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Nojirimycin suppresses inflammation via regulation of NF-κB signaling pathways

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Nojirimycin (NJ) is a compound in which the oxygen of the ring is replaced with an NH group in the D-glucose structure. NJ, which has a structure similar to D-glucose, is a powerful glucosidase inhibitor and an interesting compound. However, no anti-inflammatory effects of NJ have been reported. Therefore, to investigate its anti-inflammatory effect, the production and expression of inflammatory cytokines, as well as inflammatory mediators, such as iNOS and COX-2, were measured in LPS-stimulated RAW264.7 macrophages. In addition, the effects on the representative inflammatory signaling pathways, the suppression of NF-κB, and the activation of MAPK were studied. The production of iNOS, COX-2, and inflammatory cytokines (PGE_2, IL-6, IL-1β, and TNF-α) after NJ treatment was significantly inhibited. In addition, NJ showed anti-inflammatory effects through suppression of LPS-induced NF-κB activation. D-Glucose is structurally similar to NJ. The effects of these substances on RAW264.7 macrophages were evaluated. NJ reduced nitric oxide (NO) levels, whereas D-glucose had no significant effect. Overall, the results suggested that NJ is a potential anti-inflammatory compound.

1. Introduction

Inflammation is a common response that occurs in the human body and is an important mechanism for defending the human body from external stimuli, such as bacterial infection (Willoughby et al. 1975). The inflammatory response acts as a mechanism that protects the body from infection, killing bacteria or removing tumors. However, chronic inflammatory responses may cause disease in humans. The incidence of chronic inflammatory diseases is increasing owing to changes in living conditions and dietary habits due to the rapid industrial development of modern society and increased stress. In addition, inflammation caused by abnormal immune regulation due to various factors may persist (Kim et al. 2015). Therefore, research into controlling the inflammatory response and determining the underlying mechanisms is a current topic of interest (Lee et al. 2019).

Macrophages are the main type of cells responsible for innate immunity and are representative white blood cells that exhibit phagocytosis against infectious agents (Kim et al. 2016). Lipopolysaccharide (LPS), a protein in the outer membrane of gram-negative bacteria, is a representative inflammation-inducing factor that stimulates macrophages. The macrophages recognize LPS through Toll-like receptor-4 (TLR4), which increases nitric oxide (NO) and cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β, and prostaglandin E_2 (PGE_2) (Xie et al. 1993). They are also regulated by MAPKs, a nuclear factor of the kappaB (NF-κB) pathway (Dou et al. 2013).

The mitogen-activated protein kinase (MAPK) signaling pathway is a known mechanism of inflammation. It consists of ERK, JNK, and p38, and its activity is induced by phosphorylation (Jang et al. 2005). MAPK signaling normally regulates a variety of biological functions, including cell proliferation, differentiation, and survival. However, when activated by external stimulation, it plays an important role in inducing an inflammatory response by inducing the production of various inflammatory mediators.

The nuclear factor kappaB (NF-κB) signaling pathway, similar to the MAPK signaling pathway, plays an important role in the development of the inflammatory response. Normally, NF-κB exists in an inactivated state in a complex with IκBα, an inhibitory protein in the cytoplasm (Celec 2004). In the presence of an external stimulus, such as LPS, IκBα is phosphorylated, ubiquitinated, and separated from the complex. The isolated NF-κB dimer is freely activated and moves from the cytoplasm to the nucleus (Reber et al. 2009). Activated NF-κB induces the expression of inflammatory cytokines and inflammatory factors such as iNOS and COX-2. Therefore, the development of anti-inflammatory drugs can extend their meaning to the development of substances that inhibit the MAPKs and NF-κB pathways.

Nojirimycin (NJ) was first discovered in nature in *Streptomyces* (Inouye et al. 1968). NJ is a structure in which the oxygen of the ring is replaced with an NH group in the D-glucose structure. However, NJ is a powerful glucosidase inhibitor and has interesting physiological and biochemical effects (Reese et al.

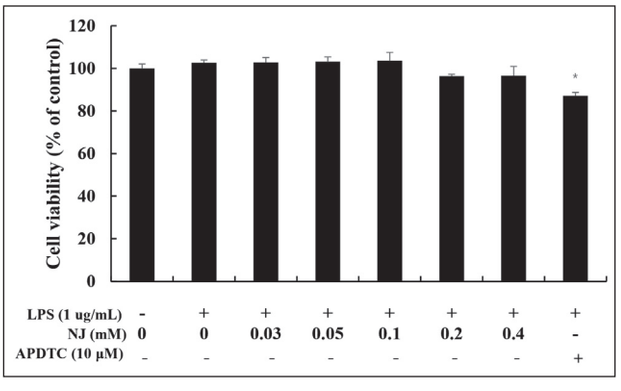

Fig. 1: Cell viability in LPS-induced RAW264.7 macrophages. The cells were treated with NJ (0.03, 0.05, 0.1, 0.2, 0.4 mM) for 24 h. Cell viability was determined by MTT assay. The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control.

1971). Recent studies have demonstrated that NJ selectively inhibits seed germination and radicle elongation in parasitic weeds (Wakabayashi et al. 2015). However, no studies have examined the anti-inflammatory effect of NJ. Therefore, in this study, we investigated the anti-inflammatory activity of NJ and its signaling mechanism in LPS-stimulated RAW264.7 macrophages. In addition, differences in the anti-inflammatory effects of D-glucose and NJ, relative to their structural differences, were also studied.

2. Investigations and results

2.1. Cell viability assay

The cell viability of RAW264.7 macrophages was examined by the MTT assay after treatment with NJ (Fig. 1). The cell viability was 90% or higher after treatment with all NJ concentrations; hence, NJ was confirmed not to be toxic to RAW264.7 macrophages. Therefore, subsequent anti-inflammatory experiments were conducted at concentrations that did not significantly affect cell viability.

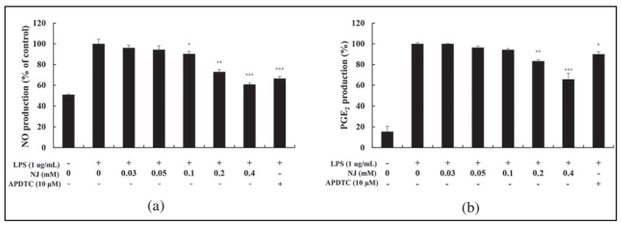

Fig. 2: (**a**) Nitric oxide and (**b**) PGE_2 production in LPS-induced RAW264.7 macrophages. The cells were each treated with NJ (0.03, 0.05, 0.1, 0.2, and 0.4 mM) for 24 h. The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the treated control.

2.2. Measurement of nitric oxide and PGE_2 production

The increase in NO and PGE_2 production in LPS-stimulated RAW264.7 macrophages decreased as the concentration of NJ increased (Fig. 2). Ammonium pyrrolidinedithiocarbamate (APDTC) was used as the control. For NJ, NO production was inhibited by 39% at a concentration of 0.4 mM. The production of NO in the presence of high concentrations of NJ was similar to that in the control sample, without LPS. It was confirmed that the treatment with NJ effectively inhibited NO production when inflammation was induced. The production of PGE_2 was inhibited by 34% at a concentration of 0.4 mM compared with the LPS-only treatment group.

Therefore, cells treated with NJ were subjected to western blotting assay to determine the concentration of iNOS, which is related to NO production (Kim et al. 2012). NJ effectively inhibited PGE_2 production. To confirm whether these results influenced the inhibition of COX-2 protein expression, which is a mediator of PGE_2 expression, this protein was also analyzed by western blotting (Kim et al. 2010).

2.3. iNOS and COX-2 expression in LPS-stimulated RAW264.7 macrophages

The increase in iNOS and COX-2 production induced by LPS decreased as the concentration of NJ increased (Fig. 3). iNOS expression was not observed in the untreated group, in which only RAW264.7 cells were incubated, but the expression of iNOS was significantly increased in the LPS-treated group compared with the untreated group. At an NJ concentration of 0.4 mM, the expression of iNOS was inhibited by 46% compared with the treated control. The expression of COX-2 was also decreased in a concentration-dependent manner; at an NJ concentration of 0.4 mM, the expression of the protein was inhibited by 74% compared with the treated control group. Therefore, it can be seen that NO and PGE_2 are decreased by iNOS and COX-2, respectively.

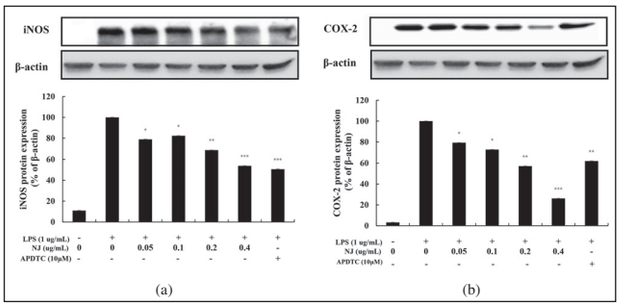

Fig. 3: Effect of NJ on the expression of iNOS and COX-2 protein in LPS-stimulated RAW264.7 macrophages (a). RAW264.7 macrophages were incubated with LPS (1 μg/mL) for 24 h in the presence or absence of NJ (0.05, 0.1, 0.2, 0.4 mM). The relative protein expression of (b) iNOS and (c) COX-2 was analyzed using Image J, compared with β-actin as an internal control. The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the treated control.

2.4. Measurement of pro-inflammatory cytokine expression

In the presence of NJ, the production of TNF-α, IL-6, and IL-1β was also inhibited in a concentration-dependent manner (Fig. 4). The production of TNF-α was inhibited by 52% at a concentration of 0.4 mM NJ compared with the LPS-only treatment group; IL-6 production was 76%, and IL-1β production was 60%.

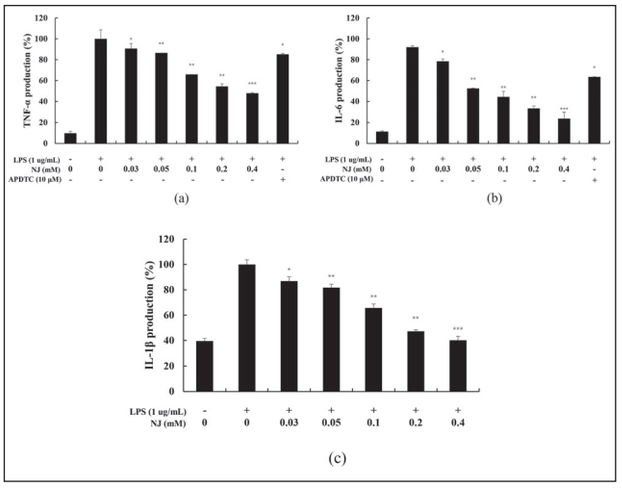

Fig. 4: Effect of NJ on LPS-induced production of (a) TNF-α, (b) IL-6, (c) and IL-1β in macrophages. RAW264.7 macrophages were incubated with LPS (1 μg/mL) for 24 h in the presence or absence of NJ (0.03, 0.05, 0.1, 0.2, 0.4 mM). The culture supernatants were collected and analyzed for the presence of TNF-α, IL-6, IL-1β using enzyme-linked immunosorbent assays. The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the treated control.

2.5. MAPK activation in LPS-stimulated RAW264.7 macrophages

Western blotting was conducted to determine the anti-inflammatory mechanism of NJ. One of the representative mechanisms of inflammation is the MAPK signaling pathway (Ramana et al. 2006). As shown in Fig. 5, the expression of the activated forms of ERK 1/2, JNK 1/2, p38, p-ERK 1/2, p-JNK 1/2, and p-p38, were increased after treatment with LPS. p-ERK 1/2, p-JNK 1/2, and p-p38 were not affected by NJ treatment.

2.6. Activation of NF-κB signaling activation in LPS-stimulated RAW264.7 macrophages

As shown in Fig. 6, the expression of the activated forms of p65, p105, p-p65, and p-p105 were increased after treatment with LPS. In the LPS-only treatment group, IκBα was the most degraded;

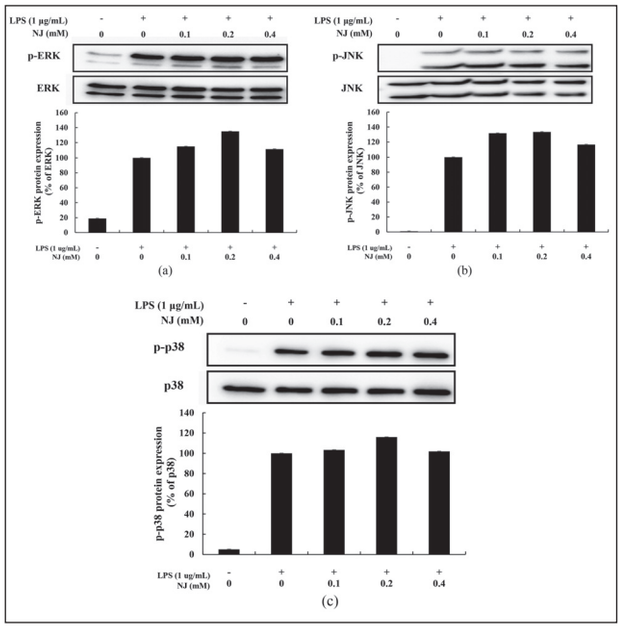

Fig. 5: Effect of NJ on the activation of ERK 1/2, JNK 1/2, and p38 in LPS-stimulated RAW264.7 macrophages. RAW264.7 macrophages were incubated with LPS (1 μg/mL) for 15 min in the presence or absence of NJ (0.05, 0.1, 0.2, 0.4 mM). (a) ERK 1/2 protein expression, (b) JNK 1/2 protein expression, (c) p38 protein expression; all measured using ImageJ. The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the treated control.

in the NJ treatment groups, degradation of IκBα was gradually inhibited. Phosphorylation of IκBα was also suppressed.

The expression of p-p65 and p-p105 was inhibited by NJ treatment. At NJ concentration of 0.4 mM, the expression of p-p65 was inhibited by 49% and that of p-p105 was inhibited by 14% compared with the control group.

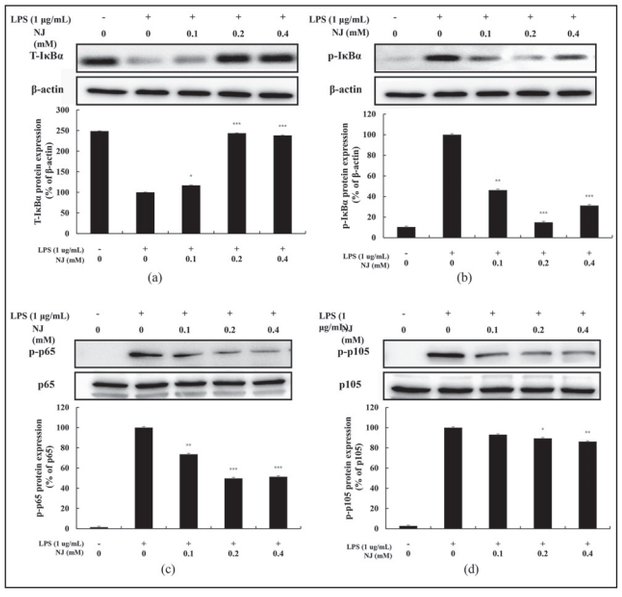

Fig. 6: Effect of NJ on the phosphorylation of IκBα, p65, p105 and T-IκBα in LPS-stimulated RAW264.7 macrophages. RAW264.7 were macrophages incubated with LPS (1 μg/mL) for 20 min in the presence or absence of NJ (0.05, 0.1, 0.2, 0.4 mM). Protein expression of (a) IκBα, (b) p-IκBα, (c) p-p65, and (d) p-p105 was determined using ImageJ. The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the treated control.

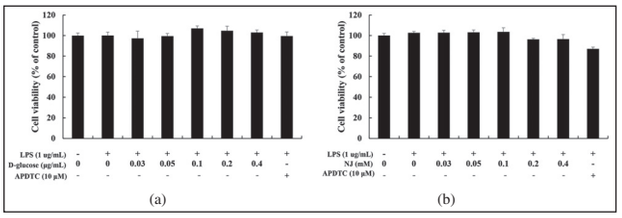

Fig. 7: Cell viability in LPS-induced RAW264.7 macrophages. The cells were treated with (a) D-glucose (0.03, 0.05, 0.1, 0.2, 0.4 mM) or (b) NJ (0.03, 0.05, 0.1, 0.2, 0.4 mM) for 24 h and the viability of treated cells was determined by MTT assay. The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ the versus control group.

2.7. Effects of nojirimycin-related structures on RAW264.7 macrophages

The cytotoxicity of D-glucose, which has a similar structure to NJ, was examined (Fig. 7). Toxicity was not observed at any concentration, and NJ tended to decrease NO production; whereas, in the case of D-glucose, the effect was insignificant (Fig. 8).

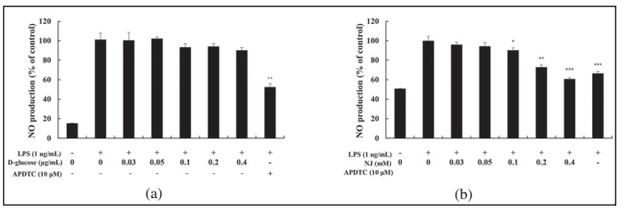

Fig. 8: Nitric oxide production in LPS-stimulated RAW264.7 macrophages. The cells were treated with (a) D-glucose (0.03, 0.05, 0.1, 0.2, 0.4 mM) or (**b**) NJ (0.03, 0.05, 0.1, 0.2, 0.4 mM) for 24 h. The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the treated control.

3. Discussion

In this study, to confirm the anti-inflammatory effects of NJ, experiments were conducted to inhibit NO production, pro-inflammatory cytokines, and PGE_2 production in RAW264.7 macrophages. Then, the changes in the MAPK and NF-κB signaling pathways were investigated. In addition, a simple experiment was conducted to compare the anti-inflammatory effects of compare D-glucose, which has a similar structure to NJ.

NO is an unstable molecule, and a major causative factor in skin damage and aging (Park et al. 2009). An excessive increase in NO due to inflammatory substances, such as LPS, leads to inflammatory diseases such as septic shock and rheumatoid arthritis (Laskin and Pendino 1995). In addition, iNOS is not present in resting cells, but increases NO production when induced by certain stimuli. In addition, COX-2 is induced by stimuli, such as LPS, to produce prostaglandins and induce an inflammatory response; moreover, it plays an important role in the development and progression of various degenerative disease (Chen et al. 2001; Noh et al. 2011). Therefore, the inhibition of NO, iNOS, and COX-2 expression may have the effect of suppressing inflammation.

In this study, final NJ concentrations of 0.03–0.4 mM were used, and these concentrations exerted no cytotoxic effects in RAW264.7 macrophages. The amount (%) of NO production was inhibited by 39% at the highest concentration of 0.4 mM. PGE_2 was inhibited in a concentration-dependent manner. In addition, NJ inhibited the protein expression of iNOS and COX-2.

Macrophages activated by inflammatory mediators produce NO and the release of prostaglandins as well as various cytokines, including the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, to amplify the early stage inflammatory response (Byun et al. 2005). In the case of NJ, TNF-α, IL-1β, and IL-6 were all inhibited in a concentration-dependent manner.

In LPS-stimulated macrophages, the NF-κB and MAPK signaling pathways are known to be the major pathways that regulate inflammatory factor expression. The MAPK signaling pathways are induced by LPS and are activated by phosphorylation. The NF-κB signaling pathway is known to be involved in the production of chronic inflammatory factors. External stimuli, such as LPS, induce decomposition by the phosphorylation of IκBα. The separated NF-κB is translocated to the nucleus and induces inflammation by binding to DNA related to chronic inflammatory factors (Dubois et al. 1994; Moynagh 2005). NF-κB exists in the form of various dimers and, when separated from IκBα, passes through the nuclear membrane and translocates into the nucleus, causing inflammation (Chae 2005). Therefore, substances that inhibit the migration of phosphorylated p65 and p105 to the nucleus are used as a treatment for diseases caused by excessive inflammation (Shin et al. 2008).

NJ had no effect on the MAPK signaling pathway. However, NJ suppressed the mechanism of NF-κB and exerted anti-inflammatory effects. By inhibiting the phosphorylation of IκBα, the activity of NF-κB was suppressed, and accordingly, the activation of p65 and p105 was also suppressed.

In addition, the structure of D-glucose is similar to NJ, and the compound is potentially interesting as a glucosidase inhibitor. In addition, NJ has a superior anti-inflammatory effect to D-glucose and induced anti-inflammatory effects through the NF-κB signaling pathway. NJ and D-glucose differ in the content of oxygen and nitrogen in the ring (Fig. 9). The data implied that the anti-inflammatory effect of NJ was due to presence of nitrogen in the ring. Although additional experiments are required to confirm the suitability of these compounds for use in medicine, we believe that we have proven the efficacy of this new antibiotic in vitro.

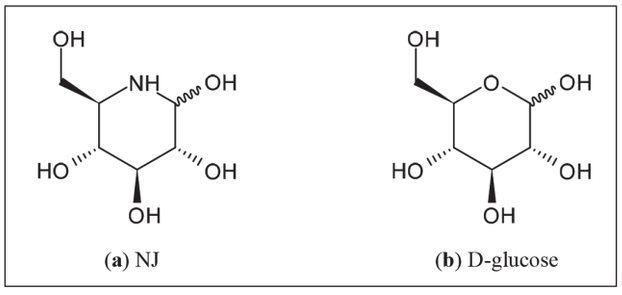

Fig. 9: Structure of (a) NJ and (b) D-glucose.

4. Experimental

4.1. Cell culture

The mouse macrophage cell line, RAW264.7, used in the experiment was purchased from the Korea Cell Line Bank (Seoul, Korea). The cells were cultured in a 5% CO_2 incubator at 37 °C, and Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA) and 1% penicillin and streptomycin (Gibco, USA) was used. The medium was changed every two days; when the cells were more than 80% confluent, they were collected using a cell scraper and cultured.

4.2. Cell viability assay

To determine the effect of NJ on cell viability, the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) was measured spectrophotometrically. The cells were resuspended in medium, and 500 μL was seeded in 24-well plates (SPL, Korea) at 1.5×10^5 cells/well, and incubated for 24 h at 37°C in a 5% CO_2 incubator. Then, LPS (Sigma, USA) and samples were added to the cells and incubated for 24 h. Thereafter, 400 μL of MTT solution was added, the cells were incubated for 4 h in an incubator, and the supernatant was removed. DMSO was added to dissolve the formazan produced by the reduction of MTT. The absorbance at 570 nm was measured using a microplate reader (Tecan Sunrise, Austria). The average absorbance values were obtained for each sample group, and the cell viability was measured in comparison with the absorbance values with those of the control group.

4.3. Measurement of nitric oxide production

To measure the anti-inflammatory effect of different concentrations of NJ in cells, Griess reagent was used (Sigma, USA) as the NO^{2-} form present in the cell culture medium. After the cells were suspended in medium, 500 μL was seeded in 24-well plates (SPL, Korea) to a cell count of 1.5×10^5 cells/well, and incubated for 24 h at 37 °C in a 5% CO_2 incubator. Then, LPS (Sigma, USA) and samples were treated at the same time and incubated for 24 h. Thereafter, 100 μL of cell culture solution and 100 μL of Griess reagent were mixed and reacted for 10 min in a 96-well plate (SPL, Korea). The absorbance at 540 nm was measured using a microplate reader. The average absorbance values were obtained for each sample group, and the amount (%) of NO produced was measured in comparison with the absorbance value of the control group treated with LPS.

4.4. Measurement of PGE_2 and inflammatory cytokine expression

To culture the cells in the same way as for the measurement of nitric oxide production, the supernatant was recovered from each well and the expression of PGE_2 and inflammatory cytokines was measured. After treatment in accordance with the suggested protocol using a mouse IL-6 ELISA kit (BD Bioscience, USA), mouse TNF-α ELISA kit (BD Bioscience, USA), mouse IL-1β ELISA kit (R&D Systems, USA), mouse PGE_2 ELISA kit (BD bioscience, USA), the absorbance was measured using a microplate reader.

4.5. Western blotting assay

After suspending the cells in medium, 3 mL of resuspended cells was seeded in a 60×15 mm culture dish (SPL, Korea) at 8×10^5 cells/dish and incubated for 24 h at 37 °C in a 5% CO_2 incubator. LPS, NJ (0.05, 0.1, 0.2, 0.4 mM), and DNJ (0.31, 0.63, 1.25, 2.5 mM) were treated simultaneously, cultured for between 15 min and 24 h, and the expression of inflammation-related proteins was measured. After incubation, the cells were washed with cold phosphate-buffered saline (PBS; Biosesang, Korea), and then lysed with RIPA lysis buffer (150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, and 2 mM EDTA, and protease inhibitor cocktail). The supernatant was obtained by centrifugation at 15,000 rpm for 20 min. The protein concentration was measured using the BCA protein assay kit (Pierce, USA). Then, 30 μg of protein was added to Laemmli sample buffer (Bio-Rad, USA) and inactivated at 100 °C for 3 min, followed by electrophoresis with 10% SDS-PAGE. The isolated protein was transferred to a PVDF membrane (Bio-Rad, USA), and non-specific binding to the membrane was blocked in 5% skim milk for 4 h. After incubation of the primary antibody overnight at room temperature, the membrane was washed six times with Tris-buffered saline in Tween-20 (TBST) and then incubated at room temperature for 2 h with HRP-conjugated secondary antibody (1:5000 dilution). After six washes with TBST, the protein was visualized on X-ray film by the application of enhanced chemiluminescence (ECL; Biosesang, Korea) solution.

4.6. Statistical analysis

All data are expressed as the mean±standard deviation (SD) from three independent experiments. Statistical analysis was performed using Student's *t*-test. *P-values* of $<$ 0.05 were considered statistically significant.

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Conflicts of interest: Non declared.

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