OPEM
www.opem.org

Oriental Pharmacy and Experimental Medicine 2008 **8(2)**, 125-129
DOI 10.3742/OPEM.2008.8.2.125

KHUP

Screening of KMU-4, 6, 7 on inflammatory responses in IFN-γ and LPS-induced mouse peritoneal macrophages

Ho-Jeong Na[1], Hyun-Ja Jeong[2], Jong-Woong Ahn[3], Jae-Young Um[4], Hyung-Min Kim[4] and Seung-Heon Hong[1,*]

[1]Department of Oriental Pharmacy, College of Pharmacy, Wonkwang University, Iksan city, Jeonbuk, 570-749, Republic of Korea; [2]Biochip Research Center, Hoseo University, Republic of Korea; [3]Division of Marine Environment & Bioscience College of Ocean Science and Technology, Korea Maritime University 1, Dongsam-dong, Youngdo-ku, Busan 606-791, Korea; [4]Department of pharmacology, Institute of Oriental Medicine, College of Oriental Medicine, Kyung Hee University, Republic of Korea

Received for publication March 07, 2008; accepted May 01, 2008

SUMMARY

Korean Marine Plants (KMU-4, 6, 7) obtained from an herb which widely used in medicine for the treatment of a variety of pathologies. In this study, using mouse peritoneal macrophages, we have examined whether KMU-4, 6, 7 affects nitric oxide (NO) and COX-2 induced IFN-γ and LPS and cell viability. KMU-6 inhibits IFN-γ and LPS-induced NO. We found that KMU-6 had a little effect on COX-2 expression. These finding means that KMU-6 can be used in controlling macrophages mediated inflammatory disease. The present results indicate that KMU-6 has an inhibitory effect on the production of NO through down-regulation of COX-2 expression in LPS stimulated mouse peritoneal macrophages.

Key words: Nitric oxide; COX-2

INTRODUCTION

KMU-4, 6, 7 are a Korean Marine Plants, which have been used for the treatment of eye disease in Republic of Korea and China. They have also been used as an herbal tea in Chinease folklore and are known as 'Ju Hua'. They have been found to possess antibacterial, antifungal, antiviral, antispirochetal, and anti-inflammatory activities (Jiangsu New Medical College, 1977). Macrophages are a first line of defence against microbial invaders and malignancies by nature of their phagocytic, cytotoxic and intracellular killing capacities (Adams and Hamilton, 1984). Macrophage activation by lipopolysaccharide (LPS), the major component of gram-negative bacteria cell wall, results in the release of several inflammatory mediators such as nitric oxide (NO) and the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 (Schimmer and Parker, 2001). The physiologic or normal production of NO from phagocytes is beneficial for the host defense against microorganism, parasites and tumor cells (Thiemermann and Vane, 1990). However, overproduction of NO can be harmful and result in septic shock, neurologic disorders, rheumatoid arthritis and autoimmune diseases (Thiemermann and Vane, 1990; Evans,

*Correspondence: Seung-Heon Hong, Department of Oriental Pharmacy, College of Pharmacy, Wonkwang University, Iksan city, Jeonbuk, 570-749, Republic of Korea. Tel: +82638506805; Fax: +82638433421; E-mail: jooklim@wonkwang.ac.kr

1995; O'Shea *et al*., 2002). Therefore, inhibition of NO production is a very important therapeutic target in the development of anti-inflammatory agents.

NO and pro-inflammatory cytokine are well known to be important mediators of acute and chronic inflammation (Park *et al*., 2000)/and are synthesized by cyclooxygenase (COX) enzymes. There are two isoform of COX, constitutively expressed COX-1 and the inducible isoform COX-2 (Kanazawa *et al*., 1995). COX-2 are upregulated in response to inflammatory and pro-inflammatory mediators and their products can influence many aspects of inflammatory cascade.

In the present study, we show that KMU-6 significantly inhibited LPS and IFN-γ-induced NO production in a dose-dependent manner. The expression of COX-2 protein was decreased. Thus, KMU may be useful in certain type of inflammation, allergy and infectious disorders.

MATERIALS AND METHODS

Reagents

Murine rIFN-γ (1×10^6 U/ml) was purchased from Pharmingen (Munchen, Germany). LPS and sodium nitrite were purchased from Sigma (St. Louis, MO). Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, MI). 0.4 μm syringe filter and tissue culture plates of 96 wells, 4 wells and 100-mm diameter dishes were purchased from Nunc (Naperville, IL). DMEM containing L-arginine (84 mg/l), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Life Technologies (Grand Island, NY). Male C57BL/6 mice were purchased from Damul Science Co. (Daejon, Republic of Korea).

Peritoneal macrophages culture

TG-elicited macrophages were harvested 3~4 days after i.p. injection of 2.5 ml TG to the mice and isolated, as reported previously (Chung *et al*., 2002). Using 8 ml of HBSS containing 10 U/ml heparin, peritoneal lavage was performed. Then, the cells were distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 4-well tissue culture plates (2.5×10^5 cells/well) incubated for 3 h at 37°C in an atmosphere of 5% CO_2, washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

Measurement of nitrite concentration

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with various concentrations of KMU-4, 6, 7. The cells were then stimulated with rIFN-γ (20 U/ml). After 6 hours, the cells were finally treated with LPS (10 μg/ml). NO synthesis in cell cultures was measured by a microplate assay method, as previously described (Chung *et al*., 2002). To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphtyl)-ethylenediamine dihydrochloride/ 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined by an automatic microplate reader. NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 9 μM of NO_2^-. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

Western blot analysis

Peritoneal macrophages (5×10^6 cells/well) were pretreated with various concentrations KMU-4, 6, 7. The cells were then incubated with for 6 h with rIFN-γ (20 U/ml). They were finally stimulated with LPS (10 μg/ml) for 24 h. Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose

paper. The membrane was then blocked with 5% skim milk in PBS-tween-20 (Sigma) for 1 h at room temperature and then incubated with anti-COX-2 antibodies. After washing in with phosphate-buffered saline (PBS) containing 0.05% tween-20 three times, the blot was incubated with secondary antibody for 1 h and the antibody-specific proteins were visualized by the enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp. Newark, NJ).

Statistical analysis

Results were expressed as the mean ± S.E.M. of independent experiments and statistical analysis was performed by one-way analysis of variance to express the difference among the groups.

RESULTS

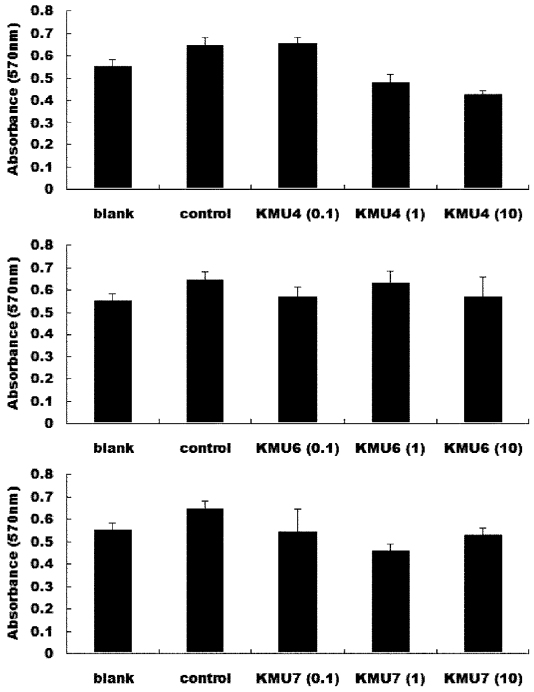

Fig. 1. Effect of KMU-4, 6, 7 on the cell viability. Cell viability was evaluated by MTT colorimetric assay 48 h after KMU treatment in peritoneal macrophages. Values are the mean ± S.E.M. of three independent experiments duplicate in each run.

Effects of KMU-4, 6, 7 on cell viability

To determine the effects of KMU-4, 6, 7 on viability of mouse peritoneal macrophages, we carried out MTT assay. When we treated the cells with KMU-4, 6, 7 (0.1, 1, 10 μg/ml), KMU-6 had no effect on cell viability (Fig. 1).

Inhibitory effects of KMU-4, 6, 7 on NO production

To determine the effect of KMU-4, 6, 7 on the production of NO by mouse peritoneal macrophages, we pretreated the cells with various concentration KMU-4, 6, 7 (0.1, 1, 10 μg/ml). And then we stimulated them with rIFN-γ (20 U/ml) and LPS (10 μg/ml).

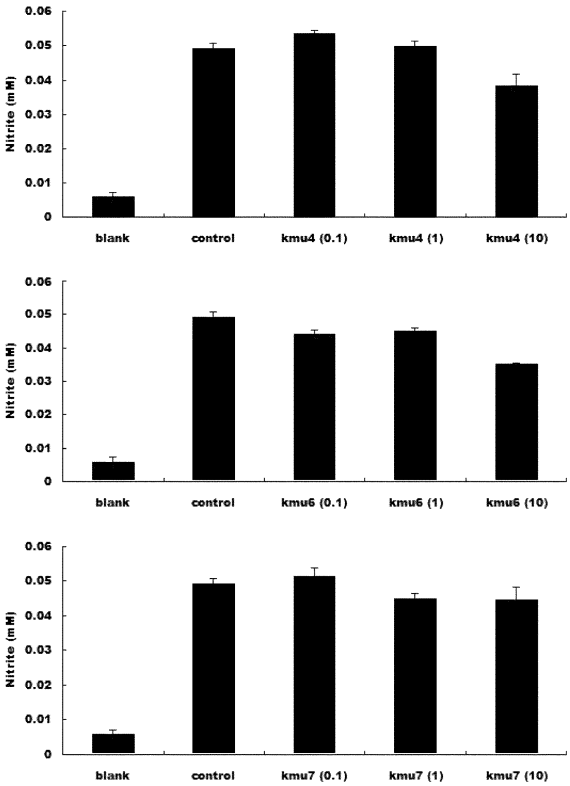

Fig. 2. Dose-dependent effects of KMU-4, 6, 7 on NO inhibition in rIFN-γ and LPS-treated peritoneal macrophages. Peritoenal macrophages (2.5×10^5 cells/well) were cultured with various concentrations KMU. The peritoneal macrophages were then stimulated with rIFN-γ (20 U/ml) and LPS (10 μg/ml). After 48 h of culture, NO release was measured by the Griess method (nitrite). NO (nitrite) released into the medium is presented as the mean ± S.E.M. of three independent experiments duplicate in each run.

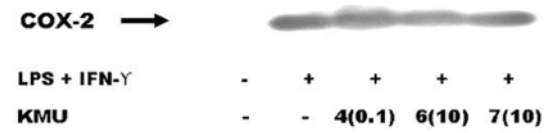

Fig. 3. Effects of KMU-4, 6, 7 on the expression of COX-2 by rIFN-γ plus LPS-induced peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were pretreated with KMU and then stimulated for 6 h with rIFN-γ (20 U/ml). The peritoneal macrophages were then stimulated with LPS (10 μg/ml) for 24 h. The protein extracts were prepared and then samples were analyzed for COX-2 expression by Western blotting as described in the method. 1: blank; 2: rIFN-γ + LPS; 3: KMU-4 (0.1 μg/ml) + rIFN-γ + LPS; 4: KMU-6 (10 μg/ml) + rIFN-γ + LPS; 5: KMU-7 (10 μg/ml) + rIFN-γ + LPS.

The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants after 48 h treatment. When mouse peritoneal macrophages were primed for 6 h with murine rIFN-γ and then treated with LPS, NO production was increased about 10 folds. It had an effect on NO production in resting mouse peritoneal macrophages compared to non-primed conditions. When KMU-4, 6, 7 were pretreated in primed cell, KMU-6 inhibits NO production dose dependently (Fig. 2).

Effects on expression of COX-2 protein

We investigate the effect of the KMU-6 (10 μg/ml) at translational level, As shown in Fig. 3, the expression of COX-2 protein were markedly increased after IFN-γ (20 U/ml) plus LPS (10 μg/ml) challenge for 24 h. This increased expression of COX-2 protein was reduced by KMU (Fig. 3).

DISCUSSION

Murine macrophage exhibits a particularly vigorous response to endotoxin, which induces production of variety of inflammatory modulators such as NO, TNF-α, IL-6 and prostaglandins by inducible COX-2.

NO has been recognized to be an important mediator of cellular communication in several preparations such as macrophages, neutrophils, smooth muscle, autonomic nervous system, and central nervous system (Blackman *et al*., 2000; Koyanagi *et al*., 2000; Sharma *et al*., 2000). In this study, exposure of macrophages to IFN-γ and LPS for 48 h was associated with an accumulation of nitrite in the medium, suggesting an enhanced NO production. This IFN-γ and LPS-induced NO production was inhibited by KMU without notable cytotoxicity.

COX-2 plays a role in the pathophysiological processes including inflammation (Meade *et al*., 1993). We documented the increased production of COX-2 protein by macrophages exposed to IFN-γ and LPS. IFN-γ and LPS in combination with KMU led to a significant reduction in COX-2 protein expression.

Here in our study, we have shown that KMU-6 exerts its anti-inflammatory effects probably by the suppression of COX-2 expression, and the final result is the inhibition of NO synthesis. Based on our present results, it is possible that KMU-6 can offer a valuable means of therapy for the treatment of inflammatory diseases by attenuating IFN-γ and LPS-induced NO synthesis controlling of COX-2 expression.

ACKNOWLEDGEMENTS

This work was financially supported by the Korea Research Foundation (KRF) grant KRF-2006-005-J00502.

REFERENCES

Adams DO, Hamilton TA. (1984) The cell biology of macrophage activation. *Annu. Rev. Immunol.* **2**, 283-318.

Blackman DJ, Morris-Thurgood JA, Atherton JJ, Ellis GR, Anderson RA, Cockcroft JR, Frenneaux MP. (2000) Endothelium-derived nitric oxide contributes to the regulation of venous tone in humans. *Circulation* **18**, 165-170.

Chung HS, Jeong HJ, Hong SH, Kim MS, Kim SJ, Song BK, Jeong IS, Lee EJ, Ahn JW, Baek SH, Kim

HM. (2002) Induction of nitric oxide synthase by Oldenlandia diffusa in mouse peritoneal macrophages. *Biol. Pharm. Bull.* **25**, 1142-1146.

Evans CH. (1995) Nitric oxide: what role does it play in inflammation and tissue destruction? *Agents Actions Suppl.* **47**, 107-116.

Jiangsu New Medical College. (1977) "The Dictionary of Chinese Medicine" (Zhong Tao Da ci Dian), Shanghai, Science and Technique Press, Shanghai, pp.2008.

Kanazawa K, Kawasaki H, Samejima K, Ashida H, Danno G. (1995) Specific desmutagen (antimutagen) in oregano against a dietary carcinogen, Trp-P-2, are galangin and quercetin. *J. Agric. Food Chem.* **43**, 404-409.

Koyanagi M, Egashira K, Kubo-Inoue M, Usui M, Kitamoto S, Tomita H, Shimokawa H, Takeshita A. (2000) Role of transforming growth factor-beta1 in cardiovascular inflammatory changes induced by chronic inhibition of nitric oxide synthesis. *Hypertension* **35**, 86-90.

Meade EA, Smith WL, DeWitt DL. (1993). Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* **268**, 6610-6614.

O'Shea JJ, Ma A, Lipsky. (2002) Cytokines and autoimmunity. *Nat. Rev. Immunol.* **2**, 37-45.

Park JG, Park JC, Hur JM, Park SJ, Choi DR, Shin DY, Park KY, Cho HW, Kim MS. (2000) Phenolic compounds from *Orostachys japonicus* having anti-HIV-1 protease activity. *Nat. Pro. Sci.* **6**, 117-121

Schimmer BP, Parker KL. (2001) Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. In: Hardman, J. G., Limbird, L.E., Goodman Gilman, A. (Eds.), *The Pharmacological Basis of Therapeutics* 10th ed. McGraw-hill, New York, 1649-1677.

Sharma R, Coats AJ, Anker SD. (2000) The role of inflammatory mediators in chronic heart failure: cytokines, nitric oxide, and endothelin-1. *Int. J. Cardiol.* **15**, 175-186.

Sradnyk AW, Gillan TL, Anderson R. (1997) Respiratory syncytial virus triggers synthesis of IL-6 in BALB/c mouse alveolar macrophages in the absence of virus replication. *Cell. Immunol.* **176**, 122-126.

Thiemermann C, Vane J. (1990) Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. *Eur. J. Pharmacol.* **182**, 591-595.