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Inhibitory effects of a fermented ginseng extract, BST204, on the expression of inducible nitric oxide synthase and nitric oxide production in lipopolysaccharide-activated murine macrophages

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Abstract

In this study, the effects of BST204, a fermented ginseng extract, on the expression of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production are looked into. Crude ginseng extract was incubated with ginsenoside-β-glucosidase to prepare BST204. BST204, unlike lipopolysaccharide (LPS) and crude ginseng extract, did not affect the level of iNOS protein and NO production in unstimulated RAW 264.7 cells. However, it suppressed the level of iNOS protein and NO production in LPS-stimulated RAW 264.7 cells but did not manifest the same effect on the iNOS mRNA level. An investigation of the activating phosphorylation of p70 S6 kinase and 4E-BP1, which are important for translation, was conducted to investigate the suppressive mechanism of iNOS protein. LPS increased the phosphorylation of p70 S6 kinase, but not 4E-BP1, in a time-dependent manner, and BST204 inhibited it in a dose-dependent manner. The expression of iNOS protein, however, was partially suppressed by rapamycin, an upstream inhibitor of p70 S6 kinase. Therefore, this paper suggests that the suppression of iNOS protein by BST204 was partially correlated with the inhibition of p70 S6 kinase activation.

Introduction

It is widely known that nitric oxide (NO), synthesized by nitric oxide synthase (NOS), is involved in diverse physiological processes (Radomski et al 1987; Moncada et al 1991; Snyder & Bredt 1992; Yap & Sher 1999). Despite the fact that NO is a pro-inflammatory and anti-inflammatory agent, excess NO production is largely thought to cause a variety of inflammatory diseases, such as sepsis, psoriasis, arthritis, multiple sclerosis and systemic lupus erythmatosus (Clancy et al 1998; Kroncke et al 1998). Three isoforms of NOS – types I, II, and III – have been identified in mammalian cells, according to the physical and biochemical characteristics of the purified enzymes. Type I (neuronal NOS, nNOS) and type III (endothelial NOS, eNOS), referred to as the constitutive NOS (cNOS), are permanent fixtures in cells. Type II, on the other hand, an inducible-type NOS (iNOS), comes about only after cells have been exposed to specific stimulants, such as cytokines, bacterial lipopolysaccharides (LPS) and calcium ionophores (Vodovotz et al 1993; Chesrown et al 1994; Denlinger et al 1996; Weisz et al 1996). Once expressed, iNOS generates significantly large and sustained amounts of NO. An excessive amount of NO is thought to have the ability to mediate acute and chronic inflammation.

The root of *Panax ginseng* (Araliaceae), commonly known as ginseng, is widely used in Asian countries as a traditional remedy. Since ancient times, various preparations of ginseng have been taken orally as health products or natural medicines. Ginsenosides, a diverse group of steroidal saponins that target a vast range of tissues, and their metabolites, are generally believed to be mainly responsible for the

pharmacological activity of ginseng (Attele et al 1999; Yun 2003). Ginsenosides have two major structural groups, namely panaxadiols (Rb1, Rb2, Rc, Rd, Rg3, Rh2 and Rh3) and panaxatriols (Re, Rf, Rg1, Rg2 and Rh1). Of these, ginsenoside Rh2 is especially well known and sought after because it can arrest the proliferation of various cultured cancer cells and can influence apoptosis (Park et al 1997; Nakata et al 1998; Fei et al 2002; Popovich & Kitts 2002). Rg3, for its part, has been demonstrated to prevent the growth of tumours and to produce an effect on drug-resistant, cultured cancer cells (Keum et al 2003; Kim et al 2003).

Although the anti-tumour and anti-inflammatory effects of Rh2 and Rg3 have been well established, the chemical synthesis of these compounds is currently very difficult. Therefore we aimed to enrich Rh2 and Rg3 with ginsenoside-β-glucosidase in crude ginseng extract. Also, the effects of the enriched ginseng ethanol extract on the expression of iNOS, production of NO and the mechanism of action were then investigated.

We found that BST204 significantly suppressed the expression of iNOS and NO production in a dose-dependent manner, but it did not produce any effect on the iNOS mRNA level. BST204 inhibited the activating phosphorylation of p70 S6 kinase in a dose-dependent manner and rapamycin, an upstream inhibitor of p70 S6 kinase, partially suppressed the expression of iNOS in a dose-dependent manner.

It can therefore be concluded that the activating phosphorylation of p70 S6 kinase is partly responsible for the suppression of iNOS protein and NO production.

Materials and Methods

Reagents

The cell culture reagents were obtained from GIBCO/Invitrogen (Carlsbad, CA, USA). The Tris-glycine polyacrylamide gels were purchased from Novex (San Diego, CA, USA). The Tris-reagent, monoclonal anti-β-actin antibody, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium (MTT) were from Sigma-Aldrich (St Louis, MO, USA). Anti-iNOS, and anti-p70 S6 kinase were from Santa Cruz (Santa Cruz, CA, USA). Anti-phospho-p70 S6 kinase, anti-4E-BP1 and anti-phospho-4E-BP1 were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Preparation of BST204 (fermented ginseng extract)

BST204, fermented ginseng extract, was obtained from Biosapogen Co., Ltd (Sungnam, Korea) and had been prepared according to its patented protocol. In brief, 0.5 g dried ginseng was extracted from 2.5 L ethanol, and was concentrated with a speedi-vac. The dry ginseng extract was then incubated with an enzyme solution containing ginsenoside-β-glucosidase. When the enzyme had reacted, it was removed through ultrafiltration (MW cut-off, 10 000 Da). The solution was concentrated and treated with 50% acetic acid. The concentrate was then loaded into an HP20 column, which was washed out with distilled water to obtain the neutral pH of the flow-through and was finally eluted with ethanol. The dried eluate was designated as BST204 (fermented ginseng extract). The BST204 contained various ginsenosides including 5% Rh2, 10% Rg3, 0.3% Rb1, 0.6% Rc, 0.3% Rd, 0.6% Rg1, 1.6% Rh1 and 2.6% F2. The contents of Rh2 and Rg3 were very high compared with those of non-detectable Rh2 and 0.0003% Rg3 in the crude ginseng extract.

Analysis of ginsenosides in the ginseng extracts

For the analysis of ginsenosides in ginseng extracts, high-performance liquid chromatography was accomplished utilizing the Capcell pack C18 UG 80 column (Shiseido, Japan) with a flow rate adjusted to 1.0 mL min^{-1}. Following sample injection (usually 20 μL), they were eluted under the gradient condition of 10–60% acetonitrile for 45 min and the eluate was monitored at 203 nm by an ultraviolet detector. The amount of each ginsenoside was determined directly from the peak area by use of a calibration curve.

Cell culture and stimulation

The murine macrophage RAW 264.7 cells were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM), containing 10% fetal bovine serum (FBS), penicillin (100 U mL^{-1}) and streptomycin sulfate (100 μg mL^{-1}), in a humidified atmosphere of 6% CO_2. The cells were treated with BST204 or rapamycin 1 h before their treatment with 1 μg mL^{-1} LPS for 4 h or for the indicated times, washed with 1 × PBS (phosphate-buffered saline) and then used for immunoblot analysis and reverse-transcription polymerase chain reaction (RT-PCR).

MTT assay for cell viability

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium (MTT) is a pale yellow substance that living cells reduce to yield a dark-blue formazan product. This process requires active mitochondria and even fresh dead cells do not reduce significant amounts of MTT. The cells were cultured in 96-well flat-bottom plates containing DMEM with 10% FBS and were incubated overnight. BST204 was added to a final volume of 200 μL medium/well. The cells were incubated at 37 °C with 50 μL MTT solution (1 mg mL^{-1} in PBS) for 4 h. Aspirations were done to remove the medium and the cells were solubilized in DMSO (100 μL) for 10 min. With the use of a microplate reader, the optical density at 540 nm was determined.

Western blot analysis

The cells were treated with crude ginseng extract, BST204 and LPS, then washed out with 1 × PBS and lysed on ice for 30 min with the use of a lysis buffer (20 mM HEPES,

pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 10% glycerol, 60 mM octyl β-glucoside, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, 0.7 μg mL^{-1} pepstatin and a protease inhibitor cocktail tablet). Lysates were centrifuged at 12 000 rev min^{-1} at 4 °C for 15 min, and the supernatants were dissolved in 2 × Laemmli buffer (Laemmli 1970). Proteins were separated by SDS-PAGE and were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dachen, Germany). The rabbit anti-iNOS antibody was used as primary antibody, along with other specific antibodies (see Reagents, above). Horseradish peroxidase-conjugated antibodies were used as secondary antibodies. Band detection was conducted using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Sweden).

RNA preparation and reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated with the use of a trizol reagent (Invitrogen), according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed using ImProm-II reverse transcriptase (Promega), following the instructions in the manufacturer's manual. Single-stranded cDNA was then amplified by PCR with specific primers of iNOS and β-actin: iNOS sense, 5′-TGCCCCTTCAATGGTTGGTA-3′; iNOS anti-sense, 5′-ACTGGAGGGACCAGCCAAAT-3′; β-actin sense, 5′-GGAGAAGATCTGGCACCACACC-3′; and β-actin anti-sense, 5′-CCTGC TTGCTGATCCACATCTGCTG G-3′. Thirty cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 30 s were applied.

Nitric oxide assay

The nitrite concentration in the culture medium was measured as an indicator of NO production based on the Griess reaction. One-hundred microlitres of each supernatant were mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) and the resulting mixture was then incubated at room temperature for 10 min. Its absorbance at 540 nm was then measured in a microplate reader. A freshly cultured medium was used as the blank in all experiments. The nitrite dilution standard curve and nitrite production were also measured.

Statistical analysis

The data were presented as the mean ± s.e.m. from three or more separate experiments. Statistical analysis was performed using a non-parametric analysis of variance (the Kruskal–Wallis test) and Dunn's test. All statistical calculations were performed using SigmaStat (Systat Software, Inc., Point Richmond, CA) software. Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.001$).

Results

Effect of BST204 on iNOS expression and NO production

There was minimal expression of iNOS protein in unstimulated RAW 264.7 cells and maximal expression in cells treated with LPS for 8 h (Figure 1A). iNOS protein then gradually decreased in the latter set of cells (data not shown). In addition, 4 h after the cells' treatment with LPS, the expression of iNOS mRNA peaked (Figure 1A). In light of the emerging evidence that crude ginseng extract stimulates immune cells to produce NO by iNOS, an investigation into whether BST204 induces the expression of iNOS and NO production was conducted. While crude ginseng extract, not treated with ginsenoside-β-glucosidase, stimulated the expression of iNOS and NO production, BST204 did not affect either (Figure 1B, data not shown for NO production). These results led to the further investigation of whether BST204 has any inhibitory activity on iNOS expression and NO production. As shown in Figure 2, BST204 dose-dependently inhibited the expression of iNOS protein and NO production, but crude ginseng extract did not. Next, we investigated whether Rh2 and Rg3, the major components of BST204, inhibited the expression of iNOS protein and NO production at the same doses as those in BST204, which contains 2.5 μg mL^{-1} Rh2 and 5.0 μg mL^{-1} Rg3. As shown in Figure 3, 5.0 μg mL^{-1} Rg3 suppressed significantly the expression of iNOS

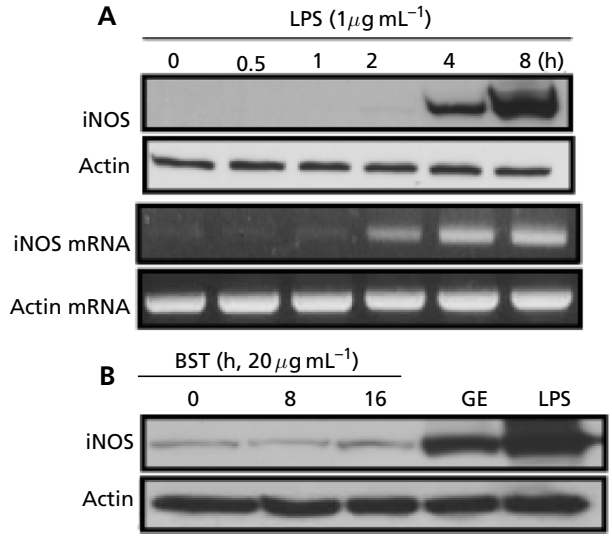

Figure 1 Effect of BST204 on iNOS expression in RAW 264.7 macrophages. A. The cells were incubated with 1 μg mL^{-1} LPS for the indicated times, and the levels of iNOS protein were determined using Western blot analysis, as described in Materials and Methods. B. The cells were incubated with 20 μg mL^{-1} BST204 for 8 or 16 h, or with crude ginseng extract (GE, 20 μg mL^{-1}) for 16 h, and the levels of iNOS protein were determined using Western blot analysis.

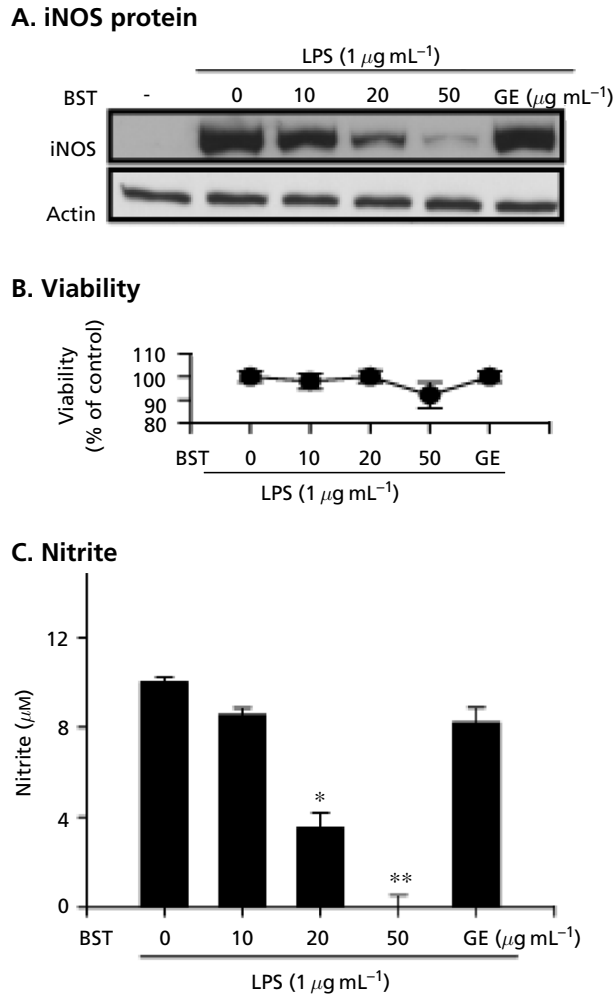

Figure 2 BST204 inhibited the expression of iNOS protein and NO production. A. The RAW 264.7 cells were treated with BST204 in the indicated doses, or with crude ginseng extract (GE, 50 μg mL^{-1}), 1 h before incubating them with 1 μg mL^{-1} LPS for 4 h, and the levels of iNOS protein were determined using Western blot analysis. B. Cell viability with BST204. C. The RAW 264.7 cells were treated with BST204 in the indicated doses, or with crude ginseng extract (GE, 50 μg mL^{-1}), 1 h before incubating them with 1 μg mL^{-1} LPS for 12 h, and the concentration of nitrite in the culture media was monitored through the Griess reaction.

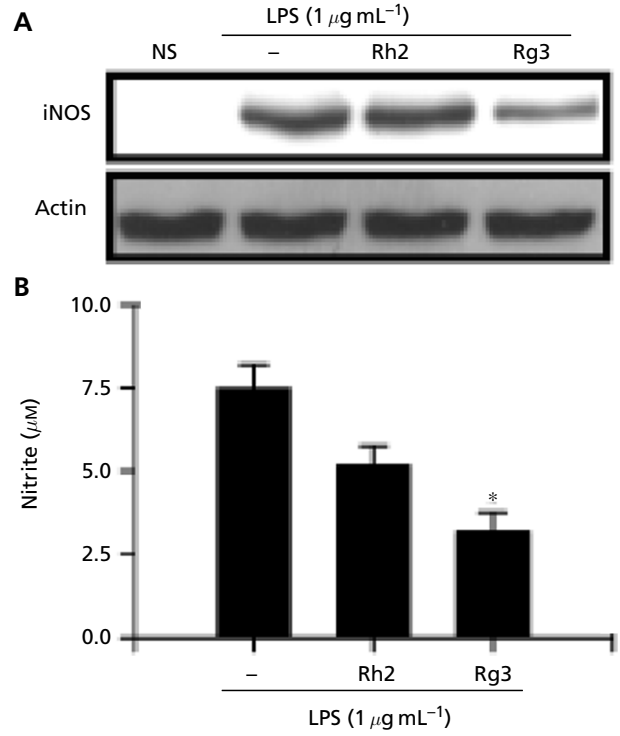

Figure 3 Effect of individual Rh2 and Rg3 on the expression of iNOS protein (A) and NO production (B). The RAW 264.7 cells were treated with 2.5 μg mL^{-1} Rh2 or 5.0 μg mL^{-1} Rg3 1 h before they were incubated with 1 μg mL^{-1} LPS for 12 h, and the levels of iNOS protein and NO production were determined using Western blot analysis and the Griess reaction, as described in Materials and Methods.

protein and NO production. However, 2.5 μg mL^{-1} Rh2 had a minimal effect.

Effect of BST204 on iNOS mRNA expression

An investigation into whether BST204 has any effect on the level of iNOS mRNA was then conducted. Surprisingly, no such effect was observed (Figure 4). BST204 did not have any effect on the transcription pathway and the mRNA stability for iNOS. Whether BST204 suppressed the expression of iNOS protein through translational inhibition was then looked into.

Effect of BST204 on the activation of p70 S6 kinase

It is widely acknowledged that p70 S6 kinase is activated through multiple phosphorylations and that its activation is critical for translation (Dunfer & Thomas 1997; Pullen & Thomas 1997). p70 S6 kinase was time-dependently activated by LPS, and after a 1-h stimulation, it showed maximal phosphorylation (Figure 5A). BST204 dose-dependently inhibited the activation (Figure 5B). Consistent with the data shown in Figure 2A, crude ginseng extract did not affect the activating phosphorylation of p70 S6 kinase (Figure 5B).

Rapamycin's partial inhibition of the expression of iNOS

To confirm the above result, whether rapamycin (an upstream inhibitor of p70 S6 kinase) suppressed the level of iNOS protein in LPS-stimulated RAW 264.7 cells was determined. It was found that the expression of iNOS protein was partially suppressed in a dose-dependent manner (Figure 6).

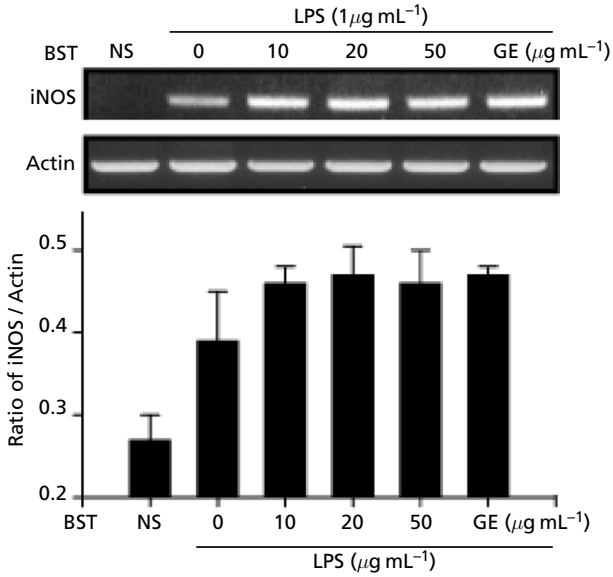

Figure 4 BST204 did not produce any effect on the iNOS mRNA level. The RAW 264.7 cells were treated with BST204 in the indicated doses, or with crude ginseng extract (GE, 50 μg mL^{-1}), 1 h before they were stimulated with 1 μg mL^{-1} LPS for 4 h, and the levels of iNOS mRNA were measured by RT-PCR, as described in Materials and Methods.

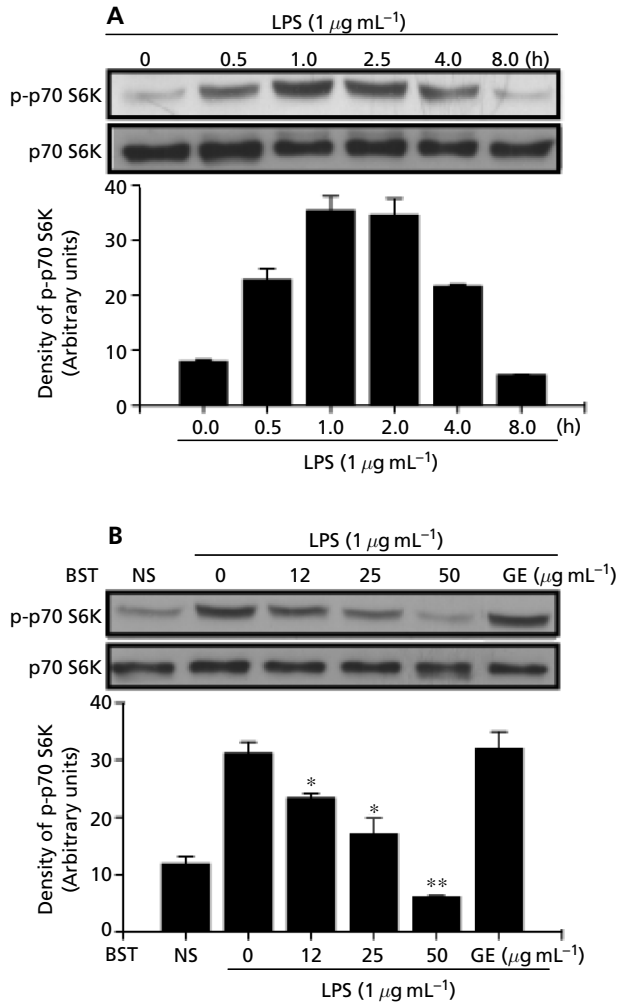

Figure 5 BST204, and not the crude ginseng extract (GE), dose-dependently inhibited the activating phosphorylation of p70 S6 kinase. A. The RAW 264.7 cells were treated with 1 μg mL^{-1} LPS for the indicated times, and the phosphorylation of p70 S6 kinase was determined using Western blot analysis. B. The RAW 264.7 cells were treated with BST204 in the indicated doses, or with crude ginseng extract (GE, 50 μg mL^{-1}), 1 h before incubating with 1 μg mL^{-1} LPS for 4 h, and the phosphorylation of p70 S6 kinase was determined using Western blot analysis, as described in Materials and Methods.

Discussion

Since increased NO production is associated with acute and chronic inflammation, and since the intracellular level of iNOS largely determines NO production rates in activated macrophages and several other cell types, iNOS regulation was given due importance for the treatment of inflammation (Szabo & Thiemermann 1995; MacMicking et al 1997). In this study, an investigation of whether BST204, a fermented ginseng extract that enriches Rh2 and Rg3, has potential therapeutic value was looked into by determining its effect on iNOS protein and NO production.

Current findings point to the possibility that ginsenosides or ginseng extract have immune-cell-modulating properties. In particular, the aqueous and acidic polysaccharide extracts of *Panax ginseng* root stimulated the production of NO through the induction of iNOS in RAW 264.7 macrophages (Friedl et al 2001; Shin et al 2002). In another study, Rh1 and Rh2 inhibited iNOS-mediated NO production (Park et al 1996). In this paper, it was shown that crude ginseng extract alone, and not BST204, induced the expression of iNOS protein and NO production, as induced by *Panax ginseng* aqueous extracts or acidic polysaccharide extracts (Figure 1B). A hypothesis was then formulated – that BST204, which is enriched with Rh2 and Rg3, suppresses the iNOS protein and NO production. An investigation into whether BST204 inhibited them in the LPS-stimulated cells was then conducted. As shown in Figures 2A and 2C, BST204 dose-dependently suppressed the expression of iNOS protein and NO production. BST204 contains various ginsenosides, such as Rh2, Rg3, Rb1, Rc, Rd, Rg1, Rh1 and F2 and probably other ginsenoside metabolites (see Materials and Methods). To prove that the effect of BST204 on iNOS and NO production was derived from Rh2 and Rg3, we measured if individual 2.5 μg mL^{-1} Rh2 and 5.0 μg mL^{-1} Rg3 at the same doses as those in BST204 suppressed the expression of iNOS protein and NO production. Though 5.0 μg mL^{-1} Rg3 had a significant effect on them, the effect of 2.5 μg mL^{-1} Rh2 was minimal (Figure 3). The more potent inhibitory effect of BST204, compared with those of individual Rh2 or Rg3 (Figure 2A), suggested to us that there are some additive or synergistic effects of other minor ginsenosides or their metabolites in BST204.

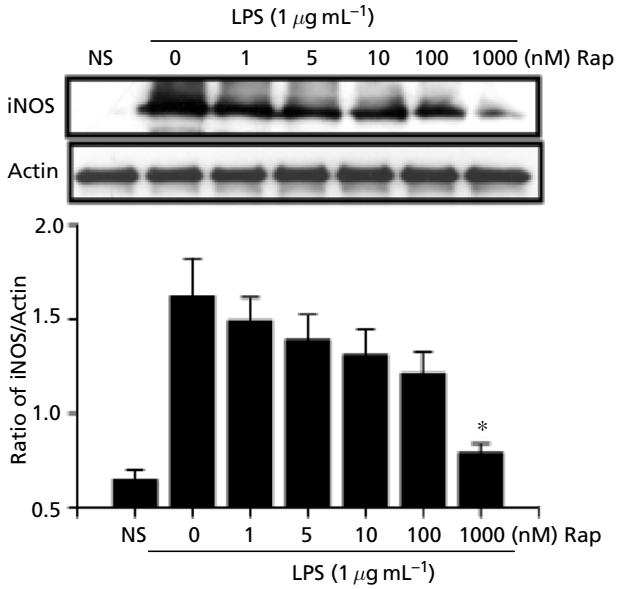

Figure 6 Effect of rapamycin on the expression of iNOS protein. The RAW 264.7 cells were treated with rapamycin (Rap) in the indicated doses 1 h before they were incubated with 1 μg mL^{-1} LPS for 24 h, and the levels of iNOS protein were determined using Western blot analysis, as described in Materials and Methods.

Next, the mechanism to inhibit iNOS expression was investigated to verify the hypothesis that BST204 suppresses the NF-κB pathway as the action mechanism with 20(*S*)-protopanaxatriol and other agents (Kim et al 2001; Ban et al 2004; Oh et al 2004). The hypothesis, interestingly, was wrong. The expression of iNOS mRNA in LPS-induced macrophages did not change with BST204 (Figure 4). Unlike the case of 20(*S*)-protopanaxatriol and other reports, this result indicated that BST204 did not produce an effect on the expression of mRNA for iNOS. Recently, several reports have suggested that NO production is mediated on the p70 S6 kinase pathway in murine macrophages (Salh et al 1998; Lim et al 2003). It was therefore interesting to know whether BST204 suppressed LPS-stimulated p70 S6 kinase-mediated translation in the cells. Whether two of the major signaling pathways for translation, p70 S6 kinase and 4E-BP1, were activated by LPS in the cells was determined. LPS time-dependently increased the activating phosphorylation of p70 S6 kinase (Figure 5A). The phosphorylation level of 4E-BP1, however, was already high in unstimulated RAW 264.7 cells, and was minimally increased by LPS in the cells (data not shown). BST204, and not crude ginseng extract, dose-dependently inhibited the activation of p70 S6 kinase (Figure 5B). It was recently reported by Lim et al (2003) that the expression of iNOS protein and NO production was mediated by activation of p70 S6 kinase. It was strongly suggested by this paper's results and those of Lim's that the inhibition of iNOS protein expression is derived from the inhibition of translation through p70 S6 kinase in RAW 264.7 cells. Rapamycin, an inhibitor of mTOR, which is upstream of p70 S6 kinase and 4E-BP1, however, partially inhibited iNOS expression in our system (Figure 6). We suggested that the inhibition with BST204 was partially mediated by the p70 S6 kinase pathway and the possibility that BST204 additionally suppressed them through other mechanisms, such as the activation of the ubiquitin/proteosome pathway, was not excluded. There are several reports that NO production in LPS-stimulated murine macrophages and rat hepatocytes is regulated by rapamycin (Salh et al 1998; Attur et al 2000; Lim et al 2003; Tunon et al 2003). However, the clear mechanism on the expression of iNOS protein is still controversial. For example, the expression of iNOS protein was inhibited by 10 nM rapamycin (Tunon et al 2003). In contrast, Salh et al (1998) reported that it was not inhibited by 27.3 μM rapamycin. Our results indicated that the expression of iNOS was partially suppressed by a low concentration and significantly inhibited by a high concentration of rapamycin (Figure 6). Although it is not clear why there has been the discrepancy, we carefully suggest that there may be some differences in experimental conditions, such as starvation of cells and LPS concentration.

It is currently difficult to chemically synthesize Rh2 and Rg3 to develop them as a therapeutic medicine. Therefore, an attempt was made to enrich Rh2 and Rg3 in crude ginseng extract by treating the extract with ginsenoside-β-glucosidase. BST204 containing 5% Rh2 and 10% Rg3 was obtained (see Materials and Methods). The effect of BST204 on the expression of iNOS was compared with that of single Rh2 or Rg3 ginsenosides. Interestingly, compared with an individual activity of Rh2 or Rg3, BST204 demonstrated a more potent activity on the iNOS expression and NO production. This indicated that ginsenosides, including Rh2 and Rg3, in BST204 might be responsible for its seemingly additive or synergistic activity. Recently, the inhibitory effect of one of the ginsenoside metabolites, protopanaxatriol, on iNOS expression was reported; it suppressed the expression of iNOS mRNA (Oh et al 2004), which was different from BST204. While BST204 suppressed iNOS protein and NO production without any effect on the mRNA level (Figure 2 and 4), protopanaxatriol inhibited the iNOS mRNA level by inhibiting the NF-κB pathway (Oh et al 2004). Therefore, it did not seem likely that the additive effect of BST204 on the expression of iNOS protein and NO production was derived from protopanaxatriol.

Conclusions

Inducible nitric oxide synthase (iNOS) is upregulated in response to inflammatory and pro-inflammatory mediators, and nitric oxide (NO) can influence many aspects of the inflammatory cascade. Abnormally excessive NO production has been associated with the pathogenesis of inflammatory diseases and certain types of human cancers. In this study, an investigation into whether a fermented ginseng extract, BST204, could regulate the expression of iNOS protein and NO production in murine macrophage cell line RAW 264.7 cells stimulated with

lipopolysaccharide (LPS) was conducted. As a result, BST204 significantly suppressed the expression of iNOS and NO production in a dose-dependent manner and the inhibitory effect seemed to have a partial correlation with the inhibition of p70 S6 kinase. It can therefore be concluded that it may be possible to develop BST204 as a useful agent for the chemoprevention of cancer or inflammatory diseases.

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