Korean J. Food & Nutr. Vol. 24. No. 2, 268～272 (2011)
<연구노트>

THE KOREAN JOURNAL OF
한국식품영양학회지
FOOD AND NUTRITION

# Anti-Inflammatory Activities of Hog Millet (*Panicum miliaceum* L.) in Murine Macrophages through IRAK-4 Signaling

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# 대식세포에서 IRAK-4 신호조절을 통한 기장(*Panicum miliaceum* L.)의 항염증능에 관한 연구

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## Abstract

전곡류의 섭취와 만성질환의 유병율은 음의 상관관계가 있는 것으로 알려져 있다. 본 연구에서는 선행 연구 결과, 지방축적억제능이 우수한 소재로 선정된 기장의 항염증능 여부를 검증하고자 하였다. 이를 위해 RAW264.7 세포에 기장열수분획(1 ㎍/㎖ 및 10 ㎍/㎖)과 lipopolysaccharide(LPS)를 함께 처리한 후, 24시간 배양시켜 염증매개인자들의 분비량 및 mRNA 발현 정도를 측정하였다. 또한 LPS 자극에 대한 첫 번째 신호전달인자로 알려져 있는 interleukin-1 receptor associated kinase-4(IRAK-4)의 단백질 발현 정도를 측정하였다. 본 연구결과, 기장의 열수분획(10 ㎍/㎖)은 LPS로 유도된 NO, $PGE_2$, TNF-α, IL-6 및 MCP-1의 생성량 및 mRNA 발현량을 유의적으로 억제하였다($p<0.05$). 특히 이들 지표 중 pro-inflammatory cytokine인 TNF-α와 IL-6의 mRNA 발현량이 효과적으로 감소하였다($p<0.01$). IRAK-4의 단백질 발현량 또한 유의적으로 감소하여 LPS 자극에 대한 기장열수분획의 항염증능은 toll-like receptor(TLR)를 통한 IRAK-4를 매개로 하는 신호전달체계 조절에 기인하는 것으로 사료된다.

Key words: *Panicum miliaceum*, water extract, inflammation, macrophages

## INTRODUCTION

Macrophages, cells that secrete molecules such as nitric oxide (NO), prostaglandin $E_2$ ($PGE_2$), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) among others, are important in the treatment of inflammation and disease (Hwang et al. 2011). This is because the downregulation of these pro-inflammatory molecules may exert anti-inflammatory effects. The release of pro-inflammatory mediators depends on inducible gene expression. Among several protein kinases, interleukin-1 receptor associated kinase-4 (IRAK-4) is known to be the initial protein kinase activated by the 'downstream' of the TLR signaling pathway (Hirayama et al. 2011). IRAK4 triggers the activation of NFκB and the phosphorylation of JNK and p38 MAPK (Asehnoune et al. 2004; Koziczak-Holbro et al. 2007).

Numerous studies have shown that high intake of whole grain foods has been linked with the reduced risk of several chronic diseases that are associated with chronic inflammation, such as obesity, metabolic syndrome, hypertension, and certain types of cancer (Misra et al. 2009). Whole grains such as foxtail millet, hog millet, sorghum, Job's tear, and barley contain high fiber and polyphenols (Gabrovská et al. 2002). In our preliminary study,

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hog millet (*Panicum miliaceum* L.) showed the highest anti-obesity activity among nine cereal types and significantly decreased the mRNA expression of peroxisome proliferator-activated receptor-$\gamma$ (PPAR$\gamma$). PPAR$\gamma$ agonists have been shown to inhibit many cytokines related to inflammatory processes. To determine whether hog millet has anti-inflammatory effects, we examined the protein release of pro-inflammatory mediators and their gene expression in LPS-stimulated RAW 264.7 cells. Additionally, we investigated whether hog millet has any influence on IRAK-4 activation.

## MATERIALS AND METHODS

### 1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, an antibiotic-antimycotic solution, and trypsin-EDTA were purchased from GIBCO co. (Grand Island, NY, USA). Lipopolysaccharide (LPS, *Escherichia coli* 0111: B4) and other chemicals otherwise indicated were purchased from Sigma (St. Louis, MO, USA).

### 2. Hot-water Extract of Hog Millet

Hog millet was obtained from National Agricultural Cooperative Federation (Shinlim, Wonju, Korea). The cereal grains were ground into fine powder. Then, the powdered material (50 g) was ultrasonically extracted with 500 mℓ of hot water extract 2 times for 3 hours at room temperature, and concentrated at 80℃ in a rotator vacuum evaporator (EYELA N-1000, Tokyo Riakikai Co., Ltd. Japan). The extracts were then freeze-dried and stored at −70℃. The extract yield of hog millet was found to be 5.3% in this study.

### 3. Cell Culture

Mouse macrophage RAW 264.7 cells obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 units/mℓ of penicillin, and 100 ㎍/mℓ of streptomycin. The cells were incubated at 37℃ and 5% $CO_2$ in a humidified cell incubator. For all assays, the RAW 264.7 macrophages were plated in a 6-well ($5\times10^5$ cells/well) for 12 hours before being treated with LPS (100 ng/mℓ) and hog millet (1 ㎍/mℓ and 10 ㎍/mℓ) for 24 more hours. The supernatant was then collected, filtered through a 0.22 ㎛ filter and stored at −70℃.

### 4. MTT Assay

The macrophages were palted at a density of $10^4$ cells/well in 96-well plates. Th cells were then treated with with hog millet (1 ㎍/mℓ and 10 ㎍/mℓ) and observed after 24 hr. After 24 hr of incubation, the macrophages were continuously cultured in tha same medium with 0.1 ㎎/mℓ 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) for 4 hr. After the culture medium was removed, dimethyl sulfoxide was added to each well, and the plates were agitated to dissolve the crystal product. Absorbances were measured at 570 ㎚ with a multiwall plate reader.

### 5. ELISA

The amount of NO, $PGE_2$, TNF-$\alpha$, IL-6 and MCP-1 in cell supernatants were determined by using a commercially available ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

### 6. RNA Isolation and RT-PCR

Total RNA was isolated from RAW264.7 macrophages using

**Table 1. Primer sequences used for real-time PCR**

| Gene | Primer sequences | GenBank™ No. |
|---|---|---|
| iNOS | 5'-ATC CAC GAA GCC TAC C-3'<br>5'-CAC ACC GTA CTT TAG CAA G-3' | NM_011144 |
| COX-2 | 5'-CCA GGC TAC AGT GGG ACA TT-3'<br>5'-GAA CTT GCC CAT GTC CTT GT-3' | NM_013495 |
| TNF-$\alpha$ | 5'-TGG ACC CAA AGT GGT CCG CA-3'<br>5'-AGT TCA GTC ACG GAC TTT AT-3' | NM_017399 |
| IL-6 | 5'-GGT CGT TTC TCC ATT AAA TTC TCA T-3'<br>5'-CTA GAA ACT TTC CCA GAA ATC TTC C-3' | NM_007988 |
| MCP-1 | 5'-TCG CCC CTA CGA CAA GAA CA-3'<br>5'-CCG GTC GTA AGC CAG GCC CA-3' | NM_009127 |

* Abbreviations: iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; TNF-$\alpha$, Tumor necrosis factor-$\alpha$; IL-6, interleukin-6; MCP-1, monocyte chemotactic protein-1.

Trizol reagent (Invitrogen, CA, USA) following the manufacturer's recommendation. Then, real-time quantitative PCR was performed with an SYBR Green$^{TM}$ kit (Quantitect$^{TM}$ SYBR Green PCR, QIAGEN). The cycling conditions were 15 min at 95℃, 40 cycles of 15 sec at 94℃, and 30 sec at 72℃. Relative quantification was calculated using the Delta-Delta method (Livak & Schmittgen 2001). The sequences of primers for the genes examined are shown in Table 1.

### 7. Western Blotting

After treatment of hog millet, RAW 264.7 cells were lysed in a buffer (20 mM Tris-HCl at pH 7.4, 10 mM EGTA, 2 mM EDTA, 250 mM sucrose) containing 2 ㎕/㎖ of protease inhibitor cocktail P8340 (Sigma-Aldrich, St. Louis, MO. USA). The soluble materials were removed by centrifugation at 12,000 rpm for 20 min. Protein samples (50 ㎍) were separated by 10% SDS-page, and the separated products were transferred to PVDF membrane. The blots were blocked with 3% skim milk and then incubated at 4℃ overnight with either IRAK-4 antibody (1:1,000; Cell Signaling Technology, USA) or β-actin (1:1,000; Cell Signaling Technology, USA) diluted with 3% skim milk. The blots were then incubated with the secondary antibody (1:1,000; Cell Signaling Technology, USA) at room temperature for 2 hr. Immunoreactive bends were detected using a chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, England, UK) according to the manufacturer's instructions. Blots were scanned and analyzed using a multiple image analyzer and the Quantity One program (Bio-Rad Laboratories).

### 8. Statistical Analysis

Data were expressed as means±S.D. The statistical significance of the differences between the mean values for the treatment groups was analyzed by Student's *t*-tests and one-way analysis of variance (ANOVA) using Origin 7 software (Microcal Software, USA). Statistical significance was defined as $p<0.05$.

## RESULTS AND DISCUSSION

Dietary intake of whole grains is known to reduce the risk of chronic diseases such as obesity, diabetes, cardiovascular disease, and cancer. To investigate whether hog millet has anti-inflammatory effects, we evaluated pro-inflammatory mediators' release and their gene expression in a macrophage cell line. In this study, we used water extract of hog millet since it was safe and practical to use. In the MTT assay, no significant difference was observed at 1 ㎍/㎖ and 10 ㎍/㎖ of the hog millet (Fig 1). We could not prepare samples at 100 ㎍/㎖ due to their viscosity. Thus, RAW 364.7 macrophages were treated with samples at 1 ㎍/㎖ and 10 ㎍/㎖ concentrations.

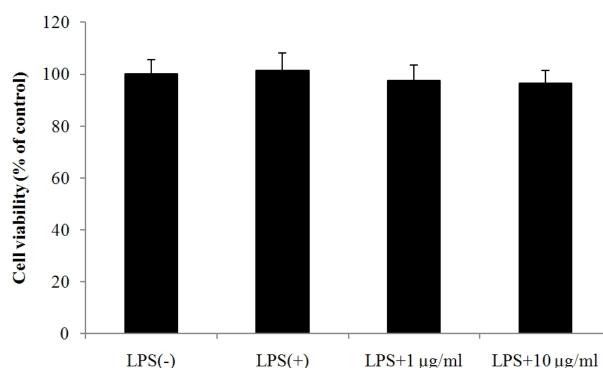


**Fig. 1. Effect of hog millet treatment on viability in RAW 264.7 cells.** Cells were co-treated with hog millet (1 ㎍/㎖ and 10 ㎍/㎖) and LPS (10 ng/㎖) for 24 hours. Each bar represents the mean±S.D. The results represent two independent experiments.

It is well known that macrophages play a critical role in both non-specific and acquired immune responses. The results of the present study indicate that hog millet inhibited LPS-induced inflammatory response in RAW 264.7 macrophages. The activation of macrophages by LPS leads to a functionally diverse series of responses, including the activation of phopholipase $A_2$, which produces lipid metabolites of arachidonic acid,such as prostaglandins. It also leads to the production of NO and to the production of pro-inflammatory cytokines (TNF-α and IL-6) (Hwang et al. 2011). In this study, hog millet effectively suppressed COX-2 and iNOS expression in LPS-stimulated macrophages, leading to the inhibition of COX-2-derived $PGE_2$ and iNOS-derived NO production (Fig. 1A). We also examined whether hog millet affects the production of proinflammatory cytokines in LPS-stimulated RAW264.7 cells. We observed that hog millet suppressed TNF-α and IL-6 generations, which are novel anti-inflammatory cytokines produced by LPS-stimulated murine macrophages (Fig. 1A) through the downregulation of the expression of their mRNA expression (Fig. 1B). TNF-α is the principal mediator of inflammation and its secretion, mainly from macrophages, enhanced local inflammation via the activation and induction of other cytokines, such as IL-6, IL-1β, IL-8, and so on. So, the several blockades of TNF-α have been used widely

due to its potential in the treatment of numerous inflammatory diseases (Carroll & Forgione 2010; Shenoi & Wallace 2010; Lee & Fedorak 2010). Among several cytokines, TNF-$\alpha$ and IL-6 play important roles in many inflammatory lesions and IL-6 production is significantly increased by stimulation with TNF-$\alpha$ (Bauer et al. 1988). Thus the decreased IL-6 level may be in part due to the reduced TNF-$\alpha$ by hog millet treatment in this study. Chemokines play a major role in selectively recruiting monocytes, neutrophils, and lymphocytes. Monocyte chemoattractant protein-1 (MCP-1) is one of the key chemokines that regulate the migration and infilteration of monocytes/macrophages (Deshmane et al. 2009). In this study, hog millet inhibited both the release and mRNA expression of MCP-1 (Fig. 1), and the data indicate that hog millet can modulate the migration of macrophages.

Toll-like receptors (TLRs) are the major pattern recognition receptors in the innate immune system used to detect conserved microbial products (Akira et al., 2006). The extracellular regions of TLRs contain varying numbers of leucine-rich repeat (LRR) motifs that form a horseshoe shape and can distinguishlipo-polysaccharides (LPS) from gram-negative bacteria. Once ligand recognition occurs, TLRs are functionally assembled and trigger downstream signaling transduction (Takada et al. 2007). TLRs involve myeloid differentiation factor 88 (MyD88) and TIR Domain-containing adaptor protein (TIRAP), and result in the activation of NF-$\kappa$B, AP-1 and MAPK via the IL-1 receptor-associated kinase (IRAK) complex. Among several protein kinases, IRAK4 is known to be the initial protein kinase activated by the 'downstream' of the TLR signaling pathway (Hirayama et al. 2011). This classical pathway ultimately leads to the synthesis of pro-inflammatory cytokines, such as TNF-$\alpha$ and IL-6 (You et al. 2008). Hog millet also inhibited the expression of the IRAK-4 protein in LPS-stimulated macrophages. This data indicated that the anti-inflammatory activity of hog millet, at least in part, was mediated by regulating IRAK-4 protein expression. There is little data about the physiological activity of hog millet. Kwak et al. (2004) only reported that hog millet has a strong in vitro antimutagenic effect. Thus, this anti-inflammatory data would provide basic information of the efficacy of hog millet and useful information regarding the further discovery of cereals with chemopreventive effects.

In summary, hog millet treatment effectively inhibited the release of pro-inflammatory mediators as well as their gene expression in LPS-stimulated macrophages. Although the active principles responsible for the anti-inflammatory activity of the hog millet were not identified in this study, the anti-inflammatory activity of hog millet appears to be related to the regulation of IRAK4 signaling. Our results provide a foundation for further

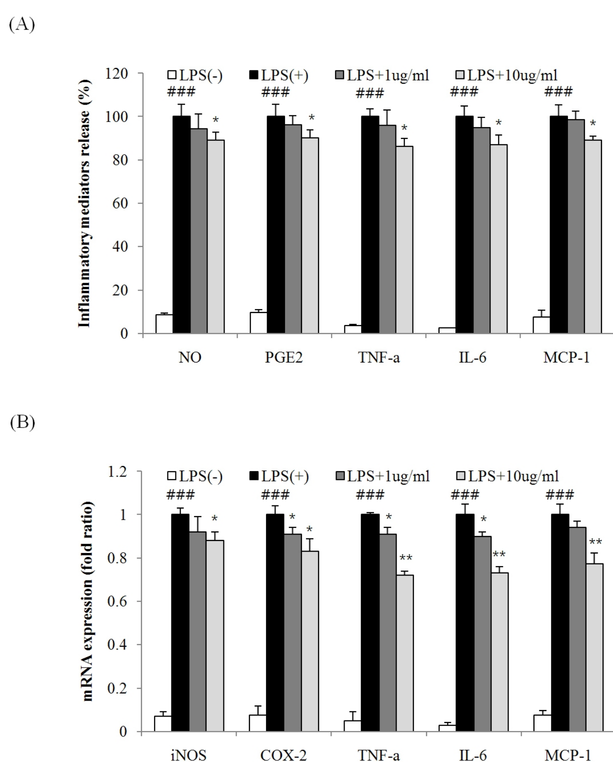


**Fig 2. Effect of hog millet treatment on the release (A) and mRNA expression (B) of pro-inflammatory mediators in RAW 264.7 cells.** Cells were co-treated with hog millet (1 ㎍/㎖ and 10 ㎍/㎖) and LPS (10 ng/㎖) for 24 hours. Each bar represents the mean±S.D. ###$p$<0.001 indicates significant differences from the unstimulated control group. *$p$<0.05 vs. LPS, **$p$<0.01 vs. LPS(+). The results represent two independent experiments.

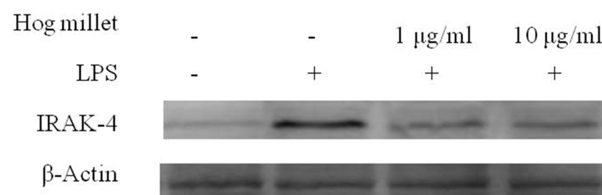


**Fig. 3. Effect of hog millet on IRAK-4 protein expression in RAW 264.7 cells.** Cells were co-treated with hog millet (1 ㎍/㎖ and 10 ㎍/㎖) and LPS (10 ng/㎖) for 24 hours. Protein levels were determined by Western blot analysis. The results represent two independent experiments.

insight into the mechanisms underlying the IRAK4- mediated reduction of LPS-induced inflammation.

## ACKNOWLEDGEMENTS

This study was supported by a grant from the Biogreen 21 Program, the Rural Development Administration of the Republic of Korea (Code pj006457).

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접 수 : 2011년 3월 14일
최종수정 : 2011년 5월 24일
채 택 : 2011년 6월 22일