group.

Figure S4 Effects of BPD on mRNA expression of the LPS-induced MyD88-dependent (A) and independent (B) signal-

ling cascades in RAW 264.7 cells. Total RNA was prepared for the qRT-PCR analysis of the gene expressions of MyD88, IRAK1 and TRIF from cells pre-treated with/without BPD (25, 50 or 100 μM) for 1 h and then stimulated LPS (1 μg·mL^{-1}) for 10 min (for MyD88) or 1 h (for IRAK1 and TRIF). mRNA levels were detected by qRT-PCR as described in Methods. The experiment was repeated three times and similar results were obtained. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the LPS-induced group.

Figure S5 Effects of BPD on transcriptional activity of NF-κB by TLR-2,3 ligands in RAW 264.7 cells. Cells were co-transfected with pNF-κB-luc reporter and phRL-TK vector. Cells were treated with PGN (10 μg·mL^{-1}) (A) or with poly(I:C) (50 μg·mL^{-1}) (B) for 18 h in absence or presence of BPD (25, 50, or 100 μM). Luciferase activities were determined using a Promega luciferase assay system. The experiment was repeated three times and similar results were obtained. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{**}P < 0.01$,$^{***}P < 0.001$ versus the PGN- or poly(I:C)-induced group.

Figure S6 Effects of BPD on NO and TNF-α productions by TLR-2,3 ligands in RAW 264.7 cells. Cells were treated with BPD (25, 50, or 100 μM) for 1 h and then stimulated with PGN (10 μg·mL^{-1}) (A) or with poly(I:C) (50 μg·mL^{-1}) (B) for 24 h. Controls were not treated with PGN, poly(I:C), or BPD. $_{L}$-NIL (10 μM) was used as a reference drug. NO and TNF-α levels were determined using the Griess reaction and an EIA kit respectively. The experiment was repeated three times, and similar results were obtained. Values shown are means ± SD of three independent experiments. $^{\#}P < 0.05$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the PGN- or poly(I:C)-stimulated group.

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