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Inhibitory Effects of Ginsenosides from the Root of *Panax ginseng* on Stimulus-Induced Superoxide Generation, Tyrosyl or Serine/Threonine Phosphorylation, and Translocation of Cytosolic Compounds to Plasma Membrane in Human Neutrophils

WENFEI HE,[†,§] GANG LIU,[†,#] XIN CHEN,[†,⊥] JINCAI LU,[⊥] HIDEHIRO ABE,[†] KEXIN HUANG,[§] MASANOBU MANABE,[†] AND HIROYUKI KODAMA*[,†]

Department of Anesthesiology and Critical Care Medicine, Kochi Medical School, Nankoku-city, Kochi 783-8505, Japan; Department of Natural Products Chemistry, School of Pharmacy, Wenzhou Medical School, Wenzhou 325035, China; Department of Anesthesiology, First Affiliated Hospital of China Medical University, Nanjing North Road 55, Shengyang 110001, China; and Department of Medical Plant Development, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110015, China

The effects of five ginsenosides (G-Rh_2, -Rd, -Rb_1, -Rb_2, -Rh_1) isolated from the root of *Panax gingseng* on stimulus-induced superoxide generation in human neutrophils were evaluated by measuring the reduction of ferricytochrome *c*. The tyrosyl or serine/threonine phosphorylation of neutrophil proteins and translocation of $p47^{phox}$, $p67^{phox}$, and Rac to the plasma membrane were detected using specific monoclonal antibodies. G-Rh_2 significantly suppressed superoxide generation induced by *N*-formylmethionyl-leucylphenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA), and arachidonic acid (AA) in a concentration-dependent manner. G-Rh_1 showed a comparably lower suppression on fMLP-induced superoxide generation. G-Rd, -Rb_1, and -Rb_2 also suppressed AA-induced superoxide generation in high concentrations. G-Rd and G-Rb_1 showed no effect on fMLP- and PMA-induced superoxide generation. FMLP-, PMA-, and AA-induced tyrosyl or serine/threonine phosphorylation and translocation of $p47^{phox}$, $p67^{phox}$, and Rac to the plasma membrane were in parallel with the suppression of the stimulus-induced superoxide generation.

KEYWORDS: ***Panax ginseng*; ginsenoside; superoxide; phosphorylation; $p47^{phox}$, $p67^{phox}$, and Rac**

INTRODUCTION

Ginseng (*Panax gingseng* C.A. Meyer, Araliaceae) root is one of the most widely used Chinese traditional medicines for the treatment of various diseases. It has long been used as a tonic and immunomodulator (*1*). Ginsenosides (ginseng saponins) are regarded as the main active ingredients and reported to possess a wide range of pharmacological actions. Among them, certain ginsenosides have been shown to exert cytotoxic or cytostatic activities against cultured tumor cells. The growth inhibitory or antiproliferative effects of certain ginsenosides appear to be associated with their ability to induce apoptosis (*2–6*). Moreover, they have also shown antiaging and estrogenic effects, improved insulin sensitivity, and antiallergic and anti-inflammatory activities (*7–10*). However, the effects of ginsenosides isolated from the root of *P. gingseng* on stimulus-induced superoxide generation in human neutrophils have not been previously investigated.

Superoxide generation in human neutrophils is stimulated not only during phagocytosis but also by treatment of the cells with various stimuli, such as certain chemoattractants and activators of protein kinase (*11*). The production of a superoxide anion (O_2^-) by the NADPH oxidase in the phagolysosome is the precursor for the generation of other reactive products, such as hydroxyl radical and hypochlorite. However, this production of O_2^- is dependent on translocation of the oxidase subunits, $p47^{phox}$, $p67^{phox}$, and Rac, from the cytosol to the plasma membrane. In response to appropriate external stimuli, polymorphonuclear leukocytes (PMNs) change from a resting, nonadhesive state to a primed, adherent phenotype, which allows for migration from the vasculature into the tissue and chemotaxis to the site of infection upon activation. Depending on the stimuli,

* Correspondening author (fax +81-88-865-5937; e-mail jm-hkodama@kochi-u.ac.jp).
[†] Kochi Medical School.
[§] Wenzhou Medical School.
[#] First Affiliated Hospital of China Medical University.
[⊥] Shenyang Pharmaceutical University.

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primed PMNs display altered structural organization of the NADPH oxidase, in that there is phosphorylation of the oxidase subunits and/or translocation of the cytosol compounds to the plasma membrane. Activation of PMNs is the complete assembly of the membrane-linked and cytosolic NADPH oxidase components on the plasma membrane. During activation, cytosolic proteins $p47^{phox}$, $p67^{phox}$, Rac, and possibly $p40^{phox}$ translocate to the plasma membrane and associate with membrane-associated flavocytochrome b_{558} to form the active superoxide-generating system (*12–16*).

We have previously reported that some triterpenoid compounds isolated from leaves of *Diaspyros kaki* suppressed the tyrosyl phosphorylation of a 45 kDa protein in parallel to the concentration-dependent suppression of superoxide generation in human neutrophils (*17*). Recently, we have reported that triterpenoid compounds isolated from root bark of *Aralia elata* suppressed tyrosyl or serine/threonine phosphorylation of proteins and translocation to the plasma membrane of $p47^{phox}$, $p67^{phox}$, and Rac in parallel to the effect of stimulus-induced superoxide genetation (*18–20*). In the present study, the effects of five ginsenosides isolated from the root of *P. ginseng* on the superoxide generation induced by various stimulus [*N*-formyl-methionyl-leucylphenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA), and arachidonic acid (AA)], tyrosyl or serine/threonine phosphorylation of proteins, and the translocation of cytosolic compounds, $p47^{phox}$, $p67^{phox}$, and Rac, to plasma membrane in human neutrophils and antioxidant activity were investigated.

MATERIALS AND METHODS

Materials. NADPH, ferricytochrome *c*, superoxide dismutase, fMLP, PMA, and AA were obtained from Sigma (St. Louis, MO). The five ginsenosides (ginsenoside Rh_2, Rd, Rb_1, Rb_2, Rh_1) were isolated from root of *P. ginseng* by silica gel column chromatography. All other reagents used were of analytical grade and purchased from Wako Chemical Industries (Osaka, Japan) unless otherwise mentioned.

Isolation of Neutrophils. Polymorphonuclear leukocytes (PMNs) were isolated from human peripheral blood of healthy volunteers by Ficoll-Hypaque (Flow Laboratories) density gradient centrifugation (*21*) and were washed twice with Krebs−Ringer phosphate solution (KRP; pH 7.4) (*22*). The cells were resuspended in KRP at a concentration of 1×10^8 cells/mL.

Assay of Superoxide Generation. The generation of superoxide was assayed by measuring the reduction of ferricytochrome *c* at 37 °C using a dual-beam spectrophotometer (Shimadzu UV-3000) under continuous stirring (*11*). The standard assay mixture consisted of 1×10^6 cells/mlL, 1 mM $CaCl_2$, 20 μM ferricytochrome *c*, 10 mM glucose, 0–200 μM ginsenosides, and stimulus (12.5 nM fMLP, 1 nM PMA, or 10 μM AA) in a final volume of 2 mL of KRP. The reaction was started by adding a stimulus after the preincubation of 3 min with each ginsenoside. Absorbance change at 550–540 nm ($\Delta A_{550-540}$) was monitored for 4 min, and the difference before and after incubation was calculated.

Detection of Tyrosyl or Serine/Threonine Phosphorylation of Neutrophil Proteins. Neutrophils (1×10^6 cells/mL) were incubated in 1 mL of KRP containing 1 mM $CaCl_2$, 10 mM glucose, and 0–200 μM ginsenoside for 3 min at 37 °C, and then they were stimulated by 12.5 nM fMLP, 1 nM PMA, or 10 μM AA and incubated for 3 min at 37 °C. Then 0.5 mL of ice-cold 45% trichloroacetic acid (final concentration of 15%) containing 1 mM sodium vanadate and phenylmethanesulfonyl fluoride (2 mM) was added to stop the reaction. After incubation for 30 min at 4 °C, the mixture was centrifuged at 10000*g* for 20 min at 4 °C. The precipitate was washed twice with diethyl ether/ethanol (1:1, v/v), dissolved in 50 μL of 62.5 mM Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 0.7 M 2-mercaptoethanol, and 10% glycerol, and then subjected to SDS−polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% gel.

The electrophoresed proteins were transferred onto Immobilon-P membrane (Nippon Millipore Ltd.) using a semidry blotting apparatus for 90 min at 20 V. Tyrosyl or serine/threonine phosphorylated proteins were probed with phosphotyrosine specific monoclonal antibody (PY-20; ICN Biochemicals) or phosphoserine/threonine specific monoclonal antibody (BD Biosciences), respectively, then probed with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibody (E. Y. Laboratories), and detected by ECL Western Blotting Detection System (Amersham). The molecular weights of the proteins were determined using prestained molecular weight standards (14300–200000 molecular weight range; Gibco) (*23*).

Translocation of $p47^{phox}$, $p67^{phox}$, and Rac to Neutrophil Membrane. The translocation of cytosolic compounds to the plasma membrane was performed as previously reported by Nauseef (*24*). Isolated PMNs were preincubated in a phosphate-buffered saline glucose solution containing 4 mM glucose, 1.2 mM $MgCl_2$, 2 mM NaN_3, and 0–200 μM ginsenoside for 6 min at 37 °C. Then, PMNs were stimulated by adding stimulus (12.5 nM fMLP, 1 nM PMA, or 10 μM AA) for 3 min at 37 °C, and the cells were spun at 1500*g* for 5 min at 4 °C and resuspended in buffer A [100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM Pipes (pH 7.3)] after standing on ice for 20 min. To separate their postnuclear supernatants (PNS), cells were first disrupted by sonication and spun at 500*g* for 5 min at 4 °C. PNS fractions were then separated into membrane and cytosol at 200000*g* for 20 min at 4 °C. The pellet was resuspended in 50 μL of 109 mM Tris-HCl (pH 7.5) containing 3.5% SDS, 0.0087% bromophenol blue, and 17.4% glycerol and sonicated for 1 h to obtain membrane fractions.

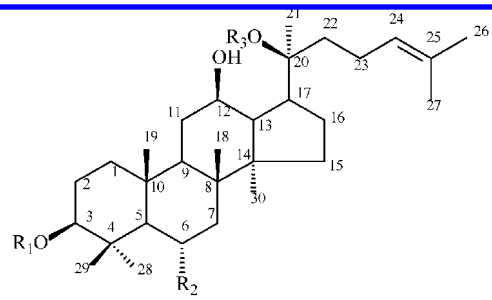
For immunoblot analysis, the membrane fraction was subjected to SDS-PAGE with 10% gel. The electrophoresed proteins were transferred onto Immobilon-P membrane (Nippon Millipore) using a semidry blotting apparatus for 90 min at 20 V. The transferred proteins were probed with a mixture of $p47^{phox}$, $p67^{phox}$, and Rac1 primary monoclonal antibody (BD Biosciences) and horseradish peroxidase-conjugated rabbit antimouse immunoglobulin G antibody (E.Y. Laboratories) detected by ECL Western Blotting Detection System (Amersham). EB-1 lysate, as the positive control, was the indicator for the location of $p47^{phox}$, $p67^{phox}$, and Rac1.

Determination of Lipid Peroxidation of Erythrocyte Membrane Ghosts by Hydroxyl Radicals. Fresh blood from a healthy human (9 parts blood/1 part 3.8% sodium citrate) was centrifuged (1000*g*, 5 min), and isolated erythrocytes were washed three times with 3 volumes of a solution composed of 150 mM NaCl and 5 mM sodium phosphate buffer (pH 8.0; PBS). Erythrocyte ghosts were obtained at 4 °C from lysed cell in 20 volumes of 5 mM sodium phosphate (pH 8.0) after centrifugation at 30000*g* for 20 min. Then, membranes were washed four times in the same buffer to obtain hemoglobin-free white ghosts, and they were finally resuspended in saline to obtain a final concentration of 1 mg of protein/mL.

Hydrogen peroxide (3 mM) and $FeSO_4$ (5 mM) were added to erythrocyte membrane ghost suspensions (1 mL) with each of the ginsenosides (0–200 μM) in five separate experiments. The suspensions were incubated for 30 min at 37 °C. Hydroxyl radical-induced lipid peroxidation of erythrocyte membrane ghosts was determined by measuring thiobarbituric acid-reactive substances (*19*).

Radical Scavenging Activity on DPPH. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was carried out according to the procedure previously described (*25*). The reaction mixture consisted of 1 mL of 100 mM acetate buffer (pH 5.5), 1.87 mL of ethanol, and 0.1 mL of 3 mM DPPH in ethanol. At 25 °C, 0.03 mL of the sample solution was added to the mixture, and the absorbance was recorded at 517 nm (DPPH, $\epsilon = 8.32 \times 10^3$) for 20 min. Water (0.03 mL) was used as control. Scavenging activity was expressed as a percent compared to control DPPH solution (100%).

Radical Scavenging Activity on Superoxide Anion Generated by Phenazine Methoxysulfate (PMS)−NADH System. Superoxide anions were generated nonenzymatically by PMS−NADH system (*26*). The reaction mixture consisted of 0.03 mL of 0.5% bovine serum albumin, 0.03 mL of 5 mM nitroblue tetrazolium (NBT), 0.03 mL of 7.8 mM NADH, 0.06 mL of sample solution (dissolved in water), and 2.82 mL of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0). After incubation at 25 °C for 3 min, 0.03 mL of 15.5 μM



ginsenoside	R_1	R_2	R_3
G-Rh2	Glc	H	H
G-Rd	Glc(2←1)Glc	H	Glc
G-Rb1	Glc(2←1)Glc	H	Glc(6←1)Glc
G-Rb2	Glc(2←1)Glc	H	Glc(6←1)Ara(p)
G-Rh1	H	O-Glc	H

Figure 1. Chemical structures of the five ginsenosides isolated from the root of *Panax ginseng*.

PMS was then added to the mixture, and the absorbance at 560 nm was recorded for 60 s (formation of blue formazan). Water (0.06 mL) was used as control. Reaction rate was calculated from the proportional increase of absorbance, and the scavenging activity was expressed as percent compared to superoxide anion generation of control solution.

Hemolysis Measurement. Fresh blood from a healthy human was collected (9 parts blood/1 part 3.8% sodium citrate) in plastic tubes, and red blood cells (RBCs) were separated by centrifugation at 1000*g* for 10 min. The RBCs were washed once with 2 volumes of 0.9% saline solution and then resuspended in 0.9% saline solution to give 10% RBC concentration. The RBC suspension (0.25 mL) was mixed with an equal volume of 0.9% saline containing ginsenoside and incubated at 37 °C for 5 min with shaking. After centrifugation at 1000*g* for 5 min, 0.2 mL of the supernatant was diluted with 3.3 mL of distilled water and the absorbance was measured at 550 nm (A_{sample}). The percent hemolysis ($H\%$) was calculated using the following equation: $H\% = A_{sample}/A_{100} \times 100$, where A_{100} is the absorbance of 100% hemolysis cells, as when 0.25 mL of 100% RBC suspension was incubated in 8.5 mL of distilled water (*20*).

Extraction and Isolation of Ginsenosides. Ginsenosides Rh_2, Rd, Rb_1, Rb_2, and Rh_1 were isolated from the root of *P. gingseng* C.A. Meyer (Araliaceae) according to the methods of Dou et al., as reported previously (*27*). The structures of five compounds were identified by comparing the FAB-MS and ^1H and ^{13}C NMR data with standard samples. The purity of each obtained compound was >98% detected by HPLC.

Statistical Analysis. The results are expressed as mean ± SD of three values. The effect on each parameter was examined using one-way analysis of variance. Individual differences between groups were evaluated using Dunnett's test, and those at $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

The chemical structures of the five ginsenosides isolated from the root of *P. gingseng* C.A. Meyer, G-Rh_2, -Rd, -Rb_1, -Rb_2, and -Rh_1, are shown in **Figure 1**.

The effects of these ginsenosides on superoxide generation, tyrosyl or serine/threonine phosphorylation of proteins, and translocation of $p47^{phox}$, $p67^{phox}$, and Rac to the plasma membrane in human neutrophils were investigated using AA, fMLP, and PMA as the stimuli. AA, fMLP, and PMA were used as the inducer of the receptor-mediated activation, membrane perturber, and an activator of Ca^{2+}- and phospholipid-dependent protein kinase C, respectively.

When neutrophils were preincubated with these ginsenosides, the AA-induced superoxide generation was suppressed in a

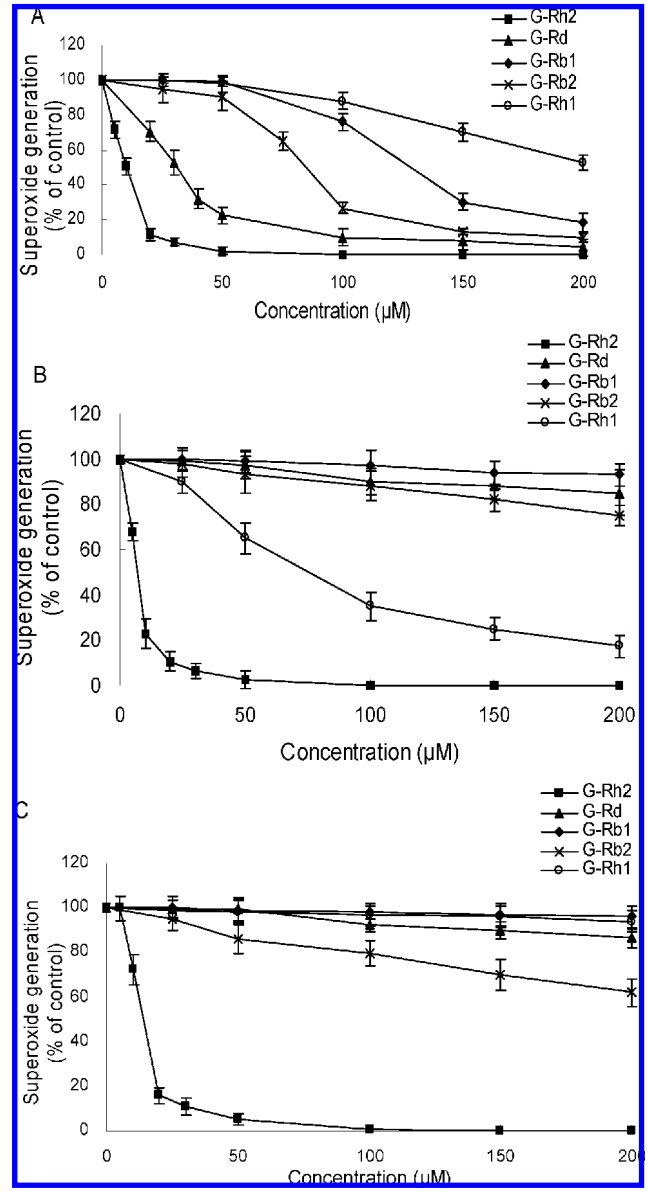


Figure 2. Effect of G-Rh_2, -Rd, -Rb_1, -Rb_2, and -Rh_1 on human neutrophil superoxide generation induced by (**A**) AA, (**B**) fMLP, and (**C**) PMA. The cells were preincubated with 0–200 μM of each ginsenoside for 3 min prior to the addition of 10 μM AA, 12.5 nM fMLP, or 1 nM PMA, for three independent experiments as described under Materials and methods. Results are expressed as means ± SD ($n = 3$).

concentration-dependent manner as shown in **Figure 2A**. The suppression of the AA-induced superoxide generation by these ginsenosides occurred in the following order: G-Rh_2 ≫ G-Rd > G-Rb_2 > G-Rb_1 > G-Rh_1. Among them, the suppression of G-Rh_2 was more effective compared with other compounds used in this experiment. G-Rh_2 also suppressed significantly fMLP- and PMA-induced superoxide generation in a manner similar to the suppression of AA-induced superoxide generation (**Figure 2B**,**C**). G-Rh_1 suppressed fMLP-induced superoxide generation in a concentration-dependent manner (**Figure 2B**). G-Rb_2 suppressed weakly fMLP- and PMA-induced superoxide generation at 200 μM. G-Rd and G-Rb_1 showed no effect on fMLP- and PMA-induced superoxide generation (**Figure 2B**,**C**). In the absence of the stimulus, these ginsenosides did not induce superoxide generation (data not shown).

Ginsenosides are tetracyclic triterpenoid saponins possessing an aglycone (protopanaxadiol or protopanaxatriol) with a

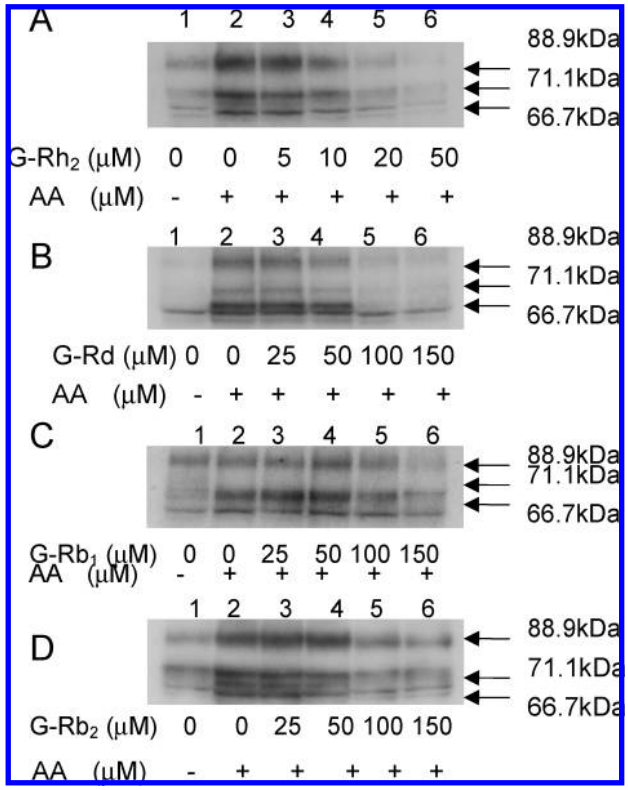


Figure 3. Effect of G-Rh_2, -Rd, -Rb_1, and -Rb_2 on AA-induced tyrosyl phosphorylation of human neutrophil proteins: lane 1, without compounds; lane 2, 10 μM AA; lanes 3–6, 10 μM AA and ginsenoside in various concentrations; (**A**) G-Rh_2; (**B**) G-Rd; (**C**) G-Rb_1; (**D**) G-Rb_2. The tyrosyl phosphorylated proteins were detected by immunoblotting using phosphotyrosine-specific monoclonal antibodies.

dammarane skeleton. G-Rh_2, -Rd, -Rb_1, and -Rb_2 are protopanaxadiol glycosides, which lack a hydroxyl group in position C-6. G-Rh1 is a protopanaxatriol glycoside, in which a glucose unit is linked to C-6. G-Rd, -Rb_1, and -Rb_2 comprise a disaccharide linked to C-3, but differ in the type and number of the sugar moieties linked at C-20. G-Rd, containing glucose at C-20, more strongly suppressed AA-induced superoxide generation than G-Rb_1 and G-Rb_2, containing an additional glucose or arabinose linked to Glc at C-20. G-Rh_2, containing only a glucose at position C-3, more strongly suppressed AA-, fMLP-, and PMA-induced superoxide generation than other compounds. On the other hand, G-Rh_1, containing only a glucose at position C-6, slightly suppressed AA- and fMLP-induced superoxide generation. Although the correlation between the chemical structure and the effect on superoxide generation of these ginsenosides is unclear at present, it might be noteworthy that the type and number of sugar moieties linking at position C-3, C-6, or C-20 in the ring of triterpenoid dammarane contribute to their suppressive effect on the stimulus-induced superoxide generation in human neutrophils.

In our previous studies on superoxide generation and inflammation, we found that various compounds affected the tyrosyl phosphorylation or serine/threonine phosphorylation of neutrophil proteins (*17–20*). Therefore, we propose that these compounds might affect the stimulus-induced superoxide generation via tyrosyl or serine/threonine phosphorylation of neutrophil proteins. In the present study, we investigated the effect of these ginsenosides on tyrosyl or serine/threonine phosphorylation of proteins in AA-, fMLP-, and PMA-treated cells, respectively.

When neutrophils were incubated with AA, tyrosyl phosphorylation of 88.9, 71.1, and 66.7 kDa proteins was induced.

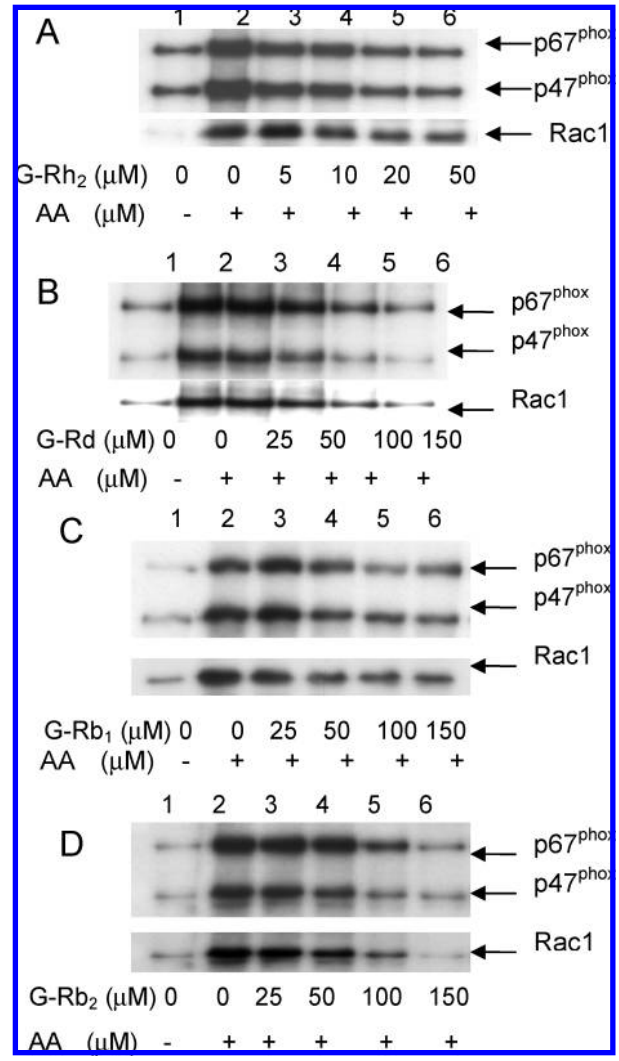


Figure 4. Effect of G-Rh_2, -Rd, -Rb_1, and -Rb_2 on translocation to the plasma membrane of $p47^{phox}$, $p67^{phox}$, and *rac* in AA-stimulated neutrophils: lane 1, without compounds; lane 2, 10 μM AA; lanes 3–6, 10 μM AA and ginsenoside in various concentrations; (**A**) G-Rh_2; (**B**) G-Rd; (**C**) G-Rb_1; (**D**) G-Rb_2. The translocation to the plasma membrane of $p47^{phox}$, $p67^{phox}$, and Rac was detected by immunoblotting using $p47^{phox}$-, $p67^{phox}$-, and Rac1-specific monoclonal antibodies as described under Materials and Methods.

The tyrosyl phosphorylation was dose-dependently suppressed in the presence of these ginsenosides (**Figure 3**). These results coincided well with the effect of superoxide generation, indicating that tyrosyl phosphorylation is involved in the ginsenoside-mediated suppression of superoxide generation in human neutrophils. The mechanism of suppression of tyrosyl phosphorylation by these ginsenosides is not known yet, but the ginsenosides may suppress the cell surface receptor-mediating system via inactivation of tyrosine kinase.

It is generally accepted that on the activation of the respiratory burst oxidase in stimulated human neutrophils, cytosolic $p47^{phox}$, $p67^{phox}$, and Rac move to the plasma membrane and associate with cytochrome b_{558}, forming an active superoxide generation system (*12–16*). Therefore, we also investigated the effect of these ginsenosides on the translocation of $p47^{phox}$, $p67^{phox}$, and Rac to the plasma membrane in AA-stimulated human neutrophils. When neutrophils were incubated with AA, the translocation of cytosolic $p47^{phox}$, $p67^{phox}$, and Rac to the plasma membrane was decreased dose-dependently as shown in **Figure 4**. These results coincided well with the suppression of these

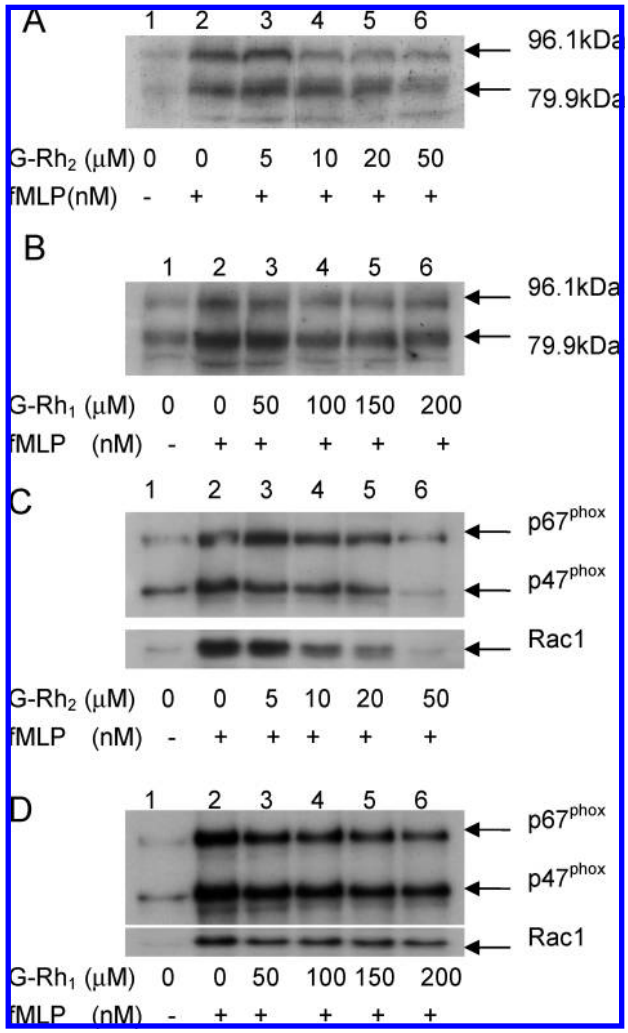


Figure 5. Effect of G-Rh$_2$ and G-Rh$_1$ on fMLP-induced tyrosyl phosphorylation of human neutrophil proteins and translocation to the plasma membrane of p47^{phox}, p67^{phox}, and Rac: lane 1, without compounds; lane 2, 12.5 nM fMLP; lanes 3–6, 12.5 nM fMLP and G-Rh$_2$ or G-Rh$_1$; (**A**) G-Rh$_2$, (**B**) G-Rh$_1$ (fMLP-induced tyrosyl phosphorylated proteins were detected by immunoblotting using phosphotyrosine-specific monoclonal antibodies); (**C**) G-Rh$_2$, (**D**) G-Rh$_1$ (translocation to the plasma membrane of p47^{phox}, p67^{phox}, and Rac was detected by immunoblotting using p47^{phox}, p67^{phox}, and Rac1-specific monoclonal antibodies as described under Materials and Methods).

ginsenosides on superoxide generation and the tyrosyl phosphorylation of neutrophil proteins in AA-stimulated neutrophils.

We also investigated the effect of these ginsenosides on tyrosyl or serine/threonine phosphorylation and translocation of cytosolic p47^{phox}, p67^{phox}, and Rac to the plasma membrane in fMLP- and PMA-stimulated human neutrophils. When neutrophils were incubated with fMLP, tyrosyl phosphorylation of 96.1 and 79.9 kDa was induced. The tyrosyl phosphorylation was dose-dependently suppressed in the presence of G-Rh$_2$ and G-Rh$_1$ (**Figure 5A**,**B**). On the other hand, serine/threonine phosphorylation did not decrease in the presence of G-Rh$_2$ and G-Rh$_1$. These results indicate that G-Rh$_2$ and G-Rh$_1$ suppressed tyrosyl phosphorylation via inactivation of tyrosine kinase, not protein kinase C. The translocation of p47^{phox}, p67^{phox}, and Rac to the plasma membrane was also decreased dose-dependently in the presence of G-Rh$_2$ and G-Rh$_1$ as shown in **Figure 5C**,**D**. In our study, effects of tyrosyl phosphorylation of 96.1 and 79.9 kDa proteins and translocation of p47^{phox}, p67^{phox}, and Rac to

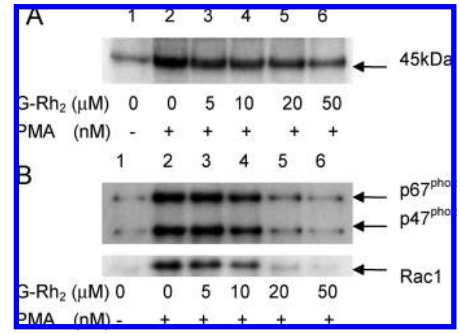


Figure 6. Effect of G-Rh$_2$ and G-Rh$_1$ on PMA-induced serine/threonine phosphorylation of human neutrophil proteins and translocation to the plasma membrane of p47^{phox}, p67^{phox}, and Rac: lane 1, without compounds; lane 2, 1 nM PMA; lanes 3–6, 1 nM PMA, and 5, 10, 20, 50 μM G-Rh$_2$; (**A**) G-Rh$_2$ (PMA-induced serine/threonine phosphorylated proteins were detected by immunoblotting using phosphoserine/threonine-specific monoclonal antibodies); (**B**) G-Rh$_2$ (translocation to the plasma membrane of p47^{phox}, p67^{phox}, and Rac was detected by immunoblotting using p47^{phox}-, p67^{phox}-, and Rac1-specific monoclonal antibodies as described under Materials and Methods).

the plasma membrane by G-Rh$_2$ and G-Rh$_1$ were in parallel to that of fMLP-induced superoxide generation.

When neutrophils were incubated with each of the five ginsenosides and PMA was added as stimuli, as shown in **Figure 2C**, a significant suppression was observed in superoxide production by G-Rh$_2$, as was a slight decrease by G-Rb$_2$, but no effect by the other compounds. Effects of the serine/threonine phosphorylation of neutrophil proteins as well as the translocation of p47^{phox}, p67^{phox}, and Rac to the plasma membrane were also in parallel to that of PMA-induced superoxide generation (**Figure 6**).

These data coincided well with our hypothesis that the five ginsenosides studied suppressed markedly superoxide generation in AA-stimulated neutrophils and some of them in fMLP- and PMA-stimulated neutrophils. The migration of cytosolic compounds p47^{phox}, p67^{phox}, and Rac to the plasma membrane and the tyrosyl or serine/threonine phosphorylation of some neutrophil proteins were correlated to the suppressive effect on superoxide generation.

The production of a superoxide anion (O_2^-) by the NADPH oxidase in the phagolysosome is the precursor for the generation of reactive oxygen species, such as hydroxyl radical and hypochlorite (*13*). They are potent oxidizing agents, fully capable of damaging protein, nucleic acid, and cellular membrane by lipid peroxidation. Therefore, the effect of five ginsenosides on hydroxyl radical induced lipid peroxidation was also investigated using erythrocyte membrane ghosts (**Figure 7**). G-Rb$_1$, G-Rb$_2$, and G-Rd reduced slightly the lipid peroxidation level (thiobarbituric acid-reactive substances) of erythrocyte membrane ghosts at the concentration of 0–200 μM in a concentration-dependent manner, but G-Rh$_1$ showed almost no effect on lipid peroxidation level. The order of inhibition of hydroxyl radical scavenging was G-Rb$_1$ > G-Rb$_2$ > G-Rd > G-Rh$_2$ > G-Rh$_1$ at a concentration of 200 μM. Moreover, Kang et al. have compared the hydroxyl radical scavenging activities of various ginsenosides from *P. ginseng* and investigated their structure and activity relationship (*28*). They reported that G-Rb$_1$ showed a >50% inhibitory activity against hydroxyl radical generation at the concentration of 2 mM, but G-Rb$_2$ and G-Rd showed a comparably lower activity. However, the scavenging effect of these ginsenosides of hydroxyl radical-induced lipid

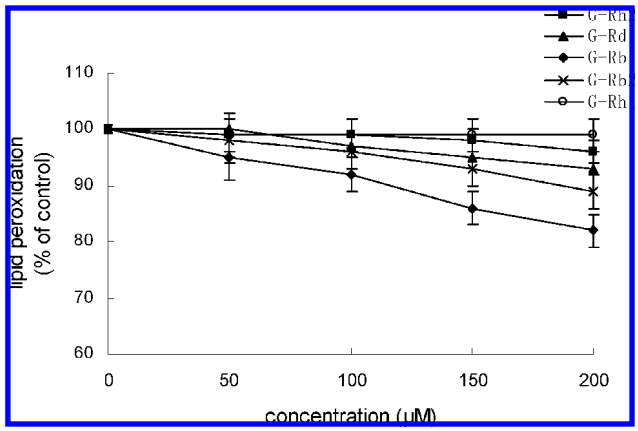


Figure 7. Effect of G-Rh_2, -Rd, -Rb_1, -Rb_2, and -Rh_1 on hydroxyl radical-derived lipid peroxidation of erythrocyte membrane ghosts. Hydroxyl radical-induced lipid peroxidation of erythrocyte membrane ghosts was determined by measuring thiobarbituric acid-reactive substances as described under Materials and Methods. Results are expressed as means ± SD ($n = 3$) of the inhibition of lipid peroxidation.

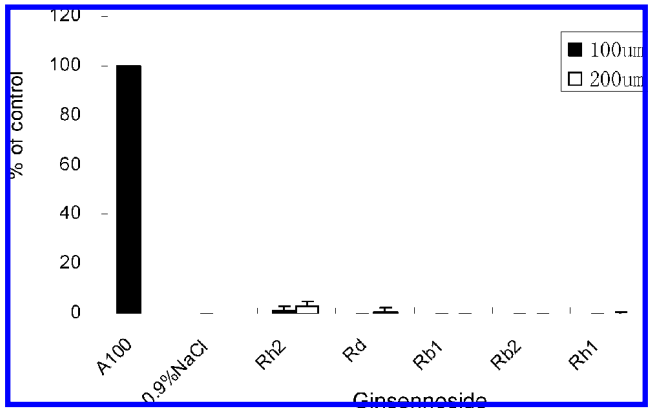


Figure 8. Hemolytic effects of G-Rh_2, -Rd, -Rb_1, -Rb_2, and -Rh_1 on human erythrocytes. The assay was carried out as described under Materials and Methods. The concentrations of the ginsenoside were 100 and 200 μM, respectively. Control means the hemolysis by distilled water, which is shown as 100%. Bars indicate mean ± SD ($n = 5$), respectively.

peroxidation was very weak compared with their inhibitory effect of superoxide generation.

The effects of the five ginsenosides on the scavenging function of generated free radicals were also investigated. In the assay of DPPH radical scavenging activity, the free radical scavenging activity of the five ginsenosides was tested by their ability to scavenge the stable radical DPPH. In this test, none of the five ginsenosides showed any scavenging effect. On the other hand, we also investigated the radical scavenging activity on superoxide anion generated by the PMS-NADH system. The PMS−NADH mixture generates superoxide anions, which have the ability to reduce NBT to formazan, and the absorbance of formazan can be measured. Superoxide generation rate under the present condition was 175 ± 13 nM/s. The five ginsenosides tested had no effect in reducing the superoxide anion levels in doses from 0 to 200 μM (data not shown).

Our results indicate that these ginsenosides do not have scavenging function for already generated free radicals. It suggests that they might work by inhibiting stimulus-induced superoxide generation of neutrophils rather than by scavenging generated superoxide anions. Our present results demonstrate clearly that these ginsenosides suppress superoxide generation mainly via suppressing tyrosyl or serine/threonine phosphorylation and translocation of $p47^{phox}$, $p67^{phox}$, and Rac to the plasma membrane and that their function is not due to scavenging of generated free radicals. Furthermore, considering the possibility of functional food or clinical applications, we examined the hemolytic effect of these ginsenosides. They showed no hemolysis at 0–200 μM (**Figure 8**). Our data may be useful to understand and elucidate the various activities of ginsenosides. However, we cannot ignore the fact that there remain many unclarified factors. Further studies on the pharmacological activities may help in the development of various applications.

ABBREVIATIONS USED

fMLP, *N*-formylmethionyl-leucylphenylalanine; PMA, phorbol 12-myristate 13-acetate; AA, arachidonic acid; PMNs, polymorphonuclear leukocytes.

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