Effect of the Geijibokryunghwan Water Extracts on Stimulus-induced Superoxide Generation and Tyrosyl Phosphorylation in Human Neutrophils

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A clinical report indicates that 'Geijibokryunghwan(GBH) is very effective in treating thrombosis in those patients who have difficulties with more conventional antithrombotic drugs. The isolation and identification of various compounds from this plant and the same genus have been reported by several groups. However, the pharmaceutical effect of the GBH on superoxide generation in human neutrophils has not been studied. In the present report, we investigated the possibility of using herbal medicine as an alternative therapy. In particular, we studied tremor in antiatheroscleosis. In this report, we shows the GBH extract can be used as a potential atherosclerosis preventive agent in human. The effect of GBH on stimulus-induced superoxide generation and phosphorylation of tyrosine residues of protein in human neutrophils was investigated. In a conclusion, GBH suppressed tyrosine phosphorylase in a dose-dependent manner, and may have pharmacoceutical applications. These data suggest that GBH extracts merits investigation as a potential anti-atherosclerogenic agent in humans.

Key words : Geijibokryunghwan(GBH), thrombosis, superoxide generation, antiatheroscleosis, neutrophils, tyrosyl phosphorylation

Introduction

The Korean herbal medicine 'Geijibokryunghwan (GBH, 桂枝茯苓丸)', which is a mixture of four crude herbal drugs, has sedative, hypnotic and antithrombosis actions. A clinical report indicates that GBH is very effective in treating thrombosis in those patients who have difficulties with more conventional antithrombotic drugs[1]. As a similar agent, the Japanese herbal medicine 'Saiko-Keishi-To', which is also a mixture of four crude herbal drugs, was also known to have sedative, hypnotic and anticonvulsant actions in central nervous system (CNS)[2], and to have effectiveness for tremor of antipsychotic-induced parkinsonism[3].

GBH has been used as a Korean folk medicine in invigorating blood circulation, normalizing menstruation and eliminating blood stasis to relieve pain[4]. The isolation and identification of various compounds from this plant and the same genus have been reported by several groups. However, the pharmaceutical effect of the GBH on superoxide generation in human neutrophils has not been studied. In the present report, we investigated the possibility of using herbal medicine as an alternative therapy. In particular, we studied tremor in antiatheroscleosis

Polymorphonuclear leukocytes (PMN) play critical roles in the defense mechanism against microorgnisms[5]; when PMN are exposed to various stimuli, one-electron reduction of molecular oxygen by NADPH oxidase leading to a "respiratory burst" is induced[6,7]. N-Formyl-methionyl-leucyl-phenylalanine (fMLP), opsoniged zymosan (OZ), arachidonic acid (AA) and phorbol 12-myristate 13-acetate (PMA) are known stimulants[8]. Several reports have shown that various cytokines and hypotonic condition enhanced the tyrosyl phosphorylation of specific proteins in neutrophils in the primed stage, suggesting the contribution of tyrosine kinase (TK) to the regulatory mechanism of priming in neutrophils[9-12].

In a previous paper, GBH was extracted with water and it was confirmed that the water GBH showed anti-thrombosis activity[13]. Also, the effect of GBH on carrageenan-induced edema inflammation in mice female (C57BL/6XC3H) F1 (B6C3F1) mice and production of cyclooxygenase metabolites was studied.

In this report, author shows the GBH extract can be used as a potential atherosclerosis preventive agent in human. The effect of GBH on superoxide generation in human neutrophils

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was investigated. It is reported that GBH significantly suppressed the superoxide generation induced by various stimuli (fMLP, PMA and AA) in human neutrophils. In the present study, it was found that GBH suppress the tyrosyl phosphorylation of 45-kDa protein in parallel to the compound-dependent suppression of superoxide generation in human neutrophils.

Materials and Methods

1. Materials

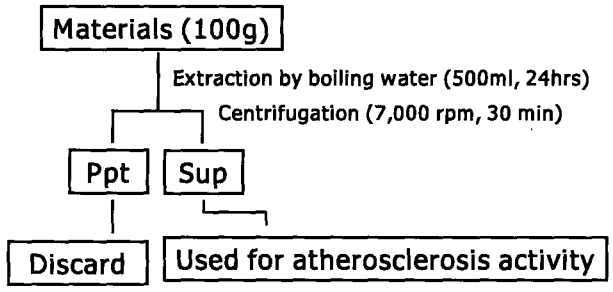
NADPH, ferricytochrome c (cyt. c), superoxide dismutase, fMLP, AA and PMA were obtained from Sigma (St. Louis, MO). All other reagents used were of analytical grade and were purchased from Nacalai Tesque (Osaka, Japan) unless otherwise noted. GBH was obtained from Dongguk oriental hospital, Kyungju, Korea(Scheme 1).

Scheme 1. Composition of GBH(桂枝茯苓丸)[14-18]

Cinnamomi Ramulus (桂枝)	1.33 g
Hoelen (茯苓)	1.33 g
Moutan Cortex Radicis (牧丹皮)	1.33 g
Paeoniae Radix (芍藥)	1.33 g
Persicae Semen (桃仁)	1.33 g
總　量	6.65 g

2. Extraction

The dried samples were homogenized using a mechanical disintegrator with Tekmar Tissue homogenizer (Tekmar Co., Cincinati, OH, USA) in distilled water, and the crude fraction was collected by centrifugation (15,000 ×g, for 20 min) at 4℃. The supernatant solution was concentrated to about 120 ml, and used for the experiments (Scheme 2).



Scheme 2. Preparation of the water-extracts

3. Isolation of neutrophil

Human peripheral blood polymorphonuclear leukocytes were isolated from the peripheral blood of healthy humans by Ficoll-Hypaque (Flow Laboratories) density gradient centrifugation[19] and were washed twice with Krebs-Binger phosphate solution, pH7.4 (KRP)[20]. The cells were counted and resuspended in KRP at a concentration of 1×10^8 cells/ml.

4. Assay of superoxide generation

Superoxide generation was assayed by measuring the reduction of cyt. c at 37℃ using a dual-beam spectrophotometer (Shimadzu UV-3000; Shimadzu, Kyoto, Japan) under constant stirring conditions[21]. The standard assay mixture consisted of 1×10^6 cells/ml, 1 mmol/ℓ $CaCl_2$, 20 mol/ℓ cyt. c, 10 mmol/ℓ glucose, 0.5 mol/ℓ flavonoids and a stimulus (12.5 nmol/ℓ fMLP, 1 nmol/ℓ PMA or 10 mol/ℓ AA) in a final volume of 2 ml of KRP. After preincubation for 3 min with a flavonoid compound, the reaction was started by adding the stimulus and the absorbance changed at 550-540 nm (A550-540) was monitored for 4 min.

5. Detection of tyrosyl phosphorylation of neutrophil proteins

Neutrophils (1×10^6 cells/ml) were incubated in 1 ml of KRP containing 1 mmol/ℓ $CaCl_2$, 10 mmol/ℓ glucose, 12.5 nmol/ℓ fMLP and 0-50 μg/ml of GBH for 3 min at 37℃, and then 0.5 ml of ice-cold 45% trichloroacetic acid (final concentration, 15%) containing 1 mmol/ℓ sodium vanadate and phenylmethylsulfonylfluoride (2 mmol/ℓ) was added to stop the reaction. After incubation for 30 min at 4℃, the mixture was centrifuged at 10,000×g for 20 min at 4℃. The precipitate was washed four times with diethyl ether-ethanol (1:1, v/v), dissolved in 50 μℓ of 62.5 mmol/ℓ Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 0.7 mol/ℓ -mercaptoethanol and 10% glycerol, then was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% gel[22].

6. Immuno-blot Western analysis

The electrophoresed proteins were transferred onto Immobilon-P membranes (Nippon Milipore) using a semidry blotting apparatus (Sartorius) for 90 min at 2 mA/cm^2, and the tyrosyl phosphorylated proteins were detected using phosphotyrosine-specific monoclonal antibody (PY-20; ICN Biochemicals), peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibody (E.Y. Laboratories) and the ECL Western Blotting Detection System (Amersham, Japan)[6]. The apparent molecular masses of the proteins were determined using prestained molecular weight standards (14,300-200,000 molecular weight range; Gibco BRL).

7. Statistical analysis

The statistical significance of difference among groups was evaluated by Student's t-test or Duncan's new multiple range test; $p<0.05$ was considered significant.

Results

1. Effect of GBH on fMLP-, PMA-, and AA-induced superoxide generation in human neutrophils

When neutrophils were preincubated with the GBH, the fMLP-induced superoxide generation was significantly suppressed by the GBH in a concentration-dependent manner (Fig. 1A). In the absence of the stimulus, GBH did not induce superoxide generation (data not shown). The PMA-induced superoxide generation was also suppressed by GBH in a concentration-dependent manner (Fig. 1B). The extent of suppression of the PMA-induced superoxide generation by the GBH: 45% inhibition at 40 μg/ml. The AA-induced superoxide generation was also suppressed by GBH (Fig. 1C). The extent of suppression of the AA-induced superoxide generation by the GBH was 62% inhibition at 40 ug/ml.

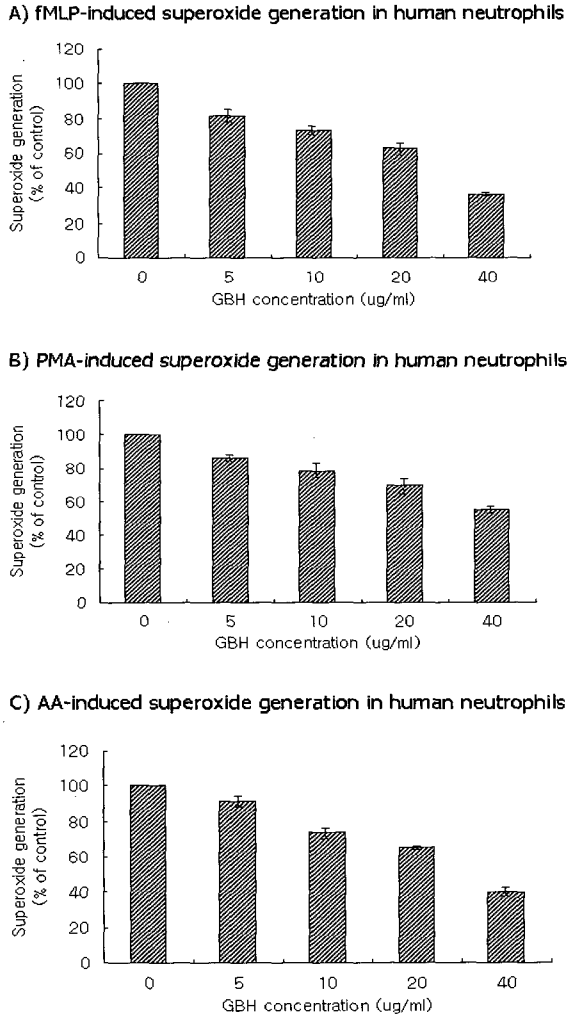


Fig. 1. Effect of GBH on (A) fMLP-, (B) PMA- and (C) AA-induced superoxide generation in human neutrophils. The cells were preincubated with 0-40 μg/ml of GBH for 3 min prior to the addition of 12.5 nmol/ℓ fMLP, 1 nmol/ℓ PMA or 10 nmol/ℓ AA. The experimental conditions were as described under Materials and methods. Results are expressed as mean±SD from three independent experiments.

2. Effect of GBH on tyrosyl phosphorylation of human neutrophil proteins

To clarify the mechanism of suppressing effect by GBH, we examined the effect of GBH on tyrosyl phosphorylation of proteins in fMLP-treated cells. When neutrophils were incubated with fMLP, tyrosyl phosphorylation of 45 kDa proteins was induced. However, in the presence of GBH, the tyrosyl phosphorylation was dose-dependently suppressed (Fig. 2). These results coincided well with the change of superoxide generation level. Suppression of superoxide generation via suppressing the tyrosyl phosphorylation of neutrophil proteins may be one of the pharmaceutical effects of this GBH.

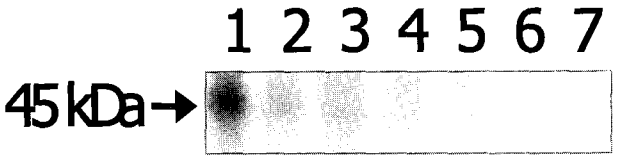


Fig. 2. Effect of GBH on fMLP-induced tyrosyl phosphorylation of human neutrophil proteins. The tyrosyl phosphorylated proteins were detected by immunoblotting using phosphotyrosine-specific monoclonal antibody. Lane 1, without GBH; Lanes 2-6, 12.5 nmol/ℓ fMLP and 0, 5, 10, 20 and 40 μg/ml GBH, respectively; Lane 7, without fMLP.

Discussion

There are obvious limitations of this study, in explaining the in vivo antithrombotic mechanism of GBH. The concentrations of the drug effective in these experiments were substantially lower than those used in our study. Thus, the in vivo antithrombotic mechanism of GBH could not be explained solely by its effects on platelets. Indeed, not only our present findings but also previous reports indicate that the antiplatelet effects of GBH in ex vivo or in vitro experiments with PRP appeared only at concentrations much higher than those effective in vivo, regardless of whether platelets were activated by chemical agonists.

Accordingly, the mechanism mediated by cells other than platelets should also be considered in explaining the enhanced in vivo antithrombotic effects of GBH. In this regard, it was suggested that the effects of GBH on endothelial cells might contribute to the enhanced antithrombotic effects by increasing the endothelial release of antithrombotic materials such as NO.

The effect of the GBH on superoxide generation in human neutrophils was investigated using fMLP, PMA and AA as the stimuli. The extent of suppression of the PMA-induced superoxide generation by the GBH was 45% inhibition at 40 μg/ml. The GBH suppressed the superoxide generations induced by fMLP, PMA and AA, but the

suppressing effect of the GBH on three stimulus-induced superoxide generations were different with each other. Although the relationship between anti-oxidative substances and suppressing effect of the GBH is not clear at present, the GBH would be responsible to its suppressive effect on the stimulus-induced superoxide generation in human neutrophils.

It was reported that antioxidative substances such as prolyproline and cystathionine metabolites induced tyrosyl phosphorylation of proteins in parallel to the enhancement of fMLP-induced superoxide generation in human neutrophils[10,11]. The tyrosyl phosphorylation was inhibited by genistein and herbimycin A, inhibitors of tyrosine protein kinase, suggesting the participation of protein tyrosine kinase in the mechanism for priming of human neutrophils by these compounds[11].

The observation that only platelet aggregation occurring under high shear rates, i.e. not that at a low shear rate, was inhibited by the same concentration of GBH, also suggested that the selective antiplatelet effects under high shear rate conditions were specific for GBH. We have demonstrated the unique antiplatelet characteristics of GBH, a selective inhibitor of platelet activation, aggregation, and thrombus formation occurring under high shear rate conditions. Results GBH on tyrosyl phosphorylation of proteins in fMLP-treated cells, tyrosyl phosphorylation of 45 kDa proteins was induced. However, GBH inhibited the tyrosyl phosphorylation by a dose-dependen manner. These results coincided well with the change of superoxide generation level. Suppression of superoxide generation via suppressing the tyrosyl phosphorylation of neutrophil proteins may be one of the pharmaceutical effects of this GBH. These results suggest that the GBH is useful as anti-inflammation, anti-atherosclerosis and anticancer reagents. Further studies on the pharmaceutical functions and immunological responses of the GBH may help in the development of clinical application.

Conclusion

The effect of an oriental medicinal prescriptions, Geijibokryunghwa (GBH) consisting of herbes of Cinnamomi Ramulus (Geiji), Poria Cocos (Bokryun), Mountan Cortex Radicis (Mokdanpi), Paeoniae Radix (Jakyak), and Persicae Semen (Doin) on stimulus-induced superoxide generation and phosphorylation of tyrosine residues of protein in human neutrophils was investigated. When the cells were preincubated with the GBH, the superoxide generation induced by N-formyl-methionyl-leucyl- phenylalanine (fMLP) was significantly suppressed in a concentration-dependent manner. The GBH also suppressed the superoxide generation induced by arachidonic acid (AA). In case of the superoxide generation induced by phorbol 12-myristate 13-acetate (PMA), GBH suppressed the superoxide generation. When the cells were incubated with fMLP in the presence of GBH, fMLP-induced tyrosyl phosphorylation of 45-kDa proteins of the cells was dose-dependently suppressed in parallel to the suppression of fMLP-induced superoxide generation. In a conclusion, GBH suppressed tyrosine phosphorylase in a dose-dependent manner, and may have pharmacoceutical applications. These data suggest that GBH extracts merits investigation as a potential anti-atherosclerogenic agent in humans.

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