J. Microbiol. Biotechnol. (2008), **18**(8), 1423–1426

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Nuruk Extract Inhibits Lipopolysaccharide-Induced Production of Nitrite and Interleukin-6 in RAW 264.7 Cells Through Blocking Activation of p38 Mitogen-Activated Protein Kinase

Kim, Jong-Eun[1,2], Sung-Keun Jung[1,2], Sang-Jin Lee[3], Ki-Won Lee[4], Gye-Won Kim[3], and Hyong-Joo Lee[1,5]*

[1]*Department of Agricultural Biotechnology,* [2]*Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Korea*
[3]*Research Laboratory, Kooksoondang Brewery Co., Ltd., Gyeonggi-do 462-120, Korea*
[4]*Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Korea*
[5]*Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea*

Received: October 24, 2007 / Accepted: February 28, 2008

***Nuruk*, which is a natural inoculator and source of amylolytic enzymes, is used in Korean traditional rice wine. A methanol extract of *nuruk* (NE) attenuated lipopolysaccharide (LPS)-induced nitrite and interleukin (IL)-6 in RAW 264.7 cells. Both the *n*-hexane and water fractions from NE (MEH and MW, respectively) inhibited the production of nitrite and IL-6 in RAW 264.7 cells. MEH and MW also inhibited the LPS-induced inducible nitric oxide synthase expression. Further, and MEH protected against the LPS-induced activation of p38 mitogen-activated protein kinase. Together, these results indicate that *nuruk* may contribute to the anti-inflammatory and cancer-preventive effects of Korean traditional rice wine.**

Keywords: Anti-inflammation, inducible nitric oxide synthase, *nuruk*

Inflammation is a defense mechanism against harmful microorganisms, but the chronic presence of this state is related to many adult diseases. In particular, it is widely accepted that inflammatory mediators such as nitric oxide and cytokines play key roles in carcinogenesis [5], and hence these molecules have become targets for drugs aimed at preventing chronic inflammatory diseases [6]. Nitric oxide, a small-molecule and membrane-permeable gas, mediates many physiological events, and chronic elevated nitric oxide is involved in the deamination of DNA and inactivation of DNA repair enzymes, mechanisms that induce cancer [15, 19]. Nitric oxide also controls other inflammatory molecules such as prostaglandin, platelet selectin intercellular adhesion molecule 1, vascular cell-adhesion molecule 1, and some cytokines. Endothelial synthase (NOS), neutral NOS, and inducible NOS (iNOS) are the three key enzymes that generate nitric oxide. iNOS is responsible for the overproduction of nitric oxide and is often present during inflammation and carcinogenesis [8]. Interleukin-6 (IL-6) is a typical pleiotropic cytokine [20]. All of these molecules also play important roles in immune responses such as endothelial activation and fever [7].

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria that enter the bloodstream during a bacterial infection and can induce lethal septic shock [14]. LPS triggers the secretion of nitric oxide and inflammatory cytokines through stimulating intracellular signaling molecules such as mitogen-activated protein kinases (MAPKs) [10]. MAPKs, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase, and p38 MAPK, are a family of Ser/Thr-specific signaling kinases activated by a variety of intracellular stimuli through a cascade of protein phosphorylations [11]. Previous studies have suggested that p38 MAPK plays a critical role in LPS-induced iNOS and IL-6 expression [2, 3, 10, 13, 16, 17].

There are multiple lines of evidence that red wine reduces the incidence of cardiovascular disease, diabetes, cancer, and inflammation [9]. The long history of Korea has produced many traditional Korean foods, but many of these have not yet been subject to scientific analysis. Korean rice wine inhibits proliferation of the B16BL6 mouse melanoma cell line and exerts an antimetastatic effect on B16BL6 mouse melanoma cells injected in C57/BL6 mouse [4]. The antiproliferative effects on murine Lewis lung carcinoma cells are much stronger for Korean rice wine than for red wine [18]. However, the active ingredients responsible for these effects of Korean rice wine remain unclear. *Nuruk* (Korean

*Corresponding author
Phone: 82-2-880-4853; Fax: 82-2-873-5095;
E-mail: leehyjo@snu.ac.kr

koji) is a natural inoculator and source of amylolytic enzymes that is used in the production of Korean traditional wine. It is made by allowing the development of numerous types of molds, bacteria, and yeasts in wheat flour or grits [21].

Nuruk extract was provided by Kooksoondang Brewery Co. Ltd. (Gyunggi, Korea). The dried and powdered *nuruk* (8 kg) was extracted three times at room temperature with 80% aqueous methanol (each 10 l). The extracts were successively partitioned with water (3 l) and EtOAc (3 l), and the EtOAc extract (231 g) was fractionated repeatedly with *n*-hexane. The yield of the hydrophobic *n*-hexane fraction (MEH) was 135 g (dry weight). The hydrophilic water fraction (MW) was fractionated from NE, with a yield of 870 g (dry weight).

We first measured the inhibitory effect of NE on nitrite production and IL-6 expression in LPS-induced RAW 264.7 murine macrophage cells. The RAW 264.7 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Macrophage cells were seeded (5×10^4) in 96-well plates and incubated for 24 h. After incubation, the cells were pretreated with each sample at the indicated concentrations for 2 h before incubation with LPS (4 μg/ml) for 24 h, and then the culture medium was harvested. The nitric oxide produced by macrophage cells was quantified by measuring nitrite (which is the metabolite of nitric oxide oxidation) as described previously [12], and IL-6 was quantified by an ELISA kit according to the manufacturer's instructions (Pharmingen, San Diego, U.S.A.). NE (at 25, 50, 100, and 200 μg/ml) dose-dependently inhibited the production of nitrite and IL-6 in the LPS-treated RAW 264.7 cells (Fig. 1). The LPS-induced generation of nitrite and IL-6 in RAW 264.7

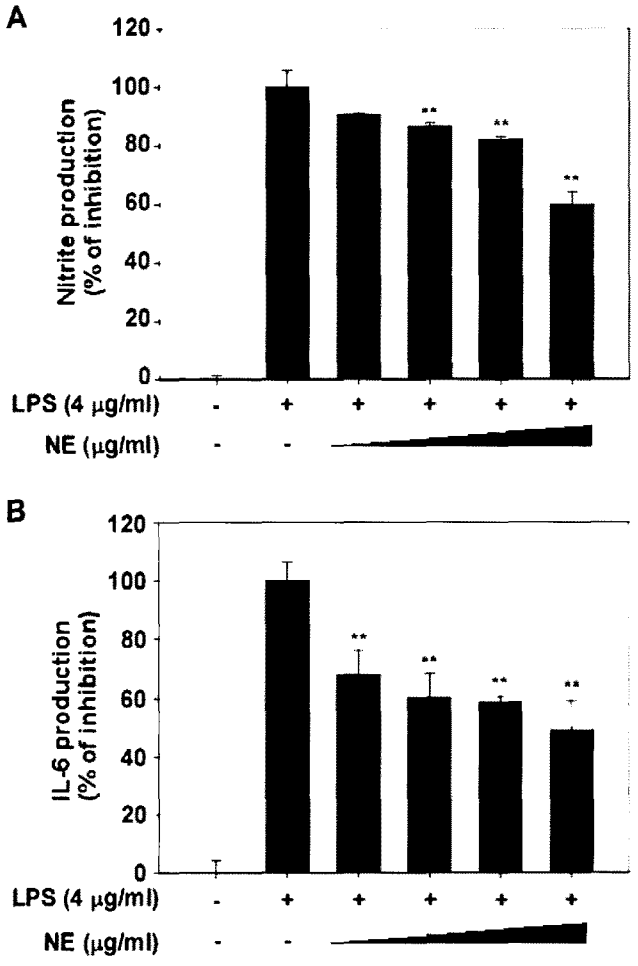

Fig. 1. Inhibitory effects of NE on LPS-induced generation of nitrite and IL-6 in RAW 264.7 cells.
The cells were pretreated with NE fractions at 25, 50, 100, and 200 μg/ml for 2 h before incubation with LPS (4 μg/ml) for 18 h. The amounts of nitrite (**A**) and IL-6 (**B**) were measured using the Griess assay and ELISA. The data are mean and SE values from triplicate tests. **P*<0.05 and ***P*<0.01 indicate statistically significant differences relative to the LPS-treated group.

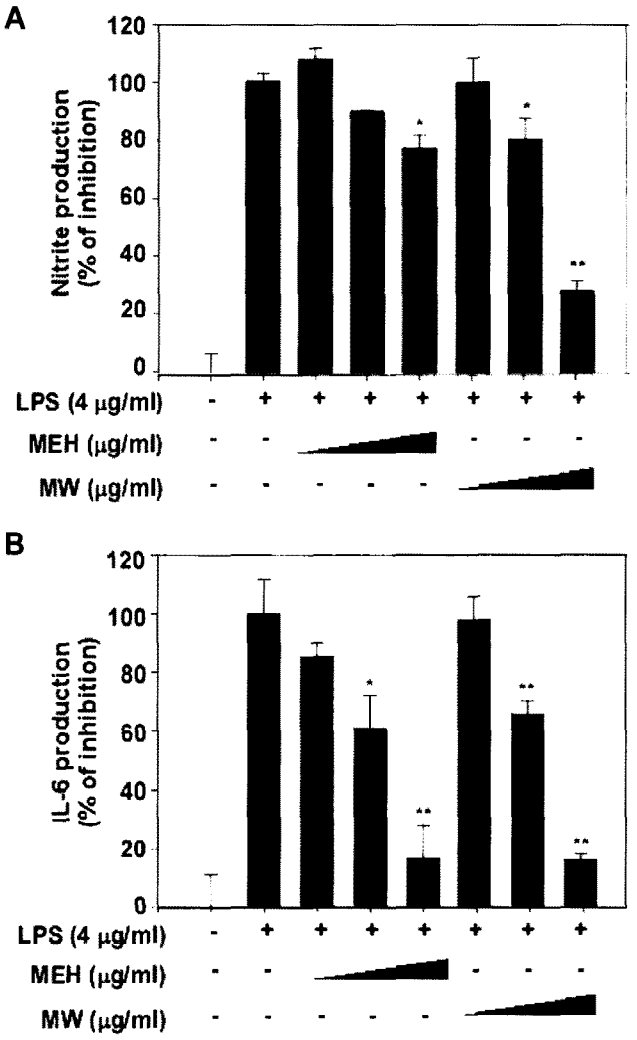

Fig. 2. Inhibitory effects of NE fractions on LPS-induced generation of nitrite and IL-6 in RAW 264.7 cells.
The cells were pretreated with each NE fraction at 50, 100, and 200 μg/ml for 2 h before incubation with LPS (4 μg/ml) for 18 h. The amounts of nitrite (**A**) and IL-6 (**B**) were measured using the Griess assay and ELISA, as described in the Materials and Methods. The data are mean and SE values from triplicate tests. **P*<0.05 and ***P*<0.01 indicate statistically significant differences relative to the LPS-treated group.

cells was also attenuated by MEH or MW treatment (Fig. 2). MW exerted more effective inhibitory effects than MEH on the LPS-induced production of nitrite, whereas these fractions exhibited similar effects on IL-6 production in LPS-stimulated RAW 264.7 cells. These results indicated that both hydrophilic and hydrophobic substances of NE could contribute to the anti-inflammatory potential of NE.

We further examined whether MEK or MW can inhibit LPS-induced iNOS expression. The cells (2×10^5) were cultured in a 3-cm-diameter dish for 48 h, starved in serum-free medium for another 12 h, and then treated with each sample for 2 h before being exposed to LPS (4 μg/ml). Western blotting data revealed that the LPS-induced upregulation of iNOS expression was inhibited by MEH or MW (Fig. 3). Multiple lines of evidence have indicated that the activation of p38 MAPK is strongly related to the process of inflammation and carcinogenesis [11]. A previous study demonstrated that SB203580 (a pharmacological inhibitor of p38 MAPK) strongly inhibited LPS-induced iNOS expression in RAW 264.7 cells, indicating that the p38 MAPK pathway is closely related to LPS-induced iNOS expression [2]. Similarly, SB203580 suppressed the LPS-induced expression of IL-6, suggesting that the p38 MAPK pathway also plays an important role in IL-6 expression [1]. The present study clearly showed that both MEH and MW inhibited the LPS-induced phosphorylation of

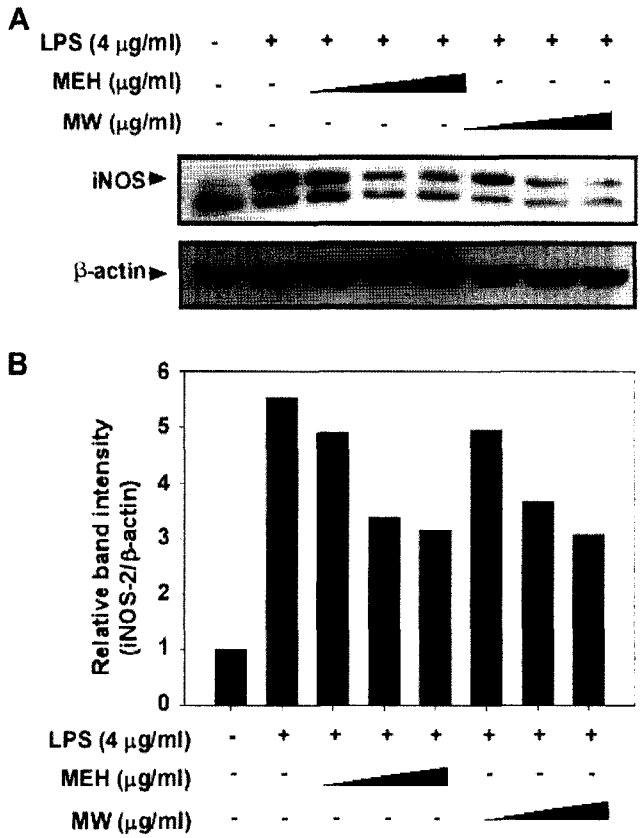

Fig. 3. Inhibitory effects of NE fractions on LPS-induced iNOS expression in RAW 264.7 cells.

A. The cells were pretreated with each NE fraction at 50, 100, and 200 μg/ml for 2 h before incubation with LPS (4 μg/ml) for 18 h. Equal amounts of total proteins (30 μg) were subjected to 10% SDS-PAGE. The expression of iNOS and β-actin protein was detected by Western blot using specific antibodies. **B.** iNOS expression was quantified using an image analyzer.

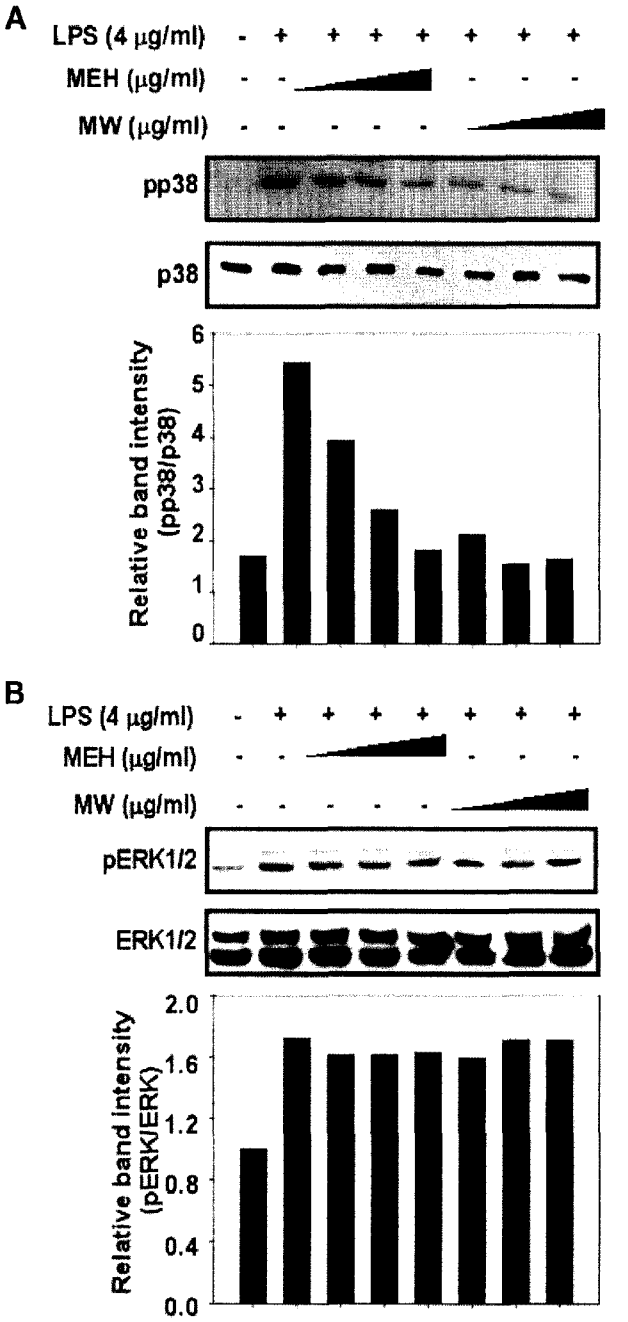

Fig. 4. Effects of NE fractions on LPS-induced phosphorylation of p38 MAPK and ERK1/2 in RAW 264.7 cells.

The cells were pretreated with each NE fraction at 50, 100, and 200 μg/ml for 2 h before incubation with LPS (4 μg/ml) for 30 min. Equal amounts of total proteins (30 μg) were subjected to 10% SDS-PAGE. The phosphorylation of p38 MAPK (**A**) and ERK1/2 (**B**) was detected by Western blot using specific antibodies. The phosphorylation of p38 MAPK and ERK1/2 was quantified using an image analyzer.

p38 MAPK but not that of ERK1/2 in RAW 264.7 cells (Fig. 4). These results indicate that the inhibition of LPS-mediated iNOS and IL-6 expression by MEH and MW was mediated by the inhibition of p38 MAPK activation.

Since inflammation is closely linked to tumor promotion, substances with potent anti-inflammatory activities are anticipated to exert chemopreventive effects on carcinogenesis, particularly in the promotion stage [5]. Our results taken together indicate that *nuruk* has potential anti-inflammatory effects, which might contribute to the aforementioned chemopreventive potential of Korean traditional rice wine. Further work is needed to investigate the major active components of NE and their underlying mechanisms.

Acknowledgments

This work was supported by research grants from the BioGreen 21 Program (No. 20070301-034-042), the Rural Development Administration, and the Biofood Research Program, the Korea Science and Engineering Foundation, Republic of Korea.

References

1. Carter, A. B., M. M. Monick, and G. W. Hunninghake. 1999. Both Erk and p38 kinases are necessary for cytokine gene transcription. *Am. J. Respir. Cell Mol. Biol.* **20:** 751–758.
2. Chen, C. C. and J. K. Wang. 1999. p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages. *Mol. Pharmacol.* **55:** 481–488.
3. Cho, S. Y., S. J. Park, M. J. Kwon, T. S. Jeong, S. H. Bok, W. Y. Choi, *et al.* 2003. Quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-κB pathway in lipopolysaccharide-stimulated macrophage. *Mol. Cell. Biochem.* **243:** 153–160.
4. Chung, K. S., W. T. Oh, S. M. Nam, B. S. Son, and Y. S. Park. 1998. Effect of Korean rice-wine (*Yakju*) on *in vitro* and *in vivo* progression of B16BL6 mouse melanoma and HRT18 human colon adenocarcinoma cells. *Korean J. Food Sci. Technol.* **30:** 1470–1475.
5. Coussens, L. M. and Z. Werb. 2002. Inflammation and cancer. *Nature* **420:** 860–867.
6. Dinarello, C. A. 2000. Proinflammatory cytokines. *Chest* **118:** 503–508.
7. Dranoff, G. 2004. Cytokines in cancer pathogenesis and cancer therapy. *Nat. Rev. Cancer* **4:** 11–22.
8. Fukumura, D., S. Kashiwagi, and R. K. Jain. 2006. The role of nitric oxide in tumour progression. *Nat. Rev. Cancer* **6:** 521–534.
9. German, J. B. and R. L. Walzem. 2000. The health benefits of wine. *Annu. Rev. Nutr.* **20:** 561–593.
10. Guha, M. and N. Mackman. 2001. LPS induction of gene expression in human monocytes. *Cell. Signal.* **13:** 85–94.
11. Hommes, D. W., M. P. Peppelenbosch, and S. J. H. van Deventer. 2003. Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. *Gut* **52:** 144–151.
12. Hur, H. J., K. W. Lee, H. Y. Kim, D. K. Chung, and H. J. Lee. 2006. *In vitro* immunopotentiating activities of cellular fractions of lactic acid bacteria isolated from *kimchi* and bifidobacteria. *J. Microbiol. Biotechnol.* **16:** 661–666.
13. Jeng, K. C. G., R. C. W. Hou, J. C. Wang, and L. I. Ping. 2005. Sesamin inhibits lipopolysaccharide-induced cytokine production by suppression of p38 mitogen-activated protein kinase and nuclear factor-κB. *Immunol. Lett.* **97:** 101–106.
14. Kim, J. E., H. J. Hur, K. W. Lee, and H. J. Lee. 2007. Anti-inflammatory effects of recombinant arginine deiminase originating from *Lactococcus lactis* ssp. *lactis* ATCC 7962. *J. Microbiol. Biotechnol.* **17:** 1491–1497.
15. Kim, J. E., J. Y. Kim, K. W. Lee, and H. J. Lee. 2007. Cancer chemopreventive effects of lactic acid bacteria. *J. Microbiol. Biotechnol.* **17:** 1227–1235.
16. Kim, J. H., D. H. Kim, S. H. Baek, H. J. Lee, M. R. Kim, H. J. Kwon, and C. H. Lee. 2006. Rengyolone inhibits inducible nitric oxide synthase expression and nitric oxide production by down-regulation of NF-κB and p38 MAP kinase activity in LPS-stimulated RAW 264.7 cells. *Biochem. Pharmacol.* **71:** 1198–1205.
17. Kim, S. H., J. Kim, and R. P. Sharma. 2004. Inhibition of p38 and ERK MAP kinases blocks endotoxin-induced nitric oxide production and differentially modulates cytokine expression. *Pharmacol. Res.* **49:** 433–439.
18. Kim, S. J., S. H. Ko, W. Y. Lee, and G. W. Kim. 2004. Cytotoxic effects of Korean rice-wine (*Yakju*) on cancer cells. *Korean J. Food Sci. Technol.* **36:** 812–817.
19. Kim, Y. J., K. W. Lee, D. O. Kim, T. W. Kim, S. K. Lee, and H. J. Lee. 2004. *In vivo* antitumor activity of hydrophilic arginine-conjugated linoleic acid complex. *J. Microbiol. Biotechnol.* **14:** 411–414.
20. Nam, K. S., M. K. Kim, and Y. H. Shon. 2007. Inhibition of proinflammatory cytokine-induced invasiveness of HT-29 cells by chitosan oligosaccharide. *J. Microbiol. Biotechnol.* **17:** 2042–2045.
21. Yu, T. S., H. S. Kim, J. Hong, H. P. Ha, T. Y. Kim, and I. W. Yoon. 1996. Bibliographical study on microorganisms of *nuruk* (until 1945). *J. Food Sci. Nutr.* **25:** 170–179.