The Immunosuppressive Effect of Gamisanghyulyunbueum through Inhibition of Mitogen-Activated Protein Kinase and Nuclear Factor Activation in MOLT-4 Cells

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Gamisanghyulyunbueum (GSHYBE) has been used clinically to treat skin related disease in South Korea. We investigated GSHYBE-mediated changes in downstream T cell signal transduction. To determine the mechanism of inhibition, we have studied many of the major pathways in phytohemagglutinin (PHA)-activated T cell. We show that among the mitogen-activated protein kinase family activation of phosphorylation of extra cellular signal-regulated kinase 1/2 (ERK1/2, p44/42) and p38, but not c-jun NH2-terminal kinase is inhibited. In activated MOLT-4 cells, the nuclear localization of calcium factor of activated T cells (NFATc) was blocked by GSHYBE (1 mg/ml). Also, degradation of inhibitor X-α and transactivation by nuclear factor-κB (NF-κB)/Rel A were impaired by GSHYBE (1 mg/ml). Furthermore, interleukin (IL)-2, IL-4 and Interferon (IFN)-γ secretion by PHA activated MOLT-4 cells and peripheral blood mononuclear cells (PBMC) were significantly diminishes following GSHYBE treatment (1 mg/ml). Also, oral administration of GSHYBE inhibited IL-2 secretion in skin allergic reaction. In conclusion, our data indicate that GSHYBE treatment of T cells inhibits ERK1/2 and p38 activation and nuclear translocation of NFATc, NF-κB, resulting in diminished secretion of IL-2.

Key words Gamisanghyulyunbueum; mitogen-activated protein kinase; NFATc; nuclear factor-κ/Rel A; interleukin-2

Typical Korean traditional medicines consist of 3 to 15 components that are mixed to minimize side effects and maximize medicinal effects. Traditional medicines appear to exert their pharmacological actions through the synergistic effects of their components and via drug interaction. Therefore, they may modulate biological responses, including immune responses, rather than acting through the direct activity of the individual components.1,2

Gamisanghyulyunbueum (GSHYBE) is a traditional Oriental herbal medicine prescription, which has been used for the treatment of allergic disorders such as atopic dermatitis and especially, skin related disease.3 However, its mechanism has not been investigated experimentally.

Activation of T cells is a very complex process that involves cell-to-cell interactions of several cell surface molecules. Engagement of the T cell antigen receptor (TcR) with the antigen-major histocompatibility complex on antigen-presenting cells triggers a complex TcR signaling cascade that leads to T cell activation and cytokine secretion.4 In consequence to early protein phosphorylation steps and calcium response, mitogen-activated protein kinases (MAPKs) are activated by phosphorylation. The three major family of MAPKs, c-Jun NH2-terminal kinases (JNK), extracellular signal-regulated kinases (ERK), and p38 MAPK, are regulated by distinct but cross-talking signaling cascades.5 Such signals culminate in the activation of transcription factors such as nuclear factor-κB (NF-κB) and the nuclear factor of activated T cells (NFAT).6–8

The transcription factor NFAT plays an essential role in IL-2 expression. Among of NFAT family members, NFAT1, NFAT2 (NFATc1), and NFAT4 are involved in the transcriptional activation of genes encoding cytokines including IL-2 and IL-4, and CD40 ligand.9 NFAT2 is induced following T cell stimulation to the same level as NFAT1; however, in nuclear extracts from activated T cells, nearly all the NFAT that bound to a prove corresponding to the distal NFAT site of the human IL-2 promoter was attributable to NFAT1.9 In contrast to normal T cells, NFAT2 seems to play the major role in driving IL-2 transcription in Jurkat T cells.10 NF-κB plays a central role in a number of signaling pathways in many cell types.11 Fundamental processes such as cell growth, apoptosis, and development are regulated by NF-κB, and NF-κB is a central mediator of immune, inflammatory, and stress response.11 The predominantly characterized NF-κB complex is a p50–p55 heterodimer, which at rest is retained in the cytoplasm and is associated with an inhibitor molecular, IκB.7 During T cell activation, IκBα is phosphorylated and becomes ubiquitinated, leading to degradation of IκBα and translocation of NF-κB to the nucleus. These transcription factors bind recognition sites within promoter sequences to induce transcription of cytokines, including IL-2 the major T lymphocyte proliferation factor.14 Thus, T cell stimulation leads to IL production and proliferation, thereby promoting the adaptive immune response.

This study attempts to find scientific understanding of the immunosuppressive effect of GSHYBE on the activated human T cell line MOLT-4 cells and peripheral blood mononuclear cells (PBMC). We investigated the mechanism of action of activated MOLT-4 cells by determining which signaling pathways were affected. We investigated whether GSHYBE inhibits T cell activation on the MAPK and nuclear factor level. Also, we investigated the effect of GSHYBE on the secretion of IL-2 in activated MOLT-4 cells and PBMC.

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MATERIALS AND METHODS

Preparation of GSHYBE  Each herb of GSHYBE was obtained from an oriental drug store, College Oriental Pharmacy (Iksan, Republic of Korea) and authenticated by Professor K. Moon, College of Oriental Medicine, Wonkwang University. A voucher specimen (number 01-37-46) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University. An extract of GSHYBE was prepared by decocting the dried prescription of herbs with boiling distilled water (57.2 g/l). The duration of decoction was about 3 h. The decoction was filtered, lyophilized and kept at 4 °C. The yield of extraction was about 14% (w/w). The GSHYBE water extract powder was dissolved in sterile saline (50 mg/ml). The ingredients of 57.2 g GSHYBE include 6 g of Asparagus tuber (Lilieaeae), 4 g of Rehmanniae radix (Scrophulariaeae), Ophiopogonis tuber (Lilieaeae), Angelicae gigantis radix (Umbelliferae), Astragali radix (Legumineae), 2 g of Scutellarias radix (Labiateae), Trichosanthis semen (Cucturiaeae), Persicae semen (Rosaceae), 0.8 g of Cimicifugae rhizoma, 0.4 g of Carthami flos (Compositae), Puerariae radix (Legumineae), 8 g of Cortex betulae platyphyllae (Betulaceae), 4 g of Ponciri fructus (Rutaceae), Fructus ponciri seu auranti (Rutaceae).

Reagents  Cell culture medium, RPMI 1640 was purchased from Gibco BRL (Grand Island, NY, U.S.A.). Phytohemagglutinin (PHA) was purchased from Calbiochem (La Jolla, CA, U.S.A.). Avidin-peroxidase, 2,2’-azio-bis(3-ethylbenzthiazoline-6-sulfonic acid), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Ficoll-Hypaque and other reagents were obtained from sigma (St. Louis, MO, U.S.A.). Anti-human (or mouse) IL-2, IL-4 and Interferon (IFN)-γ antibody (Ab), biotinylated anti-human (or mouse) IL-2, IL-4 and IFN-γ Ab, and recombinant human (or mouse) IL-2, IL-4 and IFN-γ were purchased from R&D Systems (Minneapolis, MN, U.S.A.). NFATc1 and NF-κB (p65) antibodies were purchased from Santa Cruz biotechnology (Santa Cruz, CA, U.S.A.). JNK, ERK, p38 and phosphorylated antibodies were purchased from Santa Cruz Biotechnology (Sanactrac, CA, U.S.A.).

Culture of MOLT-4 Cells  All experiments were performed on T cell line MOLT-4. MOLT-4 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in the presence of 5% CO2.

Western Blot Analysis  Cell extracts were prepared by detergent lysis procedure. Cells (5×10⁶ cells) were scraped, washed once with PBS, resuspended in lysis buffer. Samples were vortexed for lysis for a few seconds every 15 min at 4°C for 1 h and centrifuged at 15000×g for 5 min 4°C. Samples were heated at 95°C for 5 min, and briefly cooled on ice. Following the centrifugation at 15000×g for 5 min, 50 μl aliquots were resolved by 12% SDS-PAGE. Resolved proteins were transferred overnight to nitrocellulose membrane in 25 mM Tris, pH 8.5, 0.2 mm glycine, 20% methanol at 25 V. Blots were blocked for at least 2 h with 1×TBST containing 10% nonfat dry milk. Protein levels were analyzed essentially according to the manufacturer’s instructions.

Nuclear Protein Extraction  Preparation of crude nuclear extract was basically as described previously. Briefly, after cell activation for the times indicated, 1×10⁶ cells were washed in 1 ml of ice-cold PBS, centrifuged at 1000×g for 5 min, resuspended in 400 μl of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM MgCl2, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15000×g for 30 s. Pelleted nuclei were gently resuspended in 50 μl of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, and centrifuged at 15000×g for 5 min 4°C. Aliquots of the supernatant that contain nuclear proteins were frozen in liquid nitrogen and stored at −70°C. Protein was determined using a Bicinchoninic acid protein assay method (Sigma, St. Louis, MO, U.S.A.).

Transcription Factor Enzyme-Linked Immunoassay (TF-EIA)  Avidin peroxidase coated at 96 well ELISA plate. Coated plate was washed with PBST and then blocked with 3% skim milk solution. Coated plate was incubated with 1 μg/ml of 5’-biotinylated 21 single strand DNA oligonucleotide sequence for 1 h at room temperature. This sequence contains the previously described NF-κB binding motif. The sequences used here were: 5’-AGTTGAGGGAGTTT-CGCCAGG-3’. DNA binding reaction was carried out in a total volume 100 μl containing 10 μg nuclear protein extract in a buffer containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 5% glycerol, 1 mM EDTA, and 1 μM DTT, 5% BSA for 1 h. After addition of the corresponding alkaline phosphatase (AP)-coupled secondary antibody. Between each addition, wells were extensively washed in PBST. AP activity was then detected by the addition of p-nitrophenyl phosphate (PNPP) solution (Sigma). After a 10 min incubation period, the reaction was arrested by the addition of 0.5 M H2SO4. Color intensity was detected at 405 nm using ELISA reader. AP activity was normalized to control values (unstimulated cells).

PBMC Isolation and Culture  PBMC (5 healthy adults volunteers) from heparinized venous blood were isolated by Ficoll-gradient centrifugation, washed three times in phosphate-buffered saline (PBS) solution and resuspended in RPMI 1640 medium (GIBCO) supplemented with 2 mM L-glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS inactivated for 30 min at 56°C. PBMC were cultured for 24 h in 95% humidified air containing 5% CO2 (37°C), in the presence or the absence of PHA, and the supernatants were collected by centrifugation and stored at −20°C.

Passive Cutaneous Anaphylaxis (PCA)  An IgE-dependent cutaneous reaction was generated by sensitizing the skin with an i.d. injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the mice’s tail vein. The DNP-HSA was diluted in PBS. The mice were injected intradermally with 100 μg of anti-DNP IgE (a mean value) into each of 2 dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours as later each rat received an injection of 1 mg of DNP-HSA in PBS containing 4% Evans blue (1:4) via the tail vein. GSHYBE was orally administered 1 h be-
fore the challenge. Thirty minutes after the challenge, the mice were killed and the dorsal skin was removed. The dorsal skin homogenized in homogenization buffer (20 mM HEPES pH 7.5, 1.5 mM MgCl₂, 0.2 mM EDTA pH 8.0, 0.2 mM DTT, 0.5 mM Na₃VO₄, protease inhibitor cocktail). For cytokine assay, the supernatants were collected by centrifugation and stored at −20°C.

**Cytokine Assay** IL-2, IL-4 and IFN-γ secretion was measured by modification of an enzyme-linked immunosorbent assay (ELISA) as described previously.¹⁶ MOLT-4 cells and PBMC were cultured with RPMI 1640 plus 10% FBS and resuspended in Tyrode buffer A. The cells were sensitized with PHA (25 μg/ml) for 24 h in the absence or presence of GSHYBE. The ELISA was sensitive IL-2, IL-4 and IFN-γ concentrations in the medium above 0.01 ng/ml. The ELISA performed by coating 96-well plates (Nunc, Denmark) with 6.25 ng/well of murine monoclonal Ab with specificity for IL-2, IL-4 and IFN-γ. Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 and twice with PBS alone. All reagents used in this assay and the coated wells were incubated for 1 h at room temperature. For the standard curve, IL-2, IL-4 and IFN-γ was added to serum previously determined to be negative for endogenous IL-2, IL-4 and IFN-γ. After exposure to the medium, the assay plates were exposed sequentially to biotinylated anti-human IL-2, IL-4 and IFN-γ. 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) tablets substrates. Optical density readings were made within 10 min of the addition of the substrate on a Titertek Multiscan (Flow Laboratories) with a 405 nm filter.

**MTT Assay** Cell viability was determined by the MTT assay. Briefly, 500 μl of MOLT-4 cells suspension (3×10⁵ cells) was cultured in 4-well plates for 24 h after treatment by each concentration of GSHYBE. Twenty microliters of MTT solution (5 mg/ml) was added and the cells were incubated at 37°C for an additional 4 h. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, optical density of 96-well culture plates was measured using an ELISA reader at 540 nm. The optical density of formazan formed in untreated control cells was taken as 100% of viability.

**HPLC Analysis** The chromatographic system consisted of a pump (Water Assoc., U.S.A.: 600E HPLC pump), a UV detector (Linear: UVIS 200 detector), an autosampler (Water Assoc., U.S.A.: 717 plus autosampler), and a date modular (Water Assoc., U.S.A.: 746 computing integrator). A µBondapak™ C18 Waters column (3.9 mm×300 mm) was used. Water–acetonitrile–acetic acid (70:30:1) was used as the mobile phase. Detection of the peaks was made at 254 nm and the sensitivity was set of 1.0 AUFS. The injection volume was 20 μl and flow rate was 1.0 ml/min. Standard solution was prepared by dissolving in distilled water (100 μg/ml). The solution was filtered through 0.45 μm membrane filter and applied to HPLC. Acetonitrile–HPLC grade was purchased from Merck (Germany). Other chemicals were used all GR grade.

**Statistical Analysis** Each datum represents the mean±SEM of the different experiments under the same conditions. The Student’s t-test was used to make a statistical comparison between the groups. Results with p<0.05 were considered statistically significant.

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**RESULTS**

**GSHYBE Selectively Blocks ERK, p38 Activation But Not JNK** To study the impact of GSHYBE treatment on downstream T cell signaling, we first analyzed effects on the level of MAPK activation. Activation of MAPK relies on phosphorylation of specific tyrosine and threonine residues.¹⁷ Investigation of the activation of JNK1/2, ERK1/2, and p38 pathways, as determined by assaying their phosphorylation, showed that both ERK1/2 and p38 activation inhibited by GSHYBE, when stimulated for 10 min with PHA (Figs. 1B, C). However, phosphorylation of JNK1/2 induced PHA, was not altered by GSHYBE treatment (Fig. 1A). Hence, GSHYBE treatment of MOLT-4 cells inhibits ERK1/2 and p38 activation but does not interfere with phosphorylation of other MAPKs, JNK1/2.

**GSHYBE Inhibits the Nuclear Localization of NFATc1 and NF-κB/Rel A** Production of the central T cell growth factor IL-2 critically depends on activation of the transcription factors NFAT, NF-κB.¹⁴ The effect of GSHYBE on nuclear translocation of NFATc1 and NF-κB/Rel A in activated MOLT-4 cells was examined by western blot analysis. In PHA-stimulated cells, the expression level of NFATc1 increased in the nucleus. However, the expression level of NFATc1 in nucleus decreased by treatment of GSHYBE (Fig. 2A). As a marker of NF-κB activation, we detected the degradation of 1κB-α in cell lysates. Activation and nuclear translocation of NF-κB is dependent on the phosphorylation of 1κB-α, which is then rapidly degraded. In PHA-stimulated cells, the expression level of 1κB-α decreased in the cytoplasm and the level of NF-κB/Rel A increased in the nucleus. But, the expression level of NF-κB/Rel A decreased by treatment of GSHYBE (Fig. 2B). We also investigated the effect of GSHYBE on PHA-induced NF-κB transcription complex. To perform these studies, we used an NF-κB TF-EIA method. This assay has the advantage of being 10 times more sensitive than electrophoretic mobility shift assay and allows greater flexibility in the experimental step. As shown in Fig. 2D, PHA increased DNA-binding activity for NF-κB. But in

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**Fig. 1. Effect of GSHYBE on MAPK Activation**

MOLT-4 cells (5×10⁵) were treated with GSHYBE (1 mg/ml) for 30 min and then stimulated with PHA (25 μg/ml) for 10 min. A, Phospho-JNK1/2 (A). Phospho-ERK1/2 (B). Phospho-p38 (C), were determined by Western blot analysis whole cell lysates with phospho-specific Abs. ERK, p38, JNK respectively were detected from whole cell lysates.
increased binding activity was decreased by treatment of GSHYBE.

**GSHYBE Inhibits IL-2 Expression** Synthesis of IL-2 is an early characteristic in the activation of T cell. To investigate the effect of GSHYBE on protein expression, we stimulated PHA for 24 h. IL-2 expression levels in intracellular of MOLT-4 cells activated by PHA, were determined by Western blot analysis. PHA-stimulated MOLT-4 cells led to an increase in IL-2 level. But pretreatment of cells with GSHYBE (1 mg/ml) decreased IL-2 protein level (Fig. 3).

**GSHYBE Inhibits Production of IL-2, IL-4 and IFN-γ** To investigate the effect of GSHYBE on various cytokine secretion, MOLT-4 cells and PBMC were stimulated for 24 h in the presence of GSHYBE with PHA. The quantification of IL-2, IL-4 and IFN-γ in the supernatants was performed using an ELISA. GSHYBE (1 mg/ml) significantly inhibited the PHA induced IL-2, IL-4 and IFN-γ secretion from MOLT-4 cells and PBMC (Figs. 4, 5). IL-2 is a necessary cytokine for T cell proliferation in response to mitogen or alloantigen stimulation. GSHYBE inhibited PHA-induced proliferation from MOLT-4 cells and PBMC (Fig. 6). But, in the case of MOLT-4 cells, the statistical difference was weak (Fig. 6A).

**GSHYBE Inhibits Secretion of IL-2 on the Skin Allergic Reaction in Vivo** To investigate the effect of GSHYBE on IL-2 secretion in in vivo model, we used passive cutaneous anaphylaxis (PCA). PCA is one of the most important in vivo models of local skin allergic reaction. As described in the material and method, local injection of anti-DNP IgE followed by an intravenous antigenic challenge has been performed. Anti-DNP IgE was injected into dorsal skin sites. After 48 h, all animals were injected i.v. with DNP-HSA containing Evans blue dye. The quantification of IL-2 in the supernatants of homogenized the dorsal skin was performed using an ELISA. Oral administration of GSHYBE (1 g/kg)
significantly inhibited IL-2 secretion by 52.23%

DISCUSSION

In this study, we show that GSHYBE inhibit T cell activation by affecting distinct events of T cell signal transduction. GSHYBE block the nuclear localization of NFATc1 (Fig. 2A). Also, we showed that GSHYBE inhibited IκB degradation and NF-κB-dependent expression in human T cell line MOLT-4 cells. GSHYBE inhibited PHA-induced proliferation from MOLT-4 cells and PBMC (Fig. 6). GSHYBE (1 mg/ml) significantly inhibited IL-2 secretion from MOLT-4 cells and PBMC. In addition to GSHYBE inhibits secretion of IL-2 on the skin allergic reaction in vivo. Thus, inhibition of T cell proliferation by GSHYBE can be attributed both a reduction in IL-2 production and a decreased expression.

Engagement of the TcR rapidly induces multiple signal transduction pathways. These events cause the recruitment and assembly of signal complexes that trigger different signal transduction. The pathways involved included the protein kinase C, MAPK, calcineurin. All these pathways, together with those triggered by the receptor-independent stimulants, converge on activation of promitotic transcription factors such as NFAT and NF-κB, which leads to proliferation and IL-2 synthesis.20) Despite these divergent signaling pathways, GSHYBE treatment efficiently blocked IL-2 synthesis. This phenomenon suggests that GSHYBE interacts with events far upstream in the TcR signaling cascade.

In mammals, MAPK signaling cascades regulate important cellular process including gene expression, cell proliferation, cell survival and death.20) Also, MAPK signaling cascades are activated upon stimulation of T cells and are involved in the regulation of numerous transcription factors including those required for IL-2 mRNA transcription.21,22) The induction of most cytokine genes requires activation of the ERK1/2 and p38 MAPK.23) The activity of ERK1/2 and p38 has been increased in lesional psoriatic skin compared with nonlesional psoriatic skin.24) Thus p38 and ERK1/2 might be potential targets in the treatment of skin disease. Based on such clues as to the signal transduction cascade affected by activation of T cell, we investigate the intracellular signaling pathways. Our results showed that ERK1/2, p38 activation were reduced by GSHYBE whereas JNK was not affected (Fig. 1).

Two of the earliest T cell signaling events affected was the nuclear translocation of NFAT and NF-κB. It has been proposed that nuclear accumulation of NFAT may be control by a balance between Ca\(^{2+}\)/calcineurin and JNK/ERK/p38 signals.25) A common signal may be derived from the ERK MAP kinase pathway to regulate general NFAT activity. We demonstrated that nuclear translocation of NFATc1 and NF-κB/Rel A is markedly reduction in activated T cells by
GSHYBE (Figs. 2A, B). The suppression of nuclear NF-κB/Rel A could be due to either to a failure to translocate to the nucleus even after IκB-α degradation or to degradation in the nucleus. In a study of suppression of T cell function by renal cell carcinoma, failure to activate NF-κB was caused in some cases by inhibition of phosphorylation and degradation of IκB and in others by a lack of NF-κB nuclear accumulation.26,27 Our result showed that degradation of IκB has been thought to be sufficient to cause the nuclear translocation of NF-κB (Fig. 2). Indeed, roles for NF-κB have been demonstrated in experimental models of liver, kidney, and cardiac transplantation.28—30 Thus, NF-κB is a potent proinflammatory signal transduction molecule in T cells.

Many studies are performed to clarify the inhibitory mechanism of T cell activity. Azodicarbonamide is known as a new T cell immunosuppressant that acts in the early phase of T cell activation by inhibiting intracellular mobilization of calcium.31 Pyrazole compounds are a potent inhibitor of NFAT activation and T cell cytokine production.32 We tried to chase the main acting compound from GSHYBE. Hence we fractionated GSHYBE and HPLC assay (data not shown). We identified 15 separate compounds however we could not define each compound at this time. More detailed study on defining actual compound is needed.

In summary, we showed that GSHYBE attenuated PHA induced ERK and p38 MAPK, degradation of IκB-α and NF-κB-dependent activation. GSHYBE and CsA produced very similar inhibition effects on NFATc1, NF-κB activation and T cell cytokine production.32 We tried to chase the main acting compound from GSHYBE. Hence we fractionated GSHYBE and HPLC assay (data not shown). We identified 15 separate compounds however we could not define each compound at this time. More detailed study on defining actual compound is needed.

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