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Anti-inflammatory Effect of Flavonoids Kaempferol and Biochanin A-enriched Extract of Barnyard Millet (*Echinochloa crus-galli* var. *frumentacea*) Grains in LPS-stimulated RAW264.7 Cells

Ji Young Lee[1], Do Youn Jun[2], Young Ho Yoon[3], Jee Youn Ko[3], Koan Sik Woo[3], Mi Hee Woo[4] and Young Ho Kim[1]*

[1]*Laboratory of Immunobiology, School of Life Science and Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Korea*
[2]*Institute of Life Science and Biotechnology, Kyungpook National University, Daegu 702-701, Korea*
[3]*Functional Cereal Crop research Division. Department of Functional Crop, NICS, RDA, 627-803, Korea*
[4]*Department of Pharmacology, College of Pharmacology, Daegu Catholic University, Daegu 712-702, Korea*

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In order to compare the anti-inflammatory effects of five selected cereal grains－proso millet, hwanggeumchal sorghum, foxtail millet, barnyard millet, and adlay－the inhibitory activities of 80% ethanol (EtOH) extracts obtained from the individual grains on lipopolysaccharide (LPS)-induced nitric oxide (NO) generation were investigated in RAW264.7 cells. The EtOH extract of barnyard millet (*Echinochloa crus-galli* var. *frumentacea*) grains exhibited more potent anti-inflammatory activity than that of the other grains. When the EtOH extract of barnyard millet grains was sequentially fractionated with n-hexane, methylene chloride (MC), ethyl acetate (EtOAc), and n-butanol, the majority of the anti-inflammatory activity was detected in the MC fraction, followed by the EtOAc fraction. Pretreatment with the MC fraction caused downregulation of the expression levels of iNOS- and COX-2-specific transcripts and proteins, as well as proinflammatory cytokine gene transcripts (IL-1β, IL-6, and TNF-α) in LPS-stimulated RAW264.7 cells. Additionally, the MC fraction could suppress not only the LPS-induced nuclear translocation of cytosolic NF-kB, but also the LPS-induced activation of MAPKs, such as ERK, JNK, and p38MAPK. Further analysis of the MC fraction by HPLC identified kaempferol, biochanin A, and formononetin as the major phenolic components. Both kaempferol and biochanin A, but not formononetin, could exert anti-inflammatory effect at the same concentrations as those of the MC fraction. Consequently, these results indicate that kaempferol and biochanin A are among the most effective anti-inflammatory phenolic components in barnyard millet grains. This finding suggests that barnyard millet grains and the MC extract enriched in kaempferol and biochanin A could be beneficial functional food sources that have an anti-inflammatory effect.

Key words : Anti-inflammation, barnyard millet grains, cytokines, MAPKs, NF-κB

Introduction

Lipopolysaccharide (LPS) is an endotoxic component of the outer membranes of gram-negative bacteria and stimulates several pathological inflammatory responses, such as systemic inflammatory response syndrome, septic shock, disseminated intravascular coagulation and multiple organ dysfunctions [5]. These LPS-induced inflammatory diseases are known to be mediated primarily via deregulated overproduction of pro-inflammatory mediators, such as nitric oxide (NO), prostaglandin E_2 (PGE_2), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), by activated macrophages [21, 38]. The LPS-induced production of these pro-inflammatory mediators by macrophages is tightly regulated by two principal transcription factors, NF-κB and AP-1. These factors are activated by the transmembrane signaling pathway, which is triggered by the interaction of the cell surface CD14/toll-like receptor 4 (TLR4) complex with the hydrophobic lipid A portion of LPS, and then relayed by sequential activation of protein kinases [14, 22]. Among the critical protein kinases required for the signaling pathway are IL-1 receptor-associated kinase 4 (IRAK4) and IRAK1, TGF-β-activated kinase 1 (TAK1), IκB

***Corresponding author**
Tel : +82-53-950-5378, Fax : +82-53-955-5522
E-mail : ykim@knu.ac.kr

kinase (IKK), and MAPKs (p38MAPK, JNK and ERK) [2, 11].

In relation to LPS-induced TLR4-dependent activation of NF-κB in macrophages, the redox signaling mediated by reactive oxygen species (ROS) has also been implicated [9, 17, 20]. Supplementation of antioxidants in early inflammation stage has been shown to attenuate the oxidative stress by acting as ROS scavenger, and could prevent developing chronic inflammation [3]. In this context, it is likely that inhibition of oxidative stress might be one of the reliable strategies, which is beneficial for ameliorating metabolic diseases being accompanied by inflammation.

Much attention has been paid to the physiological functionality of foods, due to the increasing interest in human health, and research into the health benefits of foods has been increasing last years. Traditionally, miscellaneous cereal grains have been considered as a beneficial functional food source, which can improve the metabolic diseases [7, 8, 30]. Barnyard millet is one of the hardy crops that have adapted to sterile environments throughout the world [28]. The cultivation area of barnyard millet was gradually reduced due to various changes in current diet and cereal breeding. However, in recent years, barnyard millet grains are re-evaluated because of their high nutritional value in preventing metabolic diseases. The nutritive and biological studies about barnyard millet grains have demonstrated that the grains are rich in protein, lipid, vitamin B complex and nicotinic acid, when compared with common cereal grains. Previously, it has been reported that the extract of barnyard millet grains possess anti-oxidant activity [12, 13], a beneficial influence on metabolism of cholesterol and lipid [27], immunosuppressive activity [15], anti-microbial activity [29] and lowering glycemic index and anti-diabetes effects [23, 27, 36]. Several bioactive components have been found from barnyard millet grains including serotonin, luteolin, tricin, deoxynojirimycin and coumaric acid derivatives [33, 37], but studies on the health benefits of barnyard millet grains with respect to anti-inflammatory activity are not well-established.

In the present study, as an attempt to compare anti-inflammatory activities of five selected miscellaneous cereal grains, such as proso millet (*Panicum miliaceum*), hwanggeumchal sorghum (*Sorghum bicolor* (L.) Moench var. *hwanggeumchal*), yellow glutinous foxtail millet (*Setaria italica*), adlay (*Coix lacryma-jobi*), and barnyard millet (*Echinochloa crus-galli* var. *frumentacea*) harvested in Korea, we investigated the anti-NO production activity of the 80% ethanol (EtOH) extracts from the individual grains in LPS-stimulated murine macrophage RAW264.7 cells. The EtOH extract of barnyard millet grains, which exhibited more potent anti-inflammatory effect compared with other grain extracts, were sequentially fractionated by n-hexane, MC, EtOAc, and BuOH. Because the inhibitory effect on LPS-induced NO production in RAW264.7 cells were mainly detected in the MC fraction followed by the EtOAc fraction, the anti-inflammatory activity of the MC fraction was further examined by investigating its inhibitory action against LPS-induced inflammatory events and by detecting the active phenolic components in the MC fraction.

Materials and Methods

Reagents, chemicals, antibodies and culture medium

The ECL Western blotting kit was purchased from PerkinElmer (Boston, MA, USA), and Immobilon-P membrane was obtained from Millipore Corporation (Bedford, MA, USA). The anti-COX-2, anti-β-actin, anti-NF-κB p65, anti-p-JNK, anti-JNK, anti-Sp1 and anti-p38 MAPK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the anti-p-p38 MAPK, anti-IκBα, anti-p-IκBα and p-c-jun antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The anti-p-ERK was obtained from Millipore Corporation, and anti-ERK antibody was obtained from Zymed Laboratories (South San Francisco, CA, USA). Anti-iNOS was purchased from BD Biosciences (Chicago, IL, USA). Horse radish peroxide (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG were obtained from Cell Signaling, and HRP-conjugated anti-goat IgG was obtained from Santa Cruz Biotechnology. The murine macrophage cell line RAW264.7 was purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml gentamycin at 37°C in a humidified 5% CO_2 atmosphere. For the experiments, the cells were grown to 80-90% confluences, and were subjected to no more than 15 cell passages.

Sample extraction

Barnyard millet grains were provided by National Institute of Crop Science of Miryang, Korea, and a voucher specimen has been deposited in Laboratory of Immunology, College of Natural Sciences, Kyungpook National University, Daegu, Korea. The dried grains (250 g) were mil-

led on a Blender 7012 (Dynamics Corporation, USA) for 10 min, and then extracted with 80% ethanol (EtOH) for 3 hr at 80°C. The ethanol extract was evaporated, dissolved in water, and then sequentially extracted with n-hexane, methylene chloride (MC), ethyl acetate (EtOAc) and n-butanol (BuOH). Each organic solvent fractionation was repeated three times. Each organic solvent fraction as well as the remnant aqueous fraction was centrifuged at 7,500 rpm for 15 min to remove insoluble substances. The recovered supernatant of each fraction was then concentrated by rotary vacuum evaporator (Heidolph LR 4000, Germany). The yields of hexane fraction, MC fraction, EtOAc fraction, BuOH fraction, remnant aqueous fraction were 9.4 g, 2.5 g, 1.1 g, 0.7 g, 2.3 g and 3.1 g, respectively.

Cell viability assay

The Cytotoxic effect of samples on RAW264.7 cells was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells (0.5×10^5/well) were cultured with serial dilutions of samples in 96-well plates. After incubation for 18 hr, 50 μl of MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 2 hr. The colored formazan crystal produced from MTT was dissolved in DMSO. The absorbance was measured at 540 nm by a plate reader (Molecular Devices, Thermo Max, USA) to determine the formazan concentration, which reflects the cell viability.

Nitric oxide assay

As an indicator of NO production, the concentration of nitrite, a stable metabolite of NO, in the culture medium was assessed by Griess reagent [10]. Briefly, RAW264.7 cells (2×10^5 cells/well) were cultured overnight in 96-well plates, and then treated with LPS (0.1 μg/ml) in the absence or in the presence of various concentrations of sample for 16 hr. The culture supernatant (100 μl) was mixed with an equal volume of Griess reagent for 15 min at room temperature in dark condition, and then the absorbance of the chromophoric azo-derivative molecule was measured using a microplate reader at 540 nm. To ensure the validity of the results, experiments were done in three independent experiments with three replicates per independent experiment.

Total RNA isolation and RT-PCR

Cells were washed twice in PBS, then total RNA was isolated using the Trizol reagent from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's instructions and DNase I treatment. After RNA quantification by GE NanoVue Spectrophotometer (GE healthcare, Buckinghamshire, UK), 1 μg RNA was reversely transcribed using First strand cDNA synthesis kit (Thermo scientific, Logan, UT, USA) for cDNA synthesis. Gene expression values were normalized to housekeeping GAPDH gene. GAPDH was amplified with forward (5′-ATCCTGCGTCTGGACCTGGCT-3′) and reverse (5′-CTGATCCACATCTGCTGGAAG-3′) primers. PCR amplification was done using AccuPower™ PCR PreMix (Bioneer, Seoul, Korea) and specific primers. The following primers were used for PCR: iNOS-forward, 5′-ATGTCCGAAGCAAACATCAC-3′; iNOS-reverse, 5′-TAATGTCCAGGAAGTAGGTG-3′; COX-2-forward, 5-CAGCAAATCCTTGCTGTTCC-3; COX-2-reverse, 5′-TGGGCAAAGAATGCAAACATC-3′; TNF-α-forward, 5′-TACTGAACTTCGGGGTGATCGGTCC-3′; TNF-α-reverse, 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′; IL-6-forward, 5′-GAAATGATGGATGCTTCCAAACTGG-3′; IL-1β-forward, 5′-CAAGGAGAACCAAGCAAC-3′; IL-1β-reverse, 5′-GGGGAAGGCAATAGAAAC-3′. To ensure that the same amount of RNA was being used, the concentration of the total RNA for each sample was confirmed by spectrophotometry and normalized with GAPDH as the message of a housekeeping gene. The PCR products were electrophoresed using 1.2% agarose gel and visualized under UV light after ethidium bromide staining.

Preparation of cell lysate and western blot analysis

Cellular lysates were prepared by suspending cells (5×10^6) in 300 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 25 mM MOPS, 1 mM PMSF, and 5.0 μg/ml proteinase inhibitor E-64, 0.1% Triton X-100, pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. An equivalent amount of protein lysate (25 μg) was electrophorersed on 4~12% NuPAGE gradient gel (Invitrogen/Novex, Carlsbad, CA, USA) with MOPS buffer and then electrotransferred to Immobilon-P membranes. Detection of each protein was performed utilizing the ECL Western blotting kit following the manufacturer's instructions. Densitometry was performed using ImageQuant TL software (Amersham, Arlington Heights, IL, USA). Arbitrary densitometric units for the protein of interest were normalized to the densitometric units of β-actin.

High-performance liquid chromatography (HPLC) analysis of 80% ethanol extract and its organic solvent fractions of barnyard millet grains

The contents of phenolic compounds were analyzed as previously described [32]. Samples were filtered through a 0.45 μm syringe filter (Millipore, Billerica, MA, USA) and analyzed by HPLC (Agilent 1200, Agilent Technologies, Waldbronn, Germany). The analytical column was a ZORBAX ODS (4.6×250 mm, Agilent Technologies) with a guard column (Phenomenex, Torrance, CA, USA). The detection wavelength was set at 280 nm and the solvent flow rate was held constant at 1.0 ml/min. The mobile phase used for the separation consisted of solvent A (distilled water included 0.1% acetic acid) and solvent B (acetonitrile included 0.1% acetic acid). A gradient elution procedure was used as 0 min 92% A, 2-27 min 90% A, 27-50 min 70% A, 50-51 min 10% A, 51-60 min 0% A, and 60-62 min 92% A. The injection volume was 20 μl for analysis. The standards used were biochanin A, caffeic acid, (±)-catechin hydrate, chlorogenic acid, *trans*-cinnamic acid, formononetin, gallic acid, hesperidin, homogentisic acid, isoorientin, kaempferol, naringin, orientin, protocatechuic acid, pyrogallol, quercetin, resveratrol, rutin hydrate, syringic acid, vanillic acid, vanillin, veratric acid and all samples were analyzed in triplicate.

Statistical analysis

Unless indicated otherwise, each result in this paper is representative of at least three separate experiments. Values represent the mean standard deviation (SD) of these experiments. The statistical significance was calculated with Student's t-test. *P* values less than 0.05 were considered significant.

Results and Discussion

Anti-NO production activity of the 80% EtOH extract and its organic solvent fractions obtained from barn yard millet grains in LPS-stimulated RAW264.7 cells

In order to compare the anti-inflammatory effects of five selected miscellaneous cereal grains, including proso millet (*Panicum miliaceum*), hwanggeumchal sorghum (*Sorghum bicolor* (L.) Moench var. *hwanggeumchal*), yellow glutinous foxtail millet (*Setaria italica*), adlay (*Coix lacryma-jobi*), and barnyard millet (*Echinochloa crus-galli* var. *frumentacea*) harvested in Korea, the inhibitory activities of the 80% ethanol extracts of the individual cereal grains on LPS-induced NO production were investigated in RAW264.7 cells. As shown in Fig. 1A, the EtOH extracts of barnyard millet grains, hwanggeumchal sorghum grains, and proso millet grains appeared to exhibit more potent anti-NO production activities compared with other cereal grains tested. The LPS-induced NO productions in the presence of the EtOH extracts of barnyard millet grains and hwanggeumchal sorghum grains at concentrations of 100 μg/ml were reduced to the levels of 56.4% and 52.3%, respectively, whereas those at concentrations of 200 μg/ml were reduced to the levels of 3.4% and 10.8%, respectively. However, under the same conditions, none of these EtOH extracts could affect the cell viability of RAW264.7 cells (Fig. 1B). These results indicated that anti-inflammatory effect of barnyard millet grains was the most

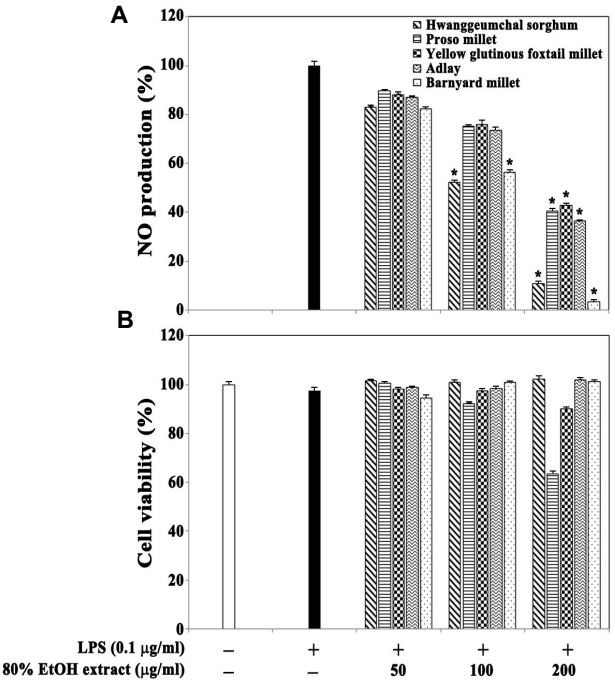

Fig. 1. Effect of the 80% EtOH extracts of five selected different miscellaneous cereal grains on LPS-induced NO production (A) and viability (B) in RAW264.7 cells. After RAW 264.7 cells (2×10^5 cells/well) were incubated in 96 well plates for 16 hr, the cells were treated with the individual 80% ethanol extracts (50, 100, and 200 μg/ml) for 1 hr and then continuously incubated with LPS (0.1 μg/ml) for 20 hr. The nitrite concentration as an indicator of NO production in culture medium was measured using Griess reagent. The cell viability was determined by the MTT assay as described in Materials and Methods. Each value is expressed as mean ± SD (n=3 with six replicates per independent experiment). *p<0.05, significant compared with vehicle- treated control.

potent among the five selected miscellaneous cereal grains tested, and that the EtOH extract of barnyard millet grains at concentrations of 100-200 μg/ml could significantly reduce the LPS-induced production of NO without affecting cell viability in RAW264.7 cells.

In order to examine further the anti-NO production property of barnyard millet grains, the 80% EtOH extract of barnyard millet grains was sequentially fractionated with n-hexane, MC, EtOAc, and BuOH, and then individual organic solvent fractions at concentrations ranging from 25-100 μg/ml were tested for the anti-NO production activity. As shown in Fig. 2A, the LPS-induced NO production was the most significantly suppressed in the presence of the MC fraction, followed by the EtOAc fraction. In addition, the LPS-induced NO productions in the presence of the MC fraction at concentrations of 25 μg/ml, 50 μg/ml and 100 μg/ml were reduced to the levels of 81.7%, 35.2% and 1.4%, respectively. At the same time, the MC fraction at concentrations of up to 100 μg/ml did not affect the cell viability (Fig. 2B).

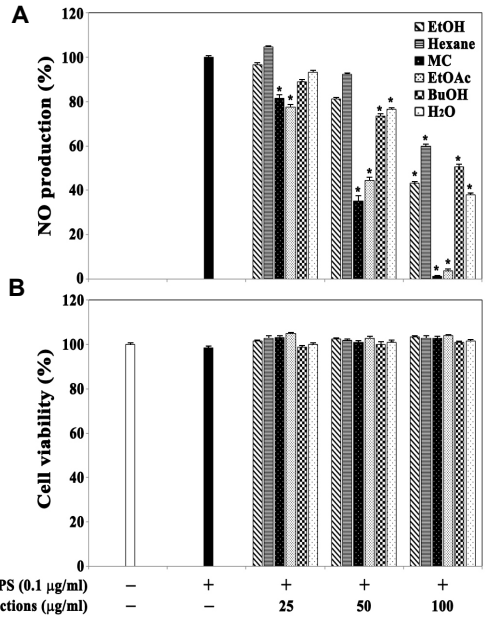

Fig. 2. Effect of the 80% EtOH extract and its organic solvent fraction of barnyard millet grains on LPS-induced NO production (A) and viability (B) in RAW264.7 cells. After RAW 264.7 cells (2×10^5 cells/well) were incubated in 96 well plates for 16 hr, the cells were treated with the individual 80% ethanol extracts (25, 50, and 100 μg/ml) for 1 hr and then continuously incubated with LPS (0.1 μg/ml) for 20 hr. The nitrite concentration as an indicator of NO production in culture medium was measured using Griess reagent. The cell viability was determined by the MTT assay as described in Materials and Methods. Each value is expressed as mean ± SD (n=3 with six replicates per independent experiment). *p<0.05, significant compared with vehicle-treated control.

Consequently, these results indicate that the MC fraction of barnyard millet grains at concentrations of 25-100 μg/ml could suppress the LPS-induced NO production in a dose-dependent manner, and that the IC_{50} value of the MC fraction was 44.8 μg/ml.

Inhibitory effect of the MC fraction of barnyard millet grains on LPS-induced expression of iNOS, COX-2, and pro-inflammatory cytokines in RAW264.7 cells

Because the production of pro-inflammatory mediators, such as NO and prostaglandin E_2 (PGE_2), in RAW64.7 cells stimulated with LPS is governed by the enzymes iNOS and COX-2, RT-PCR and western blot analysis were performed to examine whether the MC fraction of barnyard millet grains could suppress the expression levels of iNOS and COX-2 in RAW264.7 cells stimulated with LPS. As shown in Fig. 3A, RT-PCR data revealed that the expression level of mRNAs specific for iNOS and COX-2, which were not detected in unstimulated RAW264.7 cells, were significantly enhanced following LPS-stimulation; however, the presence of the MC fraction at concentration of 25-100 μg/ml down-regulated the levels of iNOS and COX-2 mRNAs. Under these conditions, western blot analysis data also revealed that although the proteins specific for iNOS and COX-2 were not detected in continuously growing RAW 264.7 cells, the levels of both proteins were enhanced by 5.8 folds and 5.2 folds, respectively, in RAW264.7 cells stimulated with LPS (Fig. 3B). However, the LPS-induced increase in the levels of iNOS and COX-2 proteins was markedly reduced by the MC fraction in a dose-dependent manner. In particular, LPS-induced expression of both iNOS and COX-2 proteins was not detected in the presence of the MC fraction at a concentration of 100 μg/ml. These results demonstrated that the MC fraction of barnyard millet grains at concentrations of 25 100 μg/ml could reduce the expression levels of the pro-inflammatory proteins (iNOS and COX-2) in RAW264.7 cells stimulated with LPS via the down-regulation of mRNA levels.

To examine whether the MC fraction could suppress the expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, RAW264.7 cells were stimulated with LPS for 4 hr following pretreatment with the MC fraction (25-100

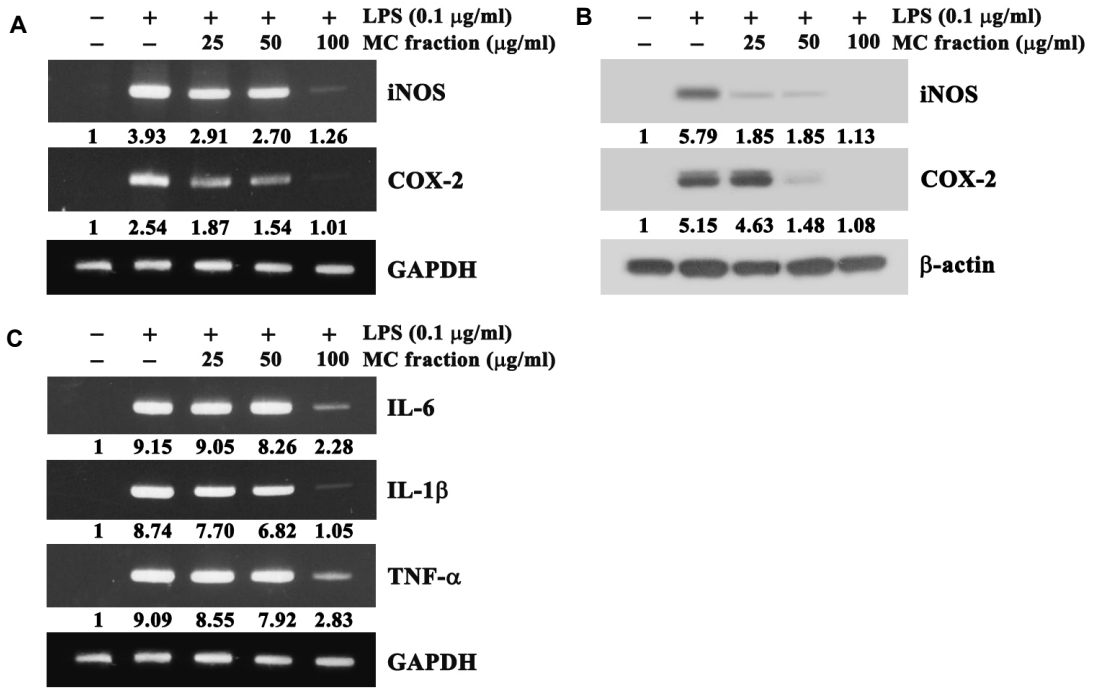

Fig. 3. Effect of the MC fraction of barnyard millet grains on the expression levels of iNOS, COX-2 and pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in RAW264.7 cells stimulated with LPS. The cells were pretreated with the MC fraction at indicated concentrations, prior to stimulation with LPS (0.1 μg/ml) for 20 hr. RT-PCR analysis of transcripts of iNOS, COX-2 and GAPDH (A) and IL-6, IL-1β, TNF-α and GAPDH (C), and western blot analysis of iNOS, COX-2 and β-actin proteins (B) were performed as described in the Materials and Methods. The expression level of GAPDH mRNA or β-actin protein was used as control. Arbitrary densitometric units for the individual transcripts and proteins of interest were normalized to the densitometric units of GAPDH transcript and β-actin protein, respectively. A representative study is shown and two additional experiments yielded similar results.

μg/ml) for 1 hr. The levels of mRNAs specific for IL-1β, IL-6, and TNF-α were assessed by RT-PCR. As shown in Fig. 3C, the mRNAs specific for IL-1β, IL-6, and TNF-α were not detected in unstimulated RAW264.7 cells, but the expression levels of these cytokine-specific mRNAs were markedly up-regulated in RAW264.7 cells following stimulation with LPS. Under the same conditions, the LPS-induced expression of IL-1β, IL-6, and TNF-α mRNA was reduced in the presence of the MC fraction, more efficiently at a concentration of 100 μg/ml. These results suggested that the MC fraction at concentrations of 25-100 μg/ml could inhibit the LPS-induced up-regulation of the expression levels of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α.

Inhibitory effect of the MC fraction of barnyard millet grains on the nuclear translocation of NF-κB complex in RAW264.7 cells

The LPS-induced expression of pro-inflammatory mediators including iNOS, COX-2, IL-1β, IL-6 and TNF-α in macrophages is known to be tightly regulated by two principal transcription factors, NF-κB and AP-1 [21, 34, 38]. Nuclear translocation of NF-κB and proteasomal degradation of IκBα have been considered as prominent markers that exhibit molecular inflammation initiation for the expression of pro-inflammatory mediators in activated macrophages.

To determine whether the MC fraction of barnyard millet grains could inhibit LPS-induced activation of NF-κB in RAW264.7 cells, LPS-induced phosphorylation of IκBα, alterations in the level of IκBα, and nuclear translocation of NF-κB (p65), all of which are known to be critical for the activation of NF-kB, were compared by western blot analysis in RAW264.7 cells stimulated with LPS with and without the MC fraction (25-100 μg/ml). As shown in Fig. 4, LPS-stimulation resulted in an increase in the phosphorylation level of IκBα and a decrease in the protein level of IκBα in RAW264.7 cells. At the same time, LPS-stimulation caused nuclear translocation of cytosolic NF-κB (p65) so that the level of nuclear NF-κB could be enhanced by approximately 7 folds compared with that of untreated RAW264.7 cells.

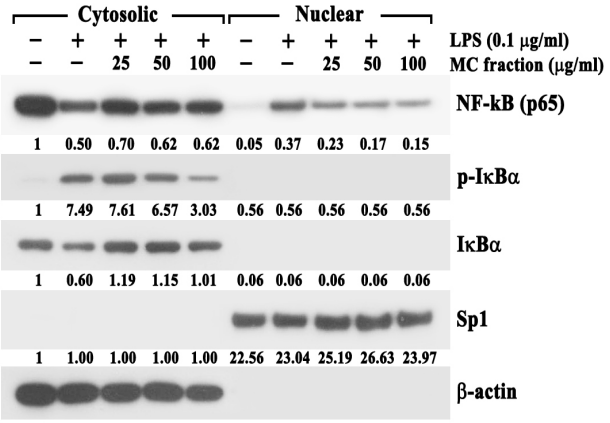

Fig. 4. Inhibition of the MC fraction of barnyard millet grains on IκBα phosphorylation, IκBα protein levels, and nuclear translocation of cytosolic NF-κB (p65) in RAW264.7 cells following LPS stimulation. The cells were treated by the MC fraction at the indicated concentrations for 1 hr, and then stimulated with LPS (0.1 μg/ml) for 4 hr to detect IκBα and its phosphorylation by western analysis. A representative study is shown and two additional experiments yielded similar results.

However, the LPS-induced phosphorylation of IκBα, down-regulation of IκBα protein levels, and nuclear translocation of cytosolic NF-κB (p65) appeared to be suppressed by the MC fraction in a dose-dependent manner. These results suggested that the MC fraction of barnyard millet grains could inhibit LPS-induced activation of NF-κB by suppressing the LPS-induced phosphorylation and degradation of IκBα, and subsequent nuclear translocation of NF-kB in RAW264.7 cells.

Inhibitory effect of the MC fraction of barnyard millet grains on LPS-induced phosphorylation of MAPKs in RAW264.7 cells

Several studies have reported that MAPKs (p38MAPK, JNK and ERK) are closely associated with the TLR4-mediated proximal signaling events that lead to the activation of transcription factors NF-kB and AP-1 in LPS-stimulated macrophages [2, 11]. Because the MC fraction of barnyard millet grains appeared to suppress the LPS-induced phosphorylation of IκBα, alterations in the level of IκBα, and nuclear translocation of NF-κB (p65), all of which are critical for activation of the transcription factor NF-kB, we decided to examine whether the LPS-induced activation of p38MAPK, JNK, and ERK could be targeted by the inhibitory action of the MC fraction of barnyard millet grains by western blot analysis using individual antibodies specific for the phosphorylated active forms of these MAPKs. Although the total protein levels of p38MAPK, JNK, and ERK all of which were easily detected in unstimulated RAW264.7 cells, were not changed regardless of the presence of the MC fractions, the active phosphorylated forms of p38MAPK, JNK, and ERK were significantly enhanced after LPS treatment (Fig. 5). Under these conditions, however, the LPS-induced phosphorylation of p38MAPK, JNK and ERK was commonly reduced by the MC fraction. In accordance with the observed reduction in the level of LPS-induced phosphorylation of JNK, the phosphorylation of c-Jun protein at Ser-63, which is catalyzed by JNK [6], was markedly reduced in the presence of the MC fractions. This finding indicated that the phosphorylated JNK that was detected in RAW264.7 cells after treatment with LPS possessed enough enzymatic activity to phosphorylate c-Jun. Consequently, these results indicated that the LPS-induced activation of p38MAPK, JNK

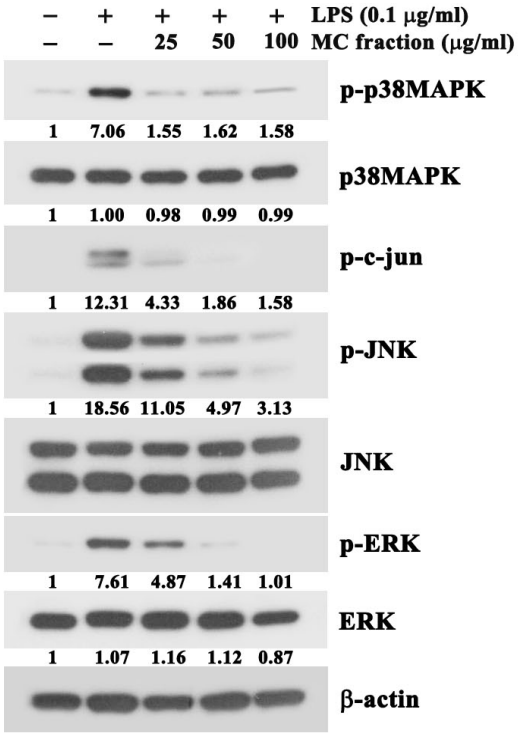

Fig. 5. Effect of the MC fraction of barnyard millet grains on LPS-induced activation of MAPKs (p38MAPK, JNK and ERK) in RAW264.7 cells. The cells were pre-treated with indicated concentrations of the MC fraction for 1 hr, and then with LPS (0.1 μg/ml) for 20 hr. The levels of the active phosphorylated forms of p38MAPK, c-Jun, JNK, and ERK were assessed by western blot analysis using antibodies specific for the phosphorylated forms of individual kinases. A representative study is shown and two additional experiments yielded similar results.

and ERK was influenced by the inhibitory action of the MC fraction. In addition, these results also suggested that inhibitory action of the MC fraction against the LPS-induced nuclear translocation of NF-kB in RAW264.7 cells might be due to the decline in the level of activation of p38MAPK, JNK and ERK.

Identification of the major anti-inflammatory phenolic compounds in the MC fraction of barnyard millet grains

Among phytochemicals, phenolic compounds have been reported to have various ameliorating effects on neurodegenerative diseases, multiple sclerosis, cardiovascular diseases, and metabolic syndrome from oxidative stress [16, 35]. In order to identify the major anti-inflammatory ingredient(s) of the MC fraction of barnyard millet grains, we decided to analyze phenolic compounds contained in the MC fraction by HPLC. As the major phenolic components, kaempferol (9.17 μg/mg), biochanin A (2.22 μg/mg), and formononectin (1.52 μg/mg), which accounted for 85% of the total phenolic compounds, were detected in the MC fraction.

Because it has previously been reported that kaempferol [4, 18], biochanin A [19], and formononectin [26] possess anti-inflammatory activity, the anti-NO production activities of these phenolic compounds were examined in LPS-stimulated RAW264.7 cells. As shown in Fig. 6A, when the inhibitory activities of kaempferol, biochanin A, and formononectin against LPS-induced NO production were examined at concentrations of 10 μM, 25 μM, and 50 μM in RAW264.7 cells, kaempferol reduced the LPS-induced NO production to the levels of 85.3%, 69.1%, and 45.7%, respectively, whereas biochanin A reduced the NO production to the levels of 79.6%, 60.9%, and 39.7%, respectively. Under these conditions, both kaempferol and biochanin A did not show a significant cytotoxic effect on RAW264.7 cells. Although formononetin has previously been reported to inhibit the inflammation in mouse lung injury model [26], it failed to inhibit the LPS-induced NO production to a remarkable level. It is noteworthy that hesperidin (0.33 μg/mg) [1, 31], naringin (0.56 μg/mg) [25], and protocatechuic acid (0.34 μg/mg) [24] as the minor components, which were reported to possess anti-inflammatory activity, were detected in the MC fraction. Consequently, these results indicated that kaempferol and biochanin A, but not formononetin, could inhibit the LPS-induced NO production

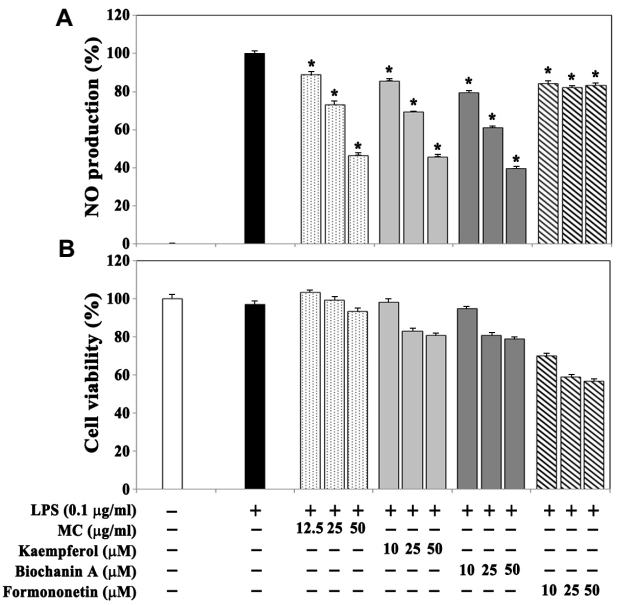

Fig. 6. Comparison of the anti-NO production activities (A) and cytotoxicity (B) of phenolic compounds (kaempferol, biochanin A, and formononetin) in LPS-stimulated RAW 264.7 cells. The cells were pre-incubated for 1 hr with the MC fraction (12.5 μg/ml, 20 μg/ml and 50 μg/ml), kaempferol (10 μM, 25 μM, and 50 μM), biochanin A (10 μM, 25 μM, and 50 μM), or formononetin (10 μM, 25 μM, and 50 μM) in triplicate and then treated with LPS (0.1 μg/ml) for 4 hr. The culture supernatants were saved and used to determine NO production. MTS were employed to check the cell viability. Each value is expressed as mean ± SD (n=3 with six replicates per independent experiment). *p<0.05, significant compared with vehicle-treated control.

in a dose-dependent manner, and that both kaempferol and biochanin A were among the most effective anti-inflammatory phenolic components in barnyard millet grains.

In conclusion, this study describes an anti-inflammatory activity of barnyard millet grains against LPS-induced inflammatory events in mouse macrophage cell line RAW 264.7, and demonstrates that this anti-inflammatory action is attributable to suppression of LPS-induced up-regulation of pro-inflammatory modulators including iNOS, COX-2, IL-1β, IL-6, and TNF-α, via inhibition of nuclear translocation of cytosolic NF-kB as well as inactivation of MAPKs. As the active phenolic ingredient in the MC fraction responsible for the inflammatory activities, kaempferol and biochanin A, which are detected as the major phenolic compounds in the MC fraction of barnyard millet grains, are identified. Current results also suggest that barnyard millet grains and the MC extract enriched in kaempferol and bio-

chanin A could be beneficial functional food sources applicable to improving inflammatory conditions.

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초록 : 마우스 대식 세포주 RAW264.7에 있어서 LPS처리에 의해 유도되는 염증반응에 대한 식용피 (*Echinochloa crus-galli* var. *frumentacea*)의 저해효과

이지영[1] · 전도연[2] · 윤영호[3] · 고지연[3] · 우관식[3] · 우미희[4] · 김영호[1]*

([1]경북대학교 자연과학대학 생명과학부, [2]경북대학교 생명공학연구소, [3]농촌진흥청 식량과학원 기능성작물부, [4]대구카톨릭대학교 약학대학 약학과)

잡곡은 대사성 질환의 예방과 치료에 우수한 기능성 식품소재로 인식되고 있다. 국내산 잡곡들(기장, 황금찰수수, 노랑차조, 율무 및 식용피)을 대상으로 항염증 활성을 비교조사하고자, 이들 잡곡의 80% 에탄올 추출물을 이용하여 마우스 대식세포주 RAW264.7을 지질다당류(LPS)로 자극할 때 생성되는 염증 매개인자인 산화질소(NO)의 생성에 미치는 저해능을 조사하였다. 그 결과, 식용피(*Echinochloa crus-galli* var. *frumentacea*) 유래의 에탄올 추출물이 LPS에 의해 유도되는 NO의 생성을 저해하는 항염증 활성에 있어서 가장 우수함을 확인하였다. 식용피의 에탄올 추출물로부터 항염증 관련 성분을 규명하기 위해, 80%에탄올 추출물을 물에 녹인 후 n-hexane, methylene chloride (MC), ethyl acetate (EtOAc), n-butanol (BuOH)과 물 분획들로 단계별 분획하고, 각 분획의 항염증 활성을 조사하였다. RAW264.7 세포에 MC 분획(100 μg/ml)을 전처리하였을 때, LPS처리에 의해 유도되는 iNOS, COX-2, 그리고 친염증성 사이토카인들(IL-1β, IL-6, 및 TNF-α)의 발현이 현저하게 저해되는 것으로 나타났다. 또한, LPS처리에 의해 유도되는 p38MAPK, JNK 및 ERK의 활성화의 경우도 MC 분획에 의해 농도-의존적으로 저해되는 것으로 나타났다. HPLC분석을 통해, 식용피 유래 MC 분획의 항염증 활성은 식용피의 MC 분획 속의 주요 성분인 kaempferol과 biochanin A에 기인함을 확인하였다. 이상의 연구 결과는 식용피 및 식용피의 MC 분획이 염증성 질환과 이와 관련된 대사성 질환의 예방과 예후개선에 유리한 기능성 식품소재 개발에 활용될 수 있음을 시사한다.