TANG HUMANITAS MEDICINE

Original Article

Evidence of hydrolyzed traditional Korean red ginseng by malted barley on activation of receptor interacting proteins 2 and IkappaB kinase-beta in mouse peritoneal macrophages

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ABSTRACT

Red ginseng, which has a variety of biological and pharmacological activities including antioxidant, anti-inflammatory, antimutagenic and anticarcinogenic effects, has been used for thousands of years as a general tonic in traditional oriental medicine. Here, we tested the immune regulatory activities of hydrolyzed red ginseng by malted barley (HRG) on the expressions of receptor interacting proteins (Rip) 2 and IκB kinase-β (IKK-β) in mouse peritoneal macrophages. We show that HRG increased the activations of Rip 2 and IKK-β for the first time. When HRG was used in combination with recombinant interferon-γ (rIFN-γ), there was a marked cooperative induction of nitric oxide (NO) production. The increased expression of inducible NO synthase from rIFN-γ plus HRG-stimulated cells was almost completely inhibited by pre-treatment with pyrrolidine dithiocarbamate (PDTC), an inhibitor of nuclear factor-κB (NF-κB). In addition, the treatment of peritoneal macrophages with rIFN-γ plus HRG caused significant increases in tumor necrosis factor (TNF)-α mRNA expression and production. Because NO and TNF-α play an important role in the immune function and host defense, HRG treatment can modulate several aspects of the host defense mechanisms as a result of the stimulations of the inducible nitric oxide synthase and NF-κB. In conclusion, our findings demonstrate that HRG increases the productions of NO and TNF-α from rIFN-γ-primed macrophages and suggest that Rip2/IKK-β plays a critical role in mediating these immune regulatory effects of HRG.

Keywords red ginseng, receptor interacting proteins 2, IκB kinase-β, nuclear factor-κB, tumor necrosis factor-α, nitric oxide

INTRODUCTION

Ginseng has been used for thousands of years in Asian countries, particularly in Korea, China, and Japan, due to its wide medicinal effects, such as tonic, immunomodulatory, and anti-aging activities (Lee, 2005). In oriental medicine, the production of red ginseng is considered a form of processing medicinal herbs under a certain traditional theory. In addition, related to the theory of how medicine works within the body, it is regarded that the minor cold character is transformed into a warm character, and that the effect of protecting the vitality is improved. Korean red ginseng has been developed for long-term storage and distribution. It has been reported that red ginseng has more powerful pharmacological activities than white ginseng (Nam, 2005; Takagu et al., 1990). The differences in the biological activities of red and white ginseng may result from a change in the chemical constituents that occur during the steaming process (Ha et al., 2005; Park et al., 2005).

Products of partially or totally hydrolyzed materials, including rice or guar gum, by enzymatic hydrolysis with various enzymes are known to have improved beneficial effects in humans (Stewart and Slavin, 2006). One of the examples of enzymatic hydrolysis is hydrolysis by malted barley in the Korean traditional method. It is well recognized that germinated barley seeds contain a variety of enzymes such as aspartic peptidases, serine peptidases and metallopeptidases, and products hydrolyzed by these enzymes exert many physiological functions (Fontanini and Jones, 2001; Gorjanovic et al., 2002).

Nuclear factor (NF)-κB plays a critical role in the expression of many genes involved in immune and inflammatory responses (Ghosh et al., 1998; Thanos and Maniatis, 1995). In unstimulated cells, *Rel* protein dimers, mainly p50 and p65 subunits, are sequestered in the cytoplasm in complex with one of the several inhibitors of NF-κB. The activation of NF-κB is the consequence of the phosphorylation of two specific serines near the N terminus of IκB-α and its degradation. The phosphorylation of IκB-α leads to ubiquitination, resulting in the degradation, which targets the protein for degradation through the 26S proteasome and the translocation of NF-κB to the nucleus (Scherer et al., 1995).

The IκB kinase (IKK)-β enzyme complex is part of the upstream NF-κB signal transduction cascade (Karin, 1999). After activation, IKK phosphorylates IκB at two conserved serine residues in the N-terminus, which then triggers the degradation of this inhibitor and allows the rapid translocation of NFκB into the nuclei where it avidly binds to DNA (Jeong et

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al., 2002). Thus, NF-κB's translocation and transactivation induce the inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF)-α expression in macrophages. Receptor interacting proteins 2 (Rip 2), a serine/threonine kinase, is also essential for NF-κB activation through the TNF receptor and Toll-like receptors (Kobayashi et al., 2002; Inohara et al., 1998).

In the present study, we show that hydrolyzed red ginseng by malted barley (HRG) synergistically induces Nitric oxide (NO) and TNF-α production by peritoneal macrophages when the cells are treated by recombinant IFN-γ (rIFN-γ). To investigate the mechanisms of HRG-induced NO and TNF-α production, we examined the effects of HRG on Rip2, IKK-β, and NF-κB activation. These findings may explain the influence of LS on NO and TNF-α production via the NF-kB signaling pathway.

MATERIALS AND METHODS

Reagents

Korean red ginseng was obtained from the Korea Ginseng Corp. (Daejeon, Republic of Korea). Murine rIFN-γ, recombinant TNF-α (rTNF-α), biotinylated TNF-α, and anti-murine TNF-α was purchased from R&D Systems (Minneapolis, Minnesota, USA). N-(1-naphtyl)- ethylenediamine dihydrochloride, lipopolysaccharide (LPS), sodium nitrite, and PDTC were purchased from Sigma (St. Louis, Missouri, USA). Rabbit polyclonal antisera to iNOS were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). N^GMMA was purchased from Calbiochem (San Diego, California, USA). Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, Michigan, USA). Dulbecco's Modified Eagle's Medium (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 Gibco BRL (Grand Island, New York, USA).

Animals

The original stock of male C57BL/6J mice were purchased from the Daehan Biolink Co., (Daejeon, Republic of Korea), and were housed at a temperature of 23 ± 1℃ with a 12/12 h light dark cycle. Food and water were available *ad libitum*. All protocols were approved by the institutional animal care and use committee of Kyung Hee University.

Preparation of HRG

The 90 g of raw rice and 10 g of red ginseng were washed and air-dried. These were precipitated in 200 ml distilled water for 2 h, and then boiled at 121℃ for 15 min. After 1 kg of malted barley was extracted with 1 l distilled water at 60℃ for 2 h, the resulted extracts were added and hydrolyzed the mixture of raw rice and red ginseng. The mixture was reacted at 60℃ for 3 h, and then boiled at 90℃ for 5 min. The reaction mixture was filtered and kept at 4℃.

Assay for endotoxin determination

The HRG used in this experiment was found to be free of endotoxin as determined within the limits of an assay E-TOXATE kit (Sigma, USA), preformed according to the manufacturers protocol. In this assay, saturation occurred at 40 EU/ml and the resolution limit was > 0.1 EU/ml.

Peritoneal macrophage cultures

TG-elicited macrophages were harvested 3 - 4 days after i.p. injection of 2.5 ml of TG to the mice and isolated, as reported previously (An et al., 2008). 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 culture plates 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.

MTT assay

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with with rIFN-γ (10 U/ml) for 6 h. The cells were then stimulated with various concentrations of HRG (0.01 - 1 mg/ml). Cell aliquots were incubated with 20 μl of a MTT solution (5 mg/ml) for 4 h at 37℃ under 5% CO_2 and 95% air. Consecutively, 250 μl of DMSO was added to extract the MTT formazan and the absorbance of each well at 540 nm was read by an automatic microplate reader (Molecular Devices Corp., Sunwayle, California, USA).

Measurement of nitrite concentration

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with rIFN-γ (10 U/ml) for 6 h. The cells were then stimulated with various concentrations of HRG. After 48 h of culture, NO synthesis in cell cultures was measured by a microplate assay method, as previously described (An et al., 2008). 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 (Molecular Devices Corp., Sunwayle, California, USA). NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 8 μM of NO_2^-. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

Assay of TNF-α release

Peritoneal macrophages (2.5×10^5 cells/well) were incubated with rIFN-γ (10 U/ml), HRG, rIFN-γ plus LPS (10 μg/ml), and rIFN-γ plus various concentrations of HRG (0.01 - 1 mg/ml) for 24 h. Then the amount of TNF-α secreted by the cells was measured by a modified enzyme-linked immunosorbent assay (ELISA), as described previously (An et al., 2008). The ELISA was devised by coating 96-well plates of murine monoclonal antibody with specificity for TNF-α. Before use and between subsequent steps in the assay, coated plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20. All reagents used in this assay were incubated for 2 h at 37°C. The rTNF-α was diluted and used as a standard. Serial dilutions starting from 10 ng/ml were used to establish the standard curve. After 2 h incubation at 37℃, the wells were washed and then each of 0.2 μg/ml of biotinylated anti-mouse TNF-α was added and the plates were incubated at 37℃ for 2 h. After washing the wells, avidin-peroxidase was added and the plates were incubated for 20 min at 37℃. Wells were again washed and ABTS was added. Color development was measured at 405 nm using an automated microplate ELISA reader (Molecular Devices Corp., Sunwayle, California, USA).

Nuclear protein extraction

Nuclear extracts were prepared as described previously (Jeong et al., 2002). Briefly, after cell activation for the times indicated cells were washed with ice-cold PBS and resuspended in 60 μl of buffer A (10 mM Hepes/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9).

The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 μl of 10% Nonidet P-40, and centrifuged at 2000 g for 10 min at 4℃. The nuclei pellet was resuspended in 40 μl of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), left on ice for 20 min, and inverted. The nuclear debris was then spun down at 15,000 g for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen and stored at -70℃ until conducting the analysis.

Western blot analysis

Peritoneal macrophages (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (R&D Systems, Minneapolis, MN, USA; 10 U/ml). The cells were then stimulated with HRG (1 mg/ml) or LPS (10 μg/ml). Whole cell lysates were made by boiling peritoneal macrophages in sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate (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 and then incubated with antibodies (Santa Cruz Biotechnology, Inc., CA, USA). After washing in PBS-tween-20 three times, the blot was incubated with 1:3000 diluted anti-rabbit or anti-mouse IgG, horseradsh peroxidase linked whole antibody (Amersham Corp. Newark, NJ, USA), and the antibody-specific proteins were visualized using the enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp. Newark, NJ, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from macrophages according to the manufacturer's specification using an easy-BLUE RNA extraction kit (iNtRON Biotech, Korea). Total RNA (2.0 μg) was heated at 75℃ for 5 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 60 min at 42℃ using a cDNA synthesis kit (iNtRON Biotech). PCR was performed with the following primers for TNF-α (5′-ATGAGAACAGAAAGCATGATC-3′; 5′-TACAGGCTTG TCACTCGAATT-3′), iNOS (5′-TCACTGGGACAGCAC AGAAT-3′; 5′-TGTGTCTGCAGATGTGCTGA-3′), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (5′-GGCATGGACTGTGGTCATGA-3′; 5′-TTCACCACCAT GGAGAAGGC-3′) which were used to verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 58℃ for iNOS, 60℃ for TNF-α, and 62℃ for GAPDH, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. GAPDH mRNA was used as a control of mRNA loading.

Statistical analysis

Results were expressed as the mean ± S.E.M. of independent experiments, and statistical analyses were performed by one-way analysis of variance (ANOVA) with Tukey, and Duncan post hoc test to express the difference between groups. All statistical analyses were performed using SPSS v11.0 statistical analysis software (SPSS INC., Chicago, Illinois, USA). A value of $p < 0.05$ was considered to indicate statistical significance.

RESULTS

Effect of HRG on activation of Rip2, IKK-β, and NF-κB

Macrophages play an important role in immune-enhancement. When the macrophage was activated, the expressions of iNOS and TNF-α increased. Rip2, IKK-β, and NF-kB are upstream proteins for the expressions of iNOS and TNF-α. To investigate the immunostimulating activities of HRG, we investigated the effects of HRG on the activations of IKK-β, Rip2, and NF-κB. As shown in Fig. 1A, stimulation of peritoneal macrophages with LPS after the treatment of rIFN-γ resulted in the increased protein synthesis of Rip2 and IKK-β. When HRG was used in combination with rIFN-γ, there was a marked co-operative activation of Rip2 and IKK-β. Since NF-κB activation requires the nuclear translocation of the *Rel A*/p65 subunit of NF-κB, we examined the effect of HRG on the nuclear pool of *Rel A*/p65 protein by Western blot analysis. As shown in Fig. 1B, rIFN-γ plus HRG treatment considerably increased the nuclear *Rel A* /p65 protein level, indicating the nuclear translocation of *Rel A*/p65. However, HRG had no effect on the activations of Rip2, IKK-β, and NF-κB by itself.

Effects of HRG on NO production in peritoneal macrophages

The downstream mediators of the pathways involving Rip2, IKK-β, and NF-κB were investigated. NO is a highly reactive molecule produced from the guanidino nitrogen of arginine by iNOS (Nathan, 1992). Over the past decade, NO as a potent

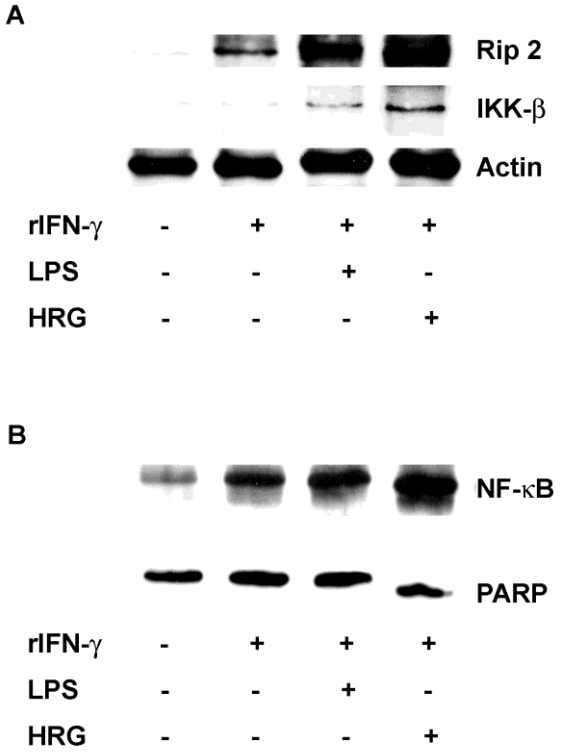

Fig. 1. Effect of HRG on the activations of Rip 2, IKK-β, and NF-κB in peritoneal macrophages. (A) The cells (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml). The cells were then stimulated with HRG (1 mg/ml) or LPS (10 μg/ml). The protein extracts were prepared, and then the samples were analyzed for Rip2 and IKK-β expressions by Western blot analysis. (B) The cells (1×10^7 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml). The cells were then stimulated with HRG (1 mg/ml) or LPS (10 μg/ml). The nuclear protein extracts were prepared, and then the samples were analyzed for NF-κB expression by Western blot analysis. PARP, Poly ADP-ribose polymerase.

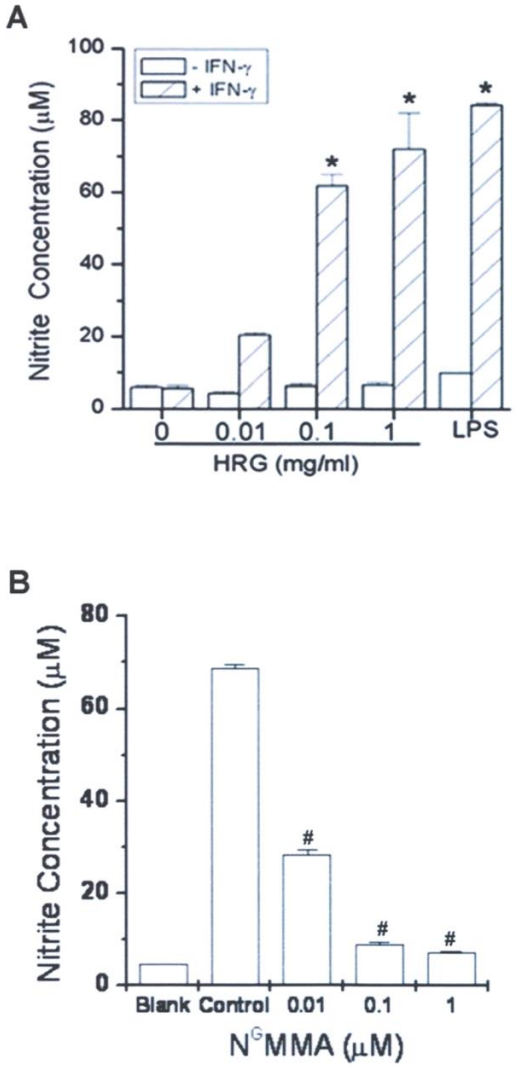

Fig. 2. Effect of HRG on NO synthesis in peritoneal macrophages. The cells (2.5×10^5 cells/well) were cultured with rIFN-γ (10 U/ml). (A) The cells were then stimulated with various concentrations of HRG for 6 h after incubation. After 48 h of culturing, NO release was measured. (B) The cells (2.5×10^5 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml) plus various concentrations of N^GMMA. The cells were then treated with HRG (1 mg/ml) and cultured for 48 h. NO (nitrite) released into the medium is presented as the mean ± S.E.M. of three independent experiments duplicated in each run. Control, rIFN-γ + HRG (1 mg/ml). $^*p < 0.05$ compared to rIFN- γ alone; $^\#p < 0.05$ compared to control (absence of N^GMMA).

macrophage-derived effector molecule against a variety of bacteria, parasites, and tumors has received increasing attention (Gantt et al., 2001). To determine the effect of HRG on the production of NO by mouse peritoneal macrophages, we treated non-primed (resting) and rIFN-γ-primed cells with HRG. The resultant NO production was determined by detecting the levels of nitrite concentration in the cell supernatants after 48 h treatment. As shown in Fig. 2A, HRG had no effect on NO production in resting mouse peritoneal macrophages. IFN-γ (10 U/ml) alone does not cause the induction of NO production in accordance with previous reports. However, when IFN-γ was used in conjunction with LPS, there was a marked augmentation of the production of NO. When mouse peritoneal macrophages were primed for 6 h with murine rIFN-γ and then treated with HRG, NO production increased compared to non-primed conditions. HRG didn't affect the viability of cells

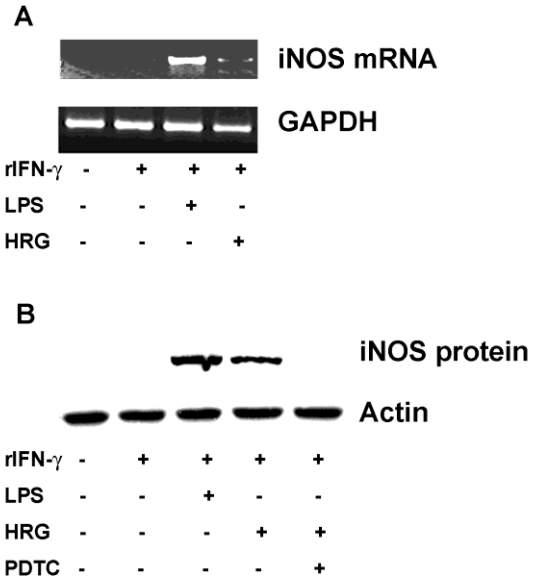

Fig. 3. Effects of HRG on the expressions of iNOS mRNA and protein in peritoneal macrophages. (A) The cells (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml). The cells were then stimulated with HRG (1 mg/ml) or LPS (10 μg/ml) for 24 h. The RNA extracts were prepared, and then the samples were analyzed for iNOS mRNA expression by RT-PCR. (B) The cells (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml) and then treated PDTC (100 μM) for 1 h. The cells were then stimulated with HRG (1 mg/ml) or LPS (10 μg/ml) for 24 h. The protein extracts were prepared and then the samples were analyzed for iNOS protein expression by Western blot analysis.

at those concentrations (data not shown). To determine if the signaling mechanism in HRG-induced NO production participates in the $_L$-arginine-dependent pathway in mouse peritoneal macrophages, the cells were incubated for 6 h in the presence of rIFN-γ plus N^GMMA. The production of nitrites by rIFN-γ plus HRG in mouse peritoneal macrophages was progressively inhibited with the increasing amounts of N^GMMA. The HRG-induced accumulation of nitrites was significantly blocked by N^GMMA (0.01 - 1 μM) (Fig. 2B, $p < 0.05$).

Effects of HRG on rIFN-γ-primed iNOS expression

Fig. 3 shows the effects of rIFN-γ plus HRG treatments on the expressions of iNOS mRNA and protein mouse peritoneal macrophages. HRG (1 mg/ml) increased synergistically the expressions of iNOS mRNA and protein in rIFN-γ-primed mouse peritoneal macrophages. It is known that PDTC, an anti-oxidant compound, inhibits NF-κB activation (Chung et al., 2004). With this understanding, as an approach to define the signaling mechanism of HRG on NO production, we examined the influence of PDTC in rIFN-γ plus HRG-treated mouse peritoneal macrophages. Adding PDTC (100 μM) to the rIFN-γ plus HRG-treated mouse peritoneal macrophages decreased the synergistic effects of HRG on iNOS expression (Fig. 3B).

Effects of HRG on rIFN-γ-primed TNF-α mRNA expression and production

We next examined the synergistic cooperative effects of HRG on rIFN-γ-induced TNF-α production. Mouse peritoneal macrophages secreted very low levels of TNF-α after 24 h incubation with a medium alone or rIFN-γ alone. However, HRG in combination with rIFN-γ markedly increased TNF-α mRNA expression and production (Fig. 4).

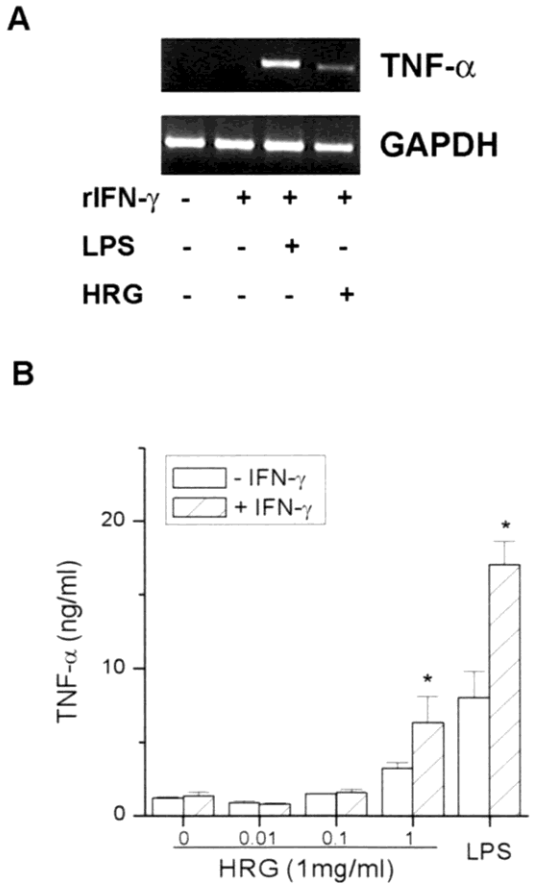

Fig. 4. Effects of HRG on TNF-α mRNA expression and production in peritoneal macrophages. (A) The cells (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml). The cells were then stimulated with HRG (1 mg/ml) or LPS (10 μg/ml). The RNA extracts were prepared, and then the samples were analyzed for TNF-α expression by RT-PCR. (B) The cells (2.5×10^5 cells/well) were incubated for 6 h with rIFN-γ (10 U/ml). The cells were then stimulated with HRG (0.01 - 1 mg/ml) or LPS (10 μg/ml). The amount of TNF-α secreted by peritoneal macrophages was measured by the ELISA method after 24 h of incubation. Values are the mean ± S.E.M. of three independent experiments duplicated in each run. $^*p < 0.05$ compared to rIFN- γ alone.

DISCUSSION

In this study, we showed that HRG induced the expressions of iNOS and TNF-α through the activation of NF-κB, IKK-β, and Rip 2.

Red ginseng is produced by steaming fresh ginseng first, and then drying the steamed ginseng; on the other hand, white ginseng is made of fresh ginseng by the drying process only. Recently, several investigators have reported the discovery of new ginsenosides from red ginseng which are not usually found in raw ginseng. These ginsenosides are Rg3, Rg5, Rg6, Rh2, Rh4, Rs3, and Rf and their levels in red ginseng are relatively low (Kim et al., 2000; Park, 1996). Ginsenosides Rg3, Rg5, and Rh2 have shown anticancer effects (Yun, 2003). Among these three ginsenosides, Rg3 has been reported to exhibit neuroprotective (Tian et al., 2005), antinociceptive (Rhim et al., 2002), hematopoietic (Joo et al., 2004), anticarcinogenic, and antimetastatic effects (Tao et al., 2002; Yun et al., 2001). Choi et al., reported that red ginseng acidic polysaccharide in combination with IFN-g enhanced the macrophage function through activation of the NF-kB pathway (2008). In this study, we demonstrated that NO production in mouse peritoneal macrophages by HRG could be highly stimulated in combination with rIFN-γ. HRG increased NO production to a higher level than red ginseng or ginsenosides. Therefore, we postulate that HRG may contain the many active compounds. However, further studies to analyze the active compounds of HRG are necessary to support the present findings.

It has been previously reported that LPS stimulation of rIFN-γ-primed macrophages induces NF-κB activation (Shin et al., 2004). Expressions of iNOS and the TNF-α gene are dependent on the activation of NF-κB (Baldwin, 1996). We found that the addition of the NF-κB modulator, PDTC, inhibits the synergistic effect of HRG with rIFN-γ on iNOS expression. These results suggest that HRG increases NO production through NF-κB activation. The NF-κB system may provide a future target in cancer therapy. Rip2 and IKK complexes may play an important role for NF-κB activation (Inohara et al., 2000). Until now, the regulatory effect of drugs in Rip 2 activation has not been reported. In this study, we showed that HRG induced the activations of Rip 2 and IKK-β for the first time. The results of this study suggest that HRG activates NF-κB via interaction with Rip 2 and IKK-β.

In conclusion, our results demonstrated that HRG acted as an accelerator of peritoneal macrophages activation by rIFN-γ via a process involving $_L$-arginine-dependent NO production and that it increased the production of TNF-α significantly via NF-κB activation by interaction with Rip 2 and IKK-β. These results suggest that the processes of HRG might explain its regulatory effect in the immune system.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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