

# Anti-inflammatory potential of peat moss extracts in lipopolysaccharide-stimulated RAW 264.7 macrophages

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**Abstract.** The aim of the present study was to identify the anti-inflammatory and anti-oxidative effects of peat moss aqueous extract (PME) on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. To demonstrate the anti-inflammatory and antioxidant effects of PME, the levels of nitric oxide (NO) and cytokines were measured using Griess reagent and cytokine ELISA kits, respectively. Reverse transcriptase-polymerase chain reaction (RT-PCR) and western blot analysis were conducted to evaluate the expression of genes and proteins. Immunofluorescence was used to measure the expression and translocation of transcription factors. Pre-treatment with PME inhibited the production of prostaglandin E<sub>2</sub> and NO by suppressing the gene expression of cyclooxygenase-2 and inducible NO synthase, respectively.

The LPS-stimulated gene expression and the production of tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$  were significantly reduced by PME. In the LPS-stimulated RAW 264.7 cells, nuclear factor- $\kappa$ B (NF- $\kappa$ B) translocated from the cytosol to the nucleus, while pre-treatment with PME induced the sequestration of NF- $\kappa$ B in the cytosol through the inhibition of I $\kappa$ B $\alpha$  degradation. In the same manner, PME contributed to the inhibition of the activation of mitogen-activated protein kinases. In addition, the PME-treated RAW 264.7 cells facilitated the activation of nuclear factor-like 2 (Nrf2), and in turn, enhanced heme oxygenase-1 (HO-1) expression. These results indicate that PME exerts anti-inflammatory and antioxidant effects, and suggest that PME may neutralize inflammation and prevent cellular damage by oxidative stress.

## Introduction

Inflammation is an immune response to recover injured or infected tissue. Under injury and infection, various factors, such as cytokines and chemokines, are secreted by immune cells and the cells are transferred to the area to resolve the abnormal condition. The characteristics of inflammatory response have been observed from the ancient era, and are still being extensively studied in order to determine the correlation between various diseases and inflammation (1). During inflammation, inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) play important roles in amplifying the inflammatory response. The enzymatic activity of iNOS converts arginine to NO (2). iNOS is expressed in response to interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  and lipopolysaccharide (LPS). NO is beneficial in eliminating microorganisms and improving the blood supply

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to injured tissue; however, it can cause tissue damage when it forms the highly reactive peroxynitrite by reacting with reactive oxygen species (ROS) (3). COX-2, an inducible form of COXs, is overexpressed in LPS-stimulated macrophages. It carries out an enzymatic role, transforming arachidonic acid to prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (4). During the inflammatory response, NO increases COX activity, resulting in an increase in the production of pro-inflammatory prostaglandins (PGs) including PGE<sub>2</sub>. Consequently, the inflammatory response is exacerbated (5).

Although inflammation is a solution to normalize troubled tissue, immune dysfunction can convert inflammation into a weapon which causes chronic inflammatory diseases. Rheumatoid arthritis (RA) is a representative chronic inflammatory disease characterized by synovial inflammation, the hyperplasia of synovial tissues and the destruction of bone and cartilage, contributing to joint disability. For patients with RA, TNF- $\alpha$  inhibitors are mainly used to inhibit inflammatory responses, resulting in the reduction of symptom aggravation (6,7). In the 19th century, the mud bath, a folk remedy, was used to ameliorate the symptoms of RA. A mud bath involves plant-derived sediments, termed humic substances. The sediments are produced through a humification process in the environment. The process synthesizes and decomposes humic substances. As a result, stable compounds remain in the sediments. Examples of plant-derived sediments include peat, sapropel and mumie. Among the plant-derived sediments, peat is a light brown to black organic material produced under marshy conditions from decomposed waterlogged vegetation, including mosses (8-10). Based on the traditional remedy, the pharmacological effects of sediments have been demonstrated *in vivo* and *in vitro*. In previous studies, humate, a derivative from bituminous or brown coal, has been shown to exert anti-inflammatory effects, including the inhibition of hypersensitivity in rats (11), of the granulation and adhesion of neutrophils (12), and of cytokine expression and complement production of mononuclear lymphocytes (13).

There are several studies available demonstrating the pharmacological effects of coal-derived sediments (11-13); however, to the best of our knowledge, the anti-inflammatory properties of peat moss extracts have not been investigated to date. In the present study, we aimed to investigate the anti-inflammatory effects of peat moss aqueous extracts (PME) on inflamed RAW 264.7 macrophages at the molecular level, as well as to identify the signal transduction pathways involved.

## Materials and methods

**Preparation of PME.** Peat moss extracts were a kind gift from Green Voltex Co. (Busan, Korea). Briefly, the peat moss was powdered, and then filtered using 6-mesh screen. Ground peat moss (100 g) and sodium bicarbonate (5 g) were mixed and fermented at 30°C for 5 to 7 days until reaching pH 7.0. The PME from the fermented peat moss was prepared with hot distilled water (60°C) for 24 h, stirring 4-5 times during this period. The infusates were filtered through Whatman No 4 paper and then centrifuged at 13,200 rpm. The total volume was measured and the extraction yield (11.1 g) determined with the dry weight of 1 ml duplicate samples. The extracts were dried at 30°C before being used in the experiments.

**Cell culture.** The RAW 264.7 cells, murine macrophage-like cells, were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin (100 U/ml)/streptomycin (100  $\mu$ g/ml) under humidified conditions of 5% CO<sub>2</sub> at 37°C. The RAW 264.7 cells were stimulated to induce an inflammatory response using LPS which as purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). DMEM, FBS and penicillin/streptomycin were purchased from Cellgro Mediatech (Manassas, VA, USA).

**Cell viability assay.** Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the RAW 264.7 cells (5x10<sup>5</sup> cells/ml) were seeded in a 96-well plate. The cells were incubated with 10, 25 and 50  $\mu$ g/ml PME for 24 h or were pre-treated with 50  $\mu$ g/ml PME for 1 h and then stimulated with 500 ng/ml LPS or 1 mM H<sub>2</sub>O<sub>2</sub> for 24 h. Following incubation with PME, LPS and H<sub>2</sub>O<sub>2</sub>, the cultured medium was changed to fresh medium and the cells were incubated with 0.5 mg/ml MTT solution (Sigma-Aldrich Chemical Co. ) for 2 h. Subsequently, the supernatant was discarded and formazan blue, which was formed in the cells, was dissolved with DMSO. The optical density was measured at 540 nm using a microplate reader (Dynatech Laboratories, Chantilly, VA, USA). The assay was performed in triplicate.

**Nitrite measurement.** The RAW 264.7 cells were seeded in each well of a 96-well plate. The cells were treated solely with PME (10, 25 and 50  $\mu$ g/ml) for 24 h or were pre-treated with 50  $\mu$ g/ml PME for 1 h and stimulated with 500 ng/ml LPS. Following incubation for 24 h, the supernatant of each well was mixed with the same volume of Griess reagent (Sigma-Aldrich Chemical Co.) for 10 min at room temperature in the dark. The absorbance of the reacted supernatant was measured at 540 nm using a microplate reader. All samples were conducted in triplicate.

**PGE<sub>2</sub>, TNF- $\alpha$  and IL-1 $\beta$  measurement.** To measure the inhibitory effects of PME on the production of PGE<sub>2</sub>, TNF- $\alpha$  and IL-1 $\beta$ , enzyme-linked immunosorbent assay (ELISA) kits were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) for PGE<sub>2</sub>, and from R&D Systems Inc. (Minneapolis, MN, USA) for TNF- $\alpha$  and IL-1 $\beta$ . The cell culture conditions were same as those for the nitrite measurement assay. Following incubation with PME and LPS for 24 h, the concentration of PGE<sub>2</sub>, TNF- $\alpha$  and IL-1 $\beta$  in the culture medium was determined by selective ELISA kits.

**Reverse transcription-polymerase chain reaction (RT-PCR).** The cells were incubated with PME (10, 25 and 50  $\mu$ g/ml) alone for 24 h or pre-treated with 50  $\mu$ g/ml PME for 1 h prior to LPS stimulation for 24 h. Total RNA was isolated from the cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was used for cDNA synthesis using AccuPower<sup>®</sup> RT premix (Bioneer, Daejeon, Korea) containing M-MLV reverse transcriptase. The iNOS, COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; used as an internal control) genes, were amplified from the cDNA by PCR. The PCR primers were as follows: iNOS

(5'-ATG TCC GAA GCA AAC ATC AC-3' and 5'-TAA TGT CCA GGA AGT AGG TG-3'), COX-2 (5'-ATG GTC AGT AGA CTT TTA CGA CTA-3' and 5'-GGA GAG ACT ATC AAG ATA GTG ATC-3'), IL-1 $\beta$  (5'-GGG CTG CTT CCA AAC CTT TG-3' and 5'-GCT TGG GAT CCA CAC TCT CC-3'), TNF- $\alpha$  (5'-TCT CAT CAG TTC TAT GGC CC-3' and 5'-GGG AGT AGA CAA GGT ACA AC-3'), and GAPDH (5'-AGG CCG GTG CTG AGT ATG TC-3' and 5'-TGC CTG CTT CAC CAC CTT CT-3'). The amplified DNA was visualized on an agarose gel containing ethidium bromide (EtBr; Sigma-Aldrich Chemical Co.).

**Western blot analysis.** The cells were cultured with or without PME for 1 h prior to stimulation with 500 ng/ml LPS for 24 h. The control group was cultured in medium without PME and were not treated with LPS. For total protein extraction, the cells were lysed with lysis buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 1 mM phenyl-methylsulfonyl fluoride (PMSF) and 5 mM dithiothreitol (DTT)] for 1 h. Insoluble materials were discarded by centrifugation at 14,000 rpm for 20 min at 4°C. In a parallel experiment, nuclear and cytosolic proteins were prepared using nuclear extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The protein concentration in the cell lysate was determined using detergent-compatible protein assay from Bio-Rad Laboratories (Hercules, CA, USA). Equal amounts of protein were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The separated protein was transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) and subsequently blocked with Tris-buffered saline (10 mM Tris-Cl, pH 7.4) containing 0.5% Tween-20 and 5% non-fat dry milk for 1 h at room temperature. The protein was probed with primary antibodies overnight at 4°C. After probing with the primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG as the secondary antibody, purchased from Amersham Corp. (Arlington Heights, IL, USA). Using the enhanced chemiluminescence (ECL) detection system (Amersham Corp.), immunoreactive bands were detected and exposed to X-ray film. All primary antibodies, including antibodies to iNOS, COX-2, nuclear factor (NF)- $\kappa$ B, I $\kappa$ B $\alpha$ , p38, extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), Akt and heme oxygenase-1 (HO-1) were purchased from Cell Signaling Technology (Beverly, MA, USA) apart from nuclear factor erythroid 2-related factor 2 (Nrf2) which was from Abcam (Cambridge, UK).

**Immunofluorescence staining.** The RAW 264.7 cells were seeded on coverslip bottom dishes for 24 h. The cells pre-treated with 50  $\mu$ g/ml PME for 30 min prior to LPS stimulation for 30 min. Following incubation with PME and LPS, 4',6-diamidino-2-phenylindole (DAPI; Roche Diagnostics Corp., Indianapolis, IN, USA) staining was conducted for 15 min, and 4% paraformaldehyde (Junsei Chemical Co., Ltd., Tokyo, Japan) was used for fixing the DAPI-stained cells. The fixed cells were blocked with 5% mouse and rabbit serum (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and then antibodies for p65 or Nrf2 (1  $\mu$ g/well) and 0.3% Triton X-100 were applied for 1 h. The cells were incubated with Alexa Fluor 488-tagged anti-rabbit IgG (Cell Signaling

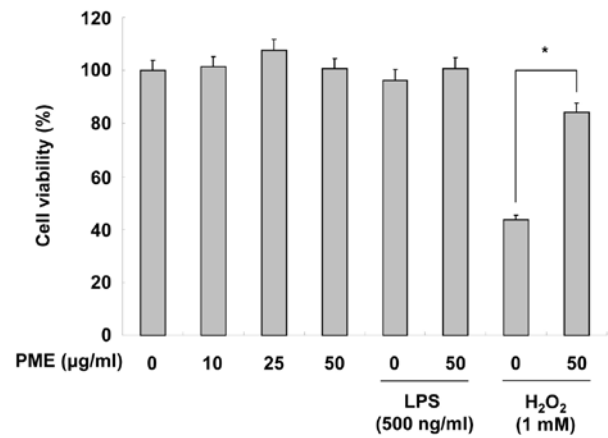


Figure 1. Effects of peat moss aqueous extract (PME) on the viability of RAW 264.7 cells. Cell viability was measured by MTT assay, and was not significantly impaired by treatment with PME (10, 25 and 50  $\mu$ g/ml) and lipopolysaccharide (LPS) (500 ng/ml) alone. Cell viability was reduced by 1 mM H<sub>2</sub>O<sub>2</sub>, whereas pre-treatment with PME suppressed H<sub>2</sub>O<sub>2</sub>-induced cell death. Values are the means  $\pm$  SD. \*P<0.05 compared with the H<sub>2</sub>O<sub>2</sub>-treated group.

Technology, Beverly, MA, USA) for 1 h, and were embedded with ProLong Antifade Reagent (Invitrogen, Eugene, OR, USA). The cells were observed using a Nikon Eclipse 50i microscope equipped with a charged-coupled device camera (Nikon, Tokyo, Japan). To determine the subcellular regions of protein co-localization, individual red-, blue- and green-stained images derived from the same field were merged using High-Content Analysis software (Cambridge Healthtech Institute, Needham, MA, USA). Densitometric analysis of the stained cells was evaluated using ImageJ software.

**Data analysis.** Results are expressed as the means  $\pm$  standard deviation (SD). Differences in mean values between groups were analyzed by a one-way analysis of variance followed by Dunnett's test. Differences were considered statistically significant with P-values <0.05.

## Results

**Cytotoxicity of peat moss extracts.** Before analyzing the anti-inflammatory effects of PME, we examined the cytotoxic effects of PME on RAW 264.7 cells by MTT assay. Treatment with PME (10, 25 and 50  $\mu$ g/ml) did not appear cytotoxic to the RAW 264.7 cells. Stimulation with LPS (500 ng/ml) also did not have any particular cytotoxic effect on the cells. When the cells were treated with H<sub>2</sub>O<sub>2</sub>, cell viability markedly decreased; however, pre-treatment with PME inhibited the cytotoxic effects of H<sub>2</sub>O<sub>2</sub> on the RAW 264.7 cells (Fig. 1). Hence, these results indicate that PME does not have any cytotoxic effects and prevents oxidative stress.

**Regulatory effects of PME on the production of NO and PGE<sub>2</sub>.** To investigate the regulatory effects of PME on the production of NO and PGE<sub>2</sub>, the RAW 264.7 cells were treated with the indicated concentrations of PME. As shown in Fig. 2A, the RAW 264.7 cells did not produce NO following treatment with PME alone. LPS stimulation increased NO production as compared to the basal levels without LPS. Pre-treatment

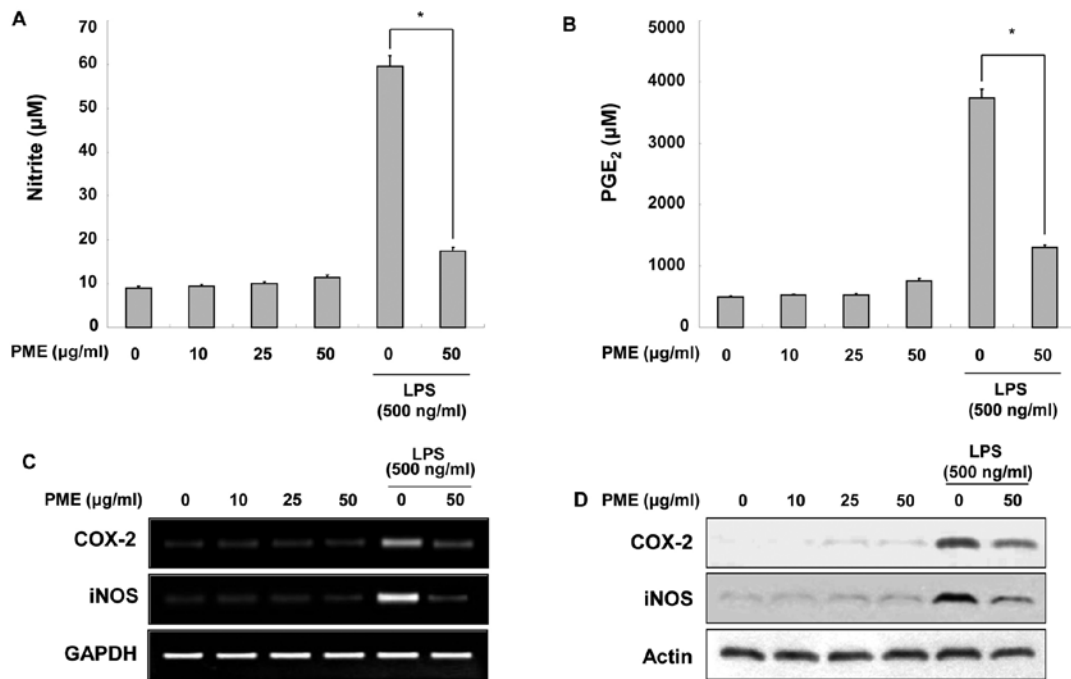


Figure 2. Effects of peat moss aqueous extract (PME) on lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production, and LPS-induced inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) expression. RAW 264.7 cells were pre-treated with PME at 10, 25 and 50 µg/ml for 1 h and then stimulated with LPS (500 ng/ml) for 24 h. (A) NO levels were measured using Griess reagent. (B) PGE<sub>2</sub> production was measured by ELISA. Values are the means ± SD. \*P<0.05 compared with the LPS-treated group. (C and D) The mRNA and protein levels of iNOS and COX-2 were determined by RT-PCR and western blot analysis, respectively. GAPDH and actin were used as internal controls for RT-PCR and western blot analysis, respectively.

with 50 µg/ml PME inhibited the production of NO in the LPS-stimulated RAW 264.7 cells. Similar to NO production, treatment with PME did not induce a particular increase in PGE<sub>2</sub> levels in the intact RAW 264.7 cells (Fig. 2B). LPS stimulation increased PGE<sub>2</sub> production; however, treatment with PME prior to LPS stimulation suppressed PGE<sub>2</sub> amplification. Since the production of NO and PGE<sub>2</sub> was inhibited by PME, we examined whether PME alters the expression of iNOS and COX-2 at the mRNA and protein levels. In the RAW 264.7 cells not stimulated with LPS, PME did not induce iNOS and COX-2 expression at the mRNA and protein levels. The stimulation of RAW 264.7 cells with LPS enhanced iNOS and COX-2 expression; however, the pre-treatment with PME inhibited iNOS and COX-2 expression (Fig. 2C). These results indicate that PME decreases NO and PGE<sub>2</sub> production in LPS-stimulated RAW 264.7 cells by inhibiting iNOS and COX-2 expression, respectively. Additionally, PME regulates the expression of iNOS and COX-2 at the transcriptional level.

**Regulatory effects of PME on the production of pro-inflammatory cytokines.** To identify the anti-inflammatory properties of PME, we quantified the production of IL-1β and TNF-α in the cultured medium. When the RAW 264.7 cells were treated solely with PME, there were not particularly changes in cytokine production. The production of IL-1β and TNF-α was increased by LPS stimulation; however, pre-treatment with PME decreased cytokine production in the LPS-stimulated RAW 264.7 cells (Fig. 3A and B). As cytokine secretion was reduced by PME treatment, we evaluated whether PME negatively regulates the mRNA synthesis of cytokines in the cells. As shown by RT-PCR, the mRNA expression of the

cytokines was downregulated following pre-treatment with PME (Fig. 3C). Based on these results, we identified that PME negatively regulates the production of pro-inflammatory cytokines, including IL-1β and TNF-α, at the transcriptional level.

**Regulatory effects of PME on NF-κB activation.** As shown by our results, the expression of iNOS, COX-2 and pro-inflammatory cytokines, including TNF-α and IL-1β, was regulated at the transcriptional level through PME treatment. Based on these results, we evaluated the expression of nuclear p65, a subunit of NF-κB and cytosolic IκBα by western blot analysis and immunofluorescence. Before evaluating the inhibitory effects of PME on p65 translocation, we investigated whether treatment with PME alone induces the activation of p65. As shown in Fig. 4A, nuclear p65 expression was not specifically amplified by PME. Stimulation with LPS gradually increased nuclear p65 expression in a time-dependent manner. However, pre-treatment with PME reduced the expression of nuclear p65, which had returned to basal levels at 30 min of treatment with PME. In the same manner, LPS stimulation decreased IκBα expression in the cytosol in a time-dependent manner; however, pre-treatment with PME somewhat amplified cytosolic IκBα expression at 30 min. In order to confirm NF-κB inactivation by PME, we visualized the location of NF-κB in the cells by immunofluorescence staining. In the control group (medium only) and PME-treated group, NF-κB was located in the cytosol. LPS stimulation facilitated the translocation of NF-κB into the nucleus. In the group pre-treated with PME and then with LPS, NF-κB was arrested in cytosolic area (Fig. 4B). Densitometric analysis of the nuclear area showed that nuclear p65 density was amplified 10-fold by LPS stimula-

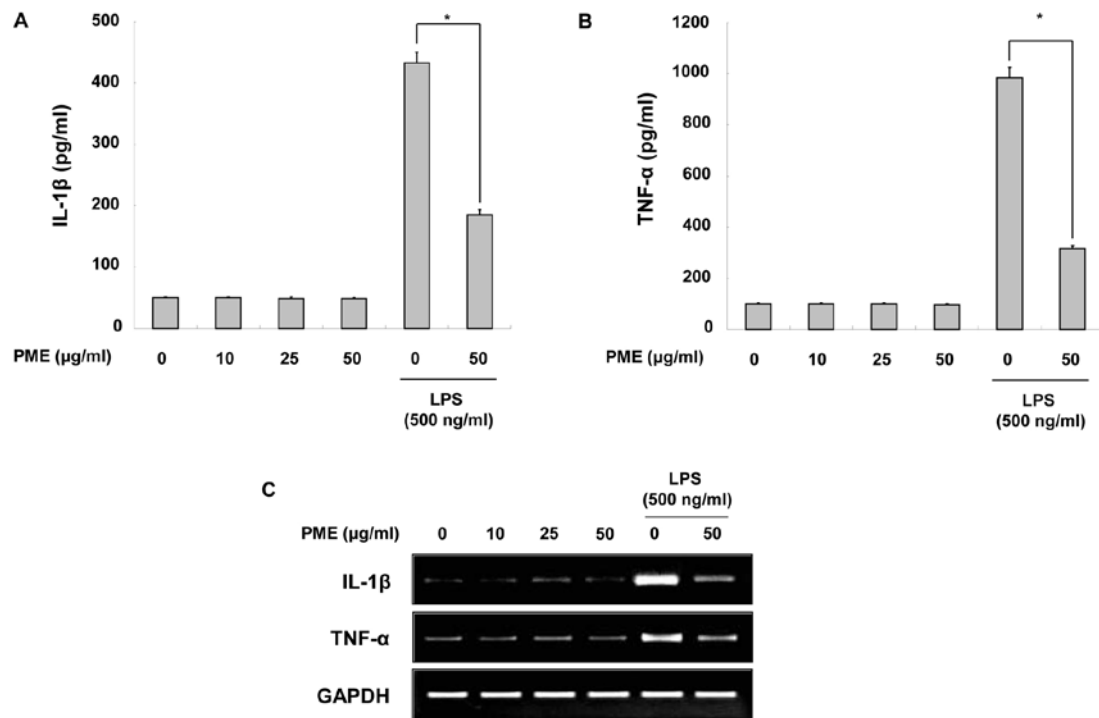


Figure 3. Effects of peat moss aqueous extract (PME) on lipopolysaccharide (LPS)-induced interleukin (IL)-1β and tumor necrosis factor (TNF)-α expression and production. RAW 264.7 cells were pre-treated with PME at the indicated concentrations for 1 h prior to LPS stimulation for 24 h. (A and B) Production of IL-1β and TNF-α was measured by ELISA. Values are the means ± SD. \*P<0.05 compared with the LPS-treated group. (C) The mRNA level of IL-1β and TNF-α was evaluated by RT-PCR. GAPDH was used as an internal control for RT-PCR.

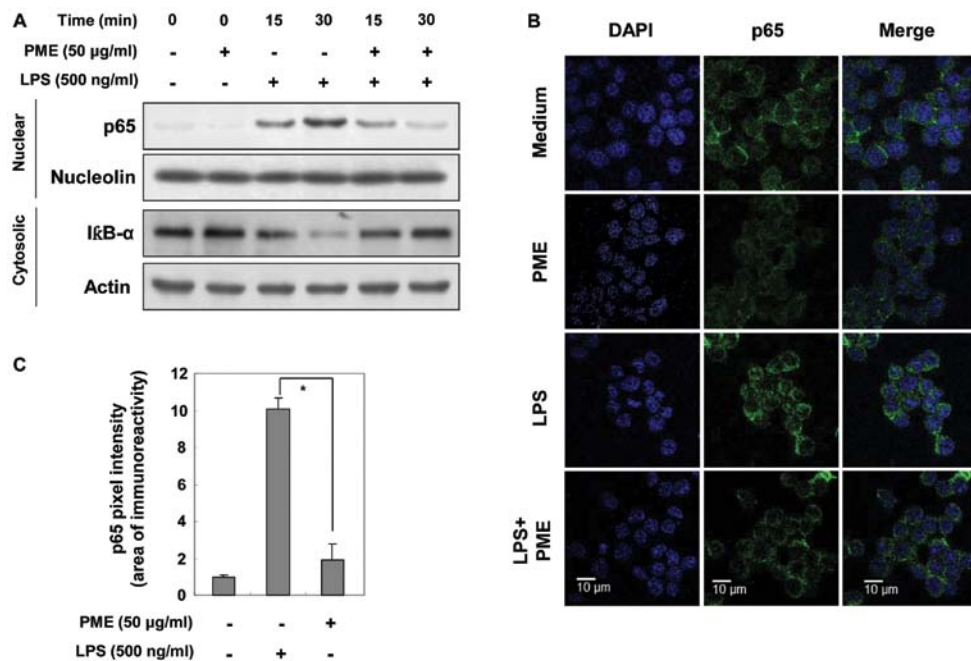


Figure 4. Effects of peat moss aqueous extract (PME) on lipopolysaccharide (LPS)-induced nuclear factor (NF)-κB translocation and expression. (A) RAW 264.7 cells were pre-treated with 50 μg/ml PME for 1 h prior to LPS stimulation for 15 and 30 min. Expression of nucleic p65, a subunit of NF-κB, and cytosolic IκBα was measured by western blot analysis. Nucleolin and actin were used as internal controls for the nuclear and cytosolic fractions, respectively. (B) RAW 264.7 cells were pre-treated with 50 μg/ml PME for 30 min prior to LPS stimulation for 30 min. p65 expression was analyzed by immunofluorescence staining. For visualization, p65 and nucleus were treated with Alexa Fluor 488-tagged anti-rabbit IgG and DAPI, respectively. (C) Density ratios of nucleic p65 were measured by densitometry. All values are the means ± standard deviation. \*P<0.05 compared with LPS-treated group.

tion (Fig. 4C). Compared with the LPS-stimulated RAW 264.7 cells, the PME-pre-treated cells maintained the basal levels of p65 intensity. Thus, PME disrupts the NF-κB transloca-

tion into the nucleus by helping to sustain IκBα expression, resulting in the transcriptional regulation of iNOS, COX-2 and pro-inflammatory cytokines.

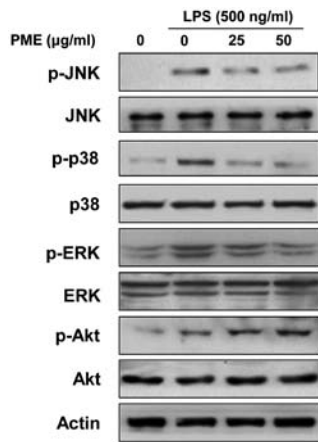


Figure 5. Effects of peat moss aqueous extract (PME) on the activation of mitogen-activated protein kinases (MAPKs) and Akt. RAW 264.7 cells were pre-treated with 50  $\mu\text{g/ml}$  PME for 1 h and then stimulated with lipopolysaccharide (LPS) for 30 min. The expression of p38 MAPK, JNK, ERK and Akt was measured by western blot analysis. Actin was used as an internal control for western blot analysis.

**Regulatory effects of PME on the activation of mitogen-activated protein kinases (MAPKs) and Akt.** We examined whether the activation of MAPKs and Akt in LPS-stimulated RAW 264.7 cells is regulated by PME. LPS stimulation for 30 min activated all members of MAPKs. Pre-treatment with PME for 1 h suppressed the phosphorylation of MAPKs in a dose-dependent manner (Fig. 5). Although PME significantly attenuated the activation of p38 MAPK and ERK, JNK was less sensitively regulated than p38 MAPK and ERK. Akt activation was increased by LPS stimulation, and treatment with PME induced the further amplification of Akt activation in a concentration-dependent manner. Our results suggest

that the inhibition of MAPK activation and the enhancement of Akt activation suppresses the inflammatory response in LPS-stimulated RAW 264.7 cells.

**Regulatory effects of PME on Nrf2 and HO-1 expression.** As PME prevented cell death from  $\text{H}_2\text{O}_2$ -induced oxidative stress (Fig. 1), we hypothesized that pre-treatment with PME may enhance the expression of antioxidant enzymes in RAW 264.7 cells. We examined whether the expression of HO-1 and Nrf2 was regulated by PME. Initially, the RAW 264.7 cells were cultured with PME at the indicated concentrations for 12 h. As shown by western blot analysis, PME gradually enhanced Nrf2 and HO-1 expression in a concentration-dependent manner (Fig. 6A). Using 50  $\mu\text{g/ml}$  PME, protein induction was evident at 6 h, and reached a maximum after 24 h of treatment with PME (Fig. 6B). We then examined the location of Nrf2 at 12 h using immunofluorescence staining. Immunofluorescent intensity in the nucleus showed that treatment with PME enhanced the translocation of Nrf2 into the nucleus (Fig. 6C and D). These results indicate that PME induces HO-1 expression by facilitating Nrf2 activation.

## Discussion

Peat moss has been used for the investigation of the absorbance efficacy of irons, such as nickel and copper, and is potentially used for purging hazardous irons from polluted water (14,15). In the biomedical field, hypersensitivity pneumonitis and chronic respiratory disorder of peat moss processing factory workers have been investigated (16,17). However, to the best of our knowledge, the anti-inflammatory or antioxidant effects of PME have not been investigated to date. In the present study, we demonstrate the anti-inflammatory and antioxidant properties of PME in LPS-stimulated RAW 264.7 cells.

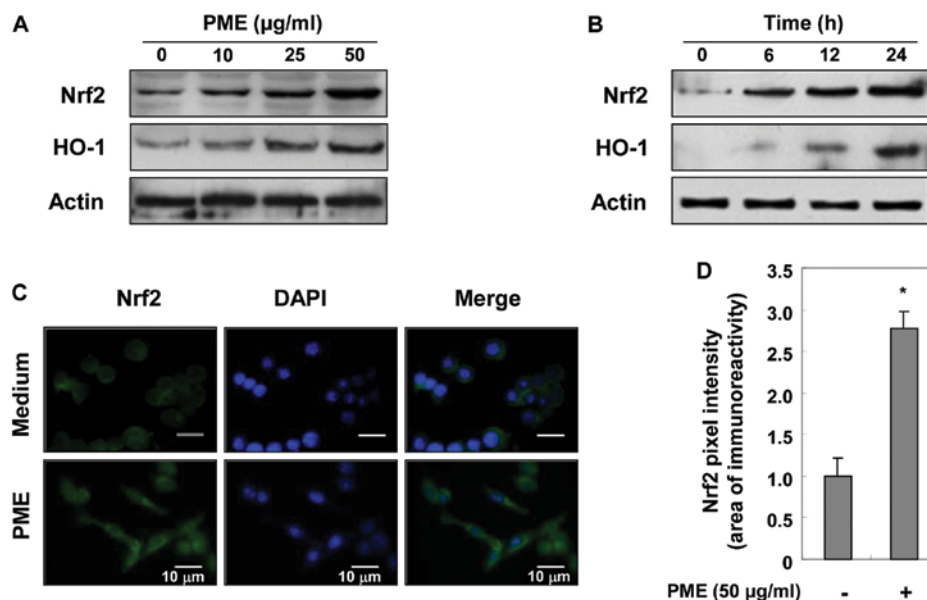


Figure 6. Effects of peat moss aqueous extract (PME) on the enhancement of nuclear factor-like 2 (Nrf2) and heme oxygenase-1 (HO-1) expression. (A and B) RAW 264.7 cells were treated PME at 10, 25 and 50  $\mu\text{g/ml}$  for 12 h (A) or 50  $\mu\text{g/ml}$  PME for different periods of time (B). Protein expression of Nrf2 and HO-1 was evaluated by western blot analysis. Actin was used as an internal control for western blot analysis. (C) RAW 264.7 cells were treated with 50  $\mu\text{g/ml}$  PME for 12 h. For visualization, Nrf2 and nucleus were treated with Alexa Fluor 488-tagged anti-rabbit IgG and DAPI, respectively. (D) Density ratios of nuclear p65 were measured by densitometry. All values are the means  $\pm$  SD. \* $P < 0.05$  compared with the control group (medium only).

In order to evaluate whether the inhibitory effects of PME on LPS-induced NO production, MTT assay was conducted. PME did not appear significantly cytotoxic to the RAW 264.7 cells, although it protected the cells against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. PME attenuated the LPS-induced NO and PGE<sub>2</sub> production, accompanied by the downregulation of iNOS and COX-2 expression. In addition, in RAW 264.7 cells PME negatively regulated the synthesis of pro-inflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , at the transcriptional level. The results of the present study provide evidence that PME induces the anti-inflammatory response in LPS-stimulated RAW 264.7 cells. The inducible enzymes (iNOS and COX-2) and their reaction products are associated with inflammatory diseases. Both enzymes are upregulated in the inflammatory response, and their reactive products, NO and PGE<sub>2</sub>, respectively, are closely related to various chronic diseases, such as ulcers and RA (18,19). TNF- $\alpha$  and IL-1 $\beta$  secreted from activated monocytes and macrophages exerts a variety of pro-inflammatory effects on many cell types (20). These cytokines are important in chronic inflammation, such as RA (21). Even though our experiments were conducted under acute inflammatory conditions, our results provide valuable information on the regulatory effects of PME on macrophages, cells critical in the process of chronic inflammation (22). There is evidence that a folk remedy, a mud bath including peat, relieves the symptoms of RA (23). Based on our results, peat moss may have anti-inflammatory abilities in acute inflammatory responses, which potentially ameliorates chronic inflammation, including rheumatoid diseases.

The LPS-induced expression of the iNOS, COX-2 pro-inflammatory cytokines was regulated by PME at the transcriptional level. In order to examine whether the inhibitory effects of PME are mediated through the inactivation of NF- $\kappa$ B, western blot analysis for nuclear p65 and cytosolic I $\kappa$ B $\alpha$  and a microscopic observation for p65 were performed in the present study. Pre-treatment with PME led to a significant decrease in nuclear p65 levels and an increase in cytosolic I $\kappa$ B $\alpha$  levels. The inhibitory effects of PME on p65 translocation into the nucleus and p65 expression were also shown by microscopic observation (Fig. 4). NF- $\kappa$ B has been implicated in the induction of the expression of iNOS and COX-2 protein. NF- $\kappa$ B is primarily composed of two proteins, p65 and p50. In the resting state, NF- $\kappa$ B can be found in the cytosol and is bound to the inhibitory protein, I $\kappa$ B. The activation of cells with various stimuli initiates I $\kappa$ B phosphorylation, which triggers proteolytic degradation. NF- $\kappa$ B is then released from the inactive complex, and translocates to the nucleus (24). Nuclear NF- $\kappa$ B binds to the  $\kappa$ B binding sites in the promoter regions of target genes. NF- $\kappa$ B response elements are present on the promoters of iNOS and COX-2 (24,25). NF- $\kappa$ B is also involved in the transcription of TNF- $\alpha$  and IL-1 $\beta$  (26). Our findings suggest that the inhibition of NF- $\kappa$ B by PME may be due to the inhibition of I $\kappa$ B $\alpha$  phosphorylation, thereby suppressing the translocation of p65. Therefore, the PME-mediated regulation of the expression of iNOS, COX-2, and pro-inflammatory cytokines was induced by the inhibition of NF- $\kappa$ B activation.

LPS-stimulated signaling events in macrophages lead to the activation of several MAPK signaling pathways. MAPKs consist of three major members, including ERK, p38 MAPK and JNK, which are activated by MAPK kinases (MEKs) in LPS-stimulated macrophages. Although LPS stimulation

activates all MAPK families, each kinase can be differentially activated in response to a particular stimuli. In the case of ERK activation, growth factors and phorbol esters play roles as stimulators. JNK and p38 MAPK are selectively activated by cellular stress, UV light and osmosis (27,28). LPS-induced MAPK activation leads to the expression of iNOS, COX-2, TNF- $\alpha$  and IL-1 $\beta$  in macrophages. The activation of ERK and p38 MAPK is related to the expression of COX-2, TNF- $\alpha$  and IL-1 $\beta$ . ERK and p38 MAPK activates cyclic AMP response element-binding protein (CREB) through mitogen- and stress-activated protein kinase (MSK)-1, resulting in the expression of COX-2 and IL-1 $\beta$ . In the case of IL-1 $\beta$ , the activation of MAP kinase-activated protein kinase 2 (MAPKAP-K2) by p38 MAPK mainly regulates IL-1 $\beta$  expression (29). iNOS expression by LPS stimulation is augmented by JNK activation. In LPS-stimulated macrophages, p38 MAPK counteracts JNK which suppresses iNOS expression. Treatment with SB203580, a p38 MAPK inhibitor, facilitates JNK phosphorylation and iNOS expression (30). In the present study, the activation of JNK, p38 MAPK and ERK was downregulated by PME in a concentration-dependent manner, suggesting that the inactivation of MAPKs by PME induces the downregulation of iNOS, COX-2, TNF- $\alpha$  and IL-1 $\beta$ .

Previous studies have indicated that PI3K/Akt-linked cascades are involved in the negative regulation of LPS-induced inflammatory responses (31,32). The present study investigated the effects of PME on Akt activation in LPS-stimulated RAW 264.7 cells. Our findings showed that Akt activation was increased by LPS stimulation, and was further enhanced by PME treatment (Fig. 5). Consistent with our results, resveratrol has been shown to enhance Akt activation which is mediated by the inhibition of inflammatory responses in LPS-stimulated RAW 264.7 cells. Treatment of LPS-stimulated RAW 264.7 cells with resveratrol augmented Akt activation, resulting in the downregulation of iNOS, COX-2, and TNF- $\alpha$  expression. However, the regulatory effects of resveratrol on the expression of inflammatory mediators were not induced under PI3K-inhibited conditions (33). As Akt activation is required for the inactivation of MAPKs, resveratrol does not appear to have anti-inflammatory effects under PI3K-inhibited conditions. Similarly, malvidin, a major red wine polyphenol, appears to have anti-inflammatory effects in LPS-stimulated macrophages through Akt activation. Treatment with malvidin amplified Akt activation in LPS-stimulated macrophages, playing a protective role in LPS-induced mitochondrial depolarization (34). In addition, Akt activation is closely associated with the induction of antioxidant effects through Nrf2 activation. The inhibition of PI3K leads to the reduction of Nrf2 activation, resulting in a decrease in HO-1 expression (35,36). Our results are consistent with these studies, in that the expression of Nrf2 and HO-1 was augmented by PME in unstimulated RAW 264.7 cells. Based on previous studies, our data suggest that the anti-inflammatory and antioxidant effects of PME are induced through the activation of Akt.

HO-1 belongs to a larger family of stress proteins whose transcriptional regulation also responds to cellular injury, including thermal or oxidant stress, and protects cells against stress (37). HO-1 plays a role as a rate-limiting enzyme in the production of bilirubin. In this reaction, heme-induced HO-1 catalyzes the conversion of heme into biliverdin which is then

changed into bilirubin, possessing antioxidant abilities (38,39). Studies have demonstrated that curcumin, a phenolic compound, prevents oxidative stress by increasing HO-1 activity (40,41). Elevated HO-1 activity induces an increase in glutathione levels in astrocytes. Furthermore, under hypoxic conditions, curcumin significantly amplifies HO-1 activity in vascular endothelial cells, protecting the cells against oxidative stress. In the case of Nrf2, it is a basic leucine zipper transcription factor activating the antioxidant response element (ARE) in the promoters of antioxidant genes. These data suggest that the expression of HO-1 is related to the antioxidative process (42). Nrf2 is sequestered in the cytosol by Keap1. Upon stimulation, Nrf2 is released from Keap1 and translocates to the nucleus to activate ARE on the HO-1 promoter (43). In addition, HO-1 negatively regulates iNOS expression in LPS-stimulated RAW 264.7 cells. A previous study on the anti-inflammatory effects of genipin, an aglycon of geniposide, proved that the inhibitory effects of genipin on iNOS expression are suppressed in HO-1-inhibited RAW 264.7 cells (44). Based on these previous studies, HO-1 and Nrf2 participate in antioxidant and anti-inflammatory processes. In the present study, pre-treatment with PME enhanced the viability of H<sub>2</sub>O<sub>2</sub>-treated RAW 264.7 cells (Fig. 1), and increased Nrf2 activation and HO-1 expression in intact RAW 264.7 cells (Fig. 6). Hence, our data indicate that PME increases Nrf2 activation and HO-1 expression which may lead to the prevention of oxidative stress-induced cell death and supports the negative regulation of iNOS expression in LPS-stimulated RAW 264.7 cells.

In conclusion, PME exerts an anti-inflammatory effects by regulating the production of pro-inflammatory mediators through the downregulation of NF- $\kappa$ B phosphorylation, and the inactivation of p38 MAPK and JNK in LPS-stimulated RAW 264.7 cells. Under oxidative conditions, PME improved cell viability by augmenting Nrf2 activation and HO-1 expression. These results suggest that PME may be a promising candidate for the treatment of inflammatory diseases by regulating inflammatory macrophages.

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