Arch. Pharm. Res. Vol. 16, No. 2, pp. 114-117, 1993

Stimulatory Effect of Ginsenosides on pp60$^{c\text{-}src}$ Protein Tyrosine Kinase

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(Received April 6, 1993)

Ginsenosides present in the roots of *panax ginseng* C.A. Meyer were shown to induce a stimulatory effect on the overexpressed cellular chicken c-src protein tyrosine kinase in NIH 3T3 cells. Among 4 ginsenosides studied (G-Rb_2, G-Rc, G-Re and G-Rg_1), G-Rg_1 showed the most stimulatory effect at 16.7 μg/ml ginsenoside concentration increasing the activity by 2-4 times. Inhibitors of either protein synthesis or RNA synthesis blocked the activation of c-src protein tyrosine kinase. These results suggest that the c-src kinase activation appears to involve an increase in the amount of protein of the kinase by transcriptional control mechanism rather than an increase in the kinase activity.

Key words: Ginsenosides, *Panax ginseng*, pp60$^{c\text{-}src}$, Protein tyrosine kinase

INTRODUCTION

It is a general opinion that the major pharmacological activity of ginseng may be due to saponin. To the present, 25 different ginsenosides have been reported and each of ginseng saponins has a variety of effects (Hong, 1978; Han, 1974; Kim, 1992). Brekhman reported that ginseng extract has antistress or adaptogenic actions (Brekhman, 1975). Oura and Hiai studied the biochemical properties of the fractions of Korea ginseng and concluded that the saponin fraction is active to promote RNA and protein biosynthesis in liver cells of rats (Oura *et al.*, 1971a; Oura *et al.*, 1971b; Hiai *et al.*, 1971). Yamamoto reported that prostisol increases the mitotic indices of the nucleated cells of bone marrow in rats (Yamamoto, 1975). There are a number of evidences that the biochemical actions of ginsenosides are mediated by cyclic AMP as a secondary messenger. Nikaido *et al.* reported that ginsenosides act as inhibitors of cyclic AMP phosphodiesterase (Nakaido *et al.*, 1984). Jin *et al.* showed that ginseng saponin stimulates the synthesis of cytosolic cAMP in isolated rabbit gastric glands (Jin *et al.*, 1986). In addition, there are numerous other effects like antineoplastic effect, hepatic regeneration effect, etc. (Hong, 1978; Han, 1974; Kim, 1992).

Since many of these biological effects of ginseng saponin are related to cell growth, we were stimulated to examine its effect on the cellular signal transduction pathway, particulary on the c-src protein tyrosine kinase (pp60$^{c\text{-}src}$). pp60$^{c\text{-}src}$ is a membrane-bound protein-tyrosine kinase. Its normal physiological function is obscure, but a number of evidences suggest that pp60$^{c\text{-}src}$ plays some fundermental role in the normal regulation of cell growth, many of which involve membrane and cytoskeletal alterations. More recent studies demonstrate that pp60$^{c\text{-}src}$ has actions in nonproliferating cells as well (Shalloway and Shenoy, 1991; Shenoy *et al.*, 1989).

Here we describe the stimulatory effect of ginsenoside on the protein tyrosine kinase activity of pp60$^{c\text{-}src}$ and the activation mechanism using inhibitors of RNA and protein biosynthesis.

MATERIALS AND METHODS

Cells and cell culture

NIH (pMc-src/focus) B cells overexpressing wild-type chicken pp60$^{c\text{-}src}$ (Johnson *et al.*, 1985) were grown at 37℃, 10% CO_2, in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Lab., Grand Island, N.Y., USA) plus 10% calf serum except as noted. Cells were plated at 1.0×10^6 cells per 10 cm plate 16-24 h before the initiation of all experiments.

Drug treatments

Cells prepared as above were treated with the speci-

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fied drugs. The treatments consist of the addition of G-Rg_1 (16.7 μg/ml) (Korea Ginseng and Tobacco Research Institute) and/or each specified drugs. After these drugs were added, cells were incubated for 18 h and then collected for *in vitro* kinase assay.

Immunoprecipitations

Cells were lysed in 0.5 ml of RIPA buffer [1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 20 mM NaH_2PO_4] (Sigma Chemical Co., ST. Louis, MO, USA) supplemented with 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 50 mM NaF, 0.2 mM Na_3VO_4, and 100 KIU of aprotinin (Sigma Chemical Co., ST. Louis, MO, USA) per ml. This RIPA buffer is a good phosphatase inhibitor. EDTA, NaF, and Na_3VO_4 were included to further inhibit protein kinases and phosphatases. Lysates were clarified at 25,000×g for 30 min. The amount of total cell protein (TCP) was determined by Bradford method and normalized to equal amount of pp60$^{c\text{-}src}$. pp60$^{c\text{-}src}$ were immunoprecipitated from a volume of lysate containing 100 μg of TCP with 1 μl of monoclonal antibody 327 (Lipsich *et al.*, 1983) for 45 min at 0℃. Immune complexes were collected on 30 μl of 10% *S. aureus* suspension that had been precoated with 1 μg of anti-mouse immunoglobulin G (heavy plus light chains) by 20 min of incubation at 0℃. It was washed with high-salt buffer (1M NaCl, 0.5% Triton X-100, 10 mM Tris hydrochloride [pH 7.2]), and washed twice with RIPA buffer.

In vitro kinase assay

For kinase reactions, pp60$^{c\text{-}src}$ immunoprecipitated with MAb 327 was resuspended in 2× phosphorylation buffer (5 mM MnCl2, 20 mM HEPES [pH 7.0], 2 mM 2-mercaptoethanol). Aliquots equivalent to 6 μg of TCP for each sample were used for the kinase reaction at room temperature for 4 min in 40 μl reaction mixture, consisting of phosphorylation buffer , acid-denatured rabbit muscle enolase (4 μg/ sample) and 1 μM [γ-^{32}P] ATP (400 Ci/mmol, Amersham , England). At the end of 4 min the reaction was stopped by adding 3× sample buffer, and analysed on 10% SDS-PAGE followed by autoradiography.

RESULTS AND DISCUSSION

NIH(pMc-src/focus) B cells overexpressing wild-type chicken pp60c-src were treated with ginsenosides at various concentrations for different incubation times and cells were collected for *in vitro* kinase assay. All the ginsenosides used for this experiment (G-Rg_1, G-Rb_2, G-Re, and G-Rc) showed a stimulatory effect on pp60$^{c\text{-}src}$ protein kinase (Data not shown). Among four ginsenosides studied, G-Rg_1 showed the most stimulatory effect at 16.7 μg/ml of ginsenoside concentration for 18 h incubation, increasing the activity by 2-4 times (Fig. 1, 2). To investigate the action mechanism of G-Rg_1 on the activation of pp60$^{c\text{-}src}$ kinase, cells were given transcription or translation inhibitors along with G-Rg_1 and incubated for 18 h. Figure 3 shows that

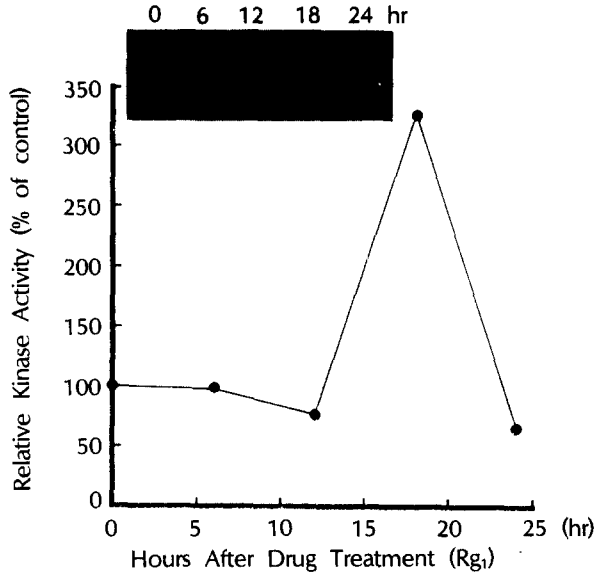

Fig. 1. Time course of pp60$^{c\text{-}src}$ kinase activity after treatment of ginsenoside Rg_1. NIH (pMc-src/focus) B cells were treated with ginsenoside Rg_1 for the incubated times and collected. pp60$^{c\text{-}src}$ was immunoprecipitated with MAb 327 from cell lysate, and incubated with [γ-^{32}p] ATP and rabbit muscle enolase as a substrate, and analysed by 10% SDS-PAGE and autoradiography.

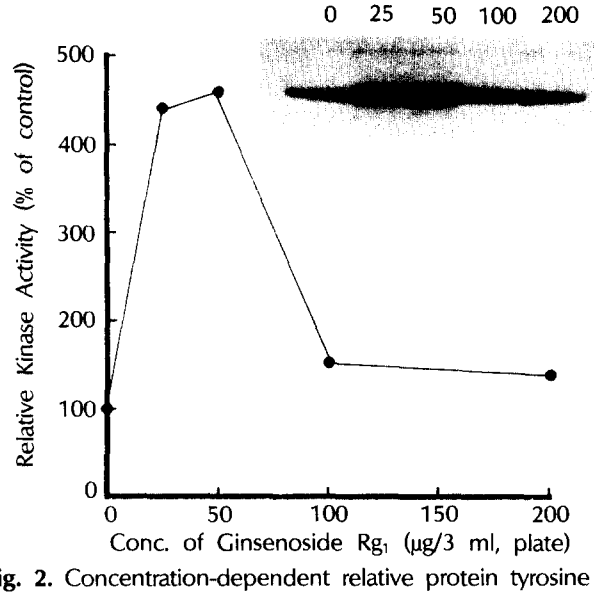

Fig. 2. Concentration-dependent relative protein tyrosine kinase activity of pp60$^{c\text{-}src}$. Cells were treated with a different concentration of G-Rg_1 for 18 h and in vitro kinase assay was performed as in Fig. 1.

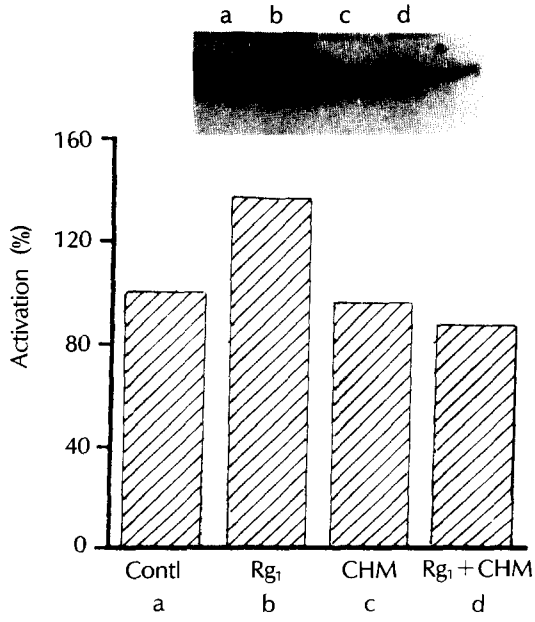

Fig. 3. Inhibitory effect of cycloheximide on the G-Rg_1-induced activation of $pp60^{c\text{-}src}$. (a) Untreated control; (b) G-Rg_1 (16.7 μg/ml); (c) Cycloheximide (25 μg/ml); (d) G-Rg_1 (16.7 μg/ml)+Cycloheximide (25 μg/ml).

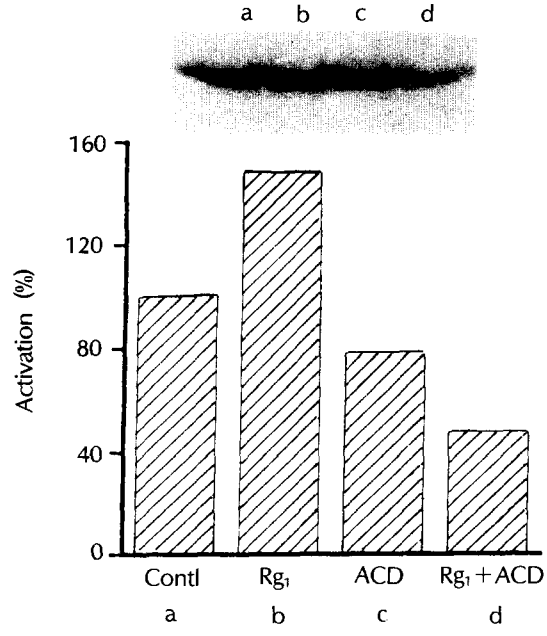

Fig. 4. Inhibitory effect of actinomycin D on the G-Rg_1-induced activation of $pp60^{c\text{-}src}$. (a) Untreated control; (b) G-Rg_1 (16.7 μg/ml); (c) Actinomycin D (5 μg/ml); (d) G-Rg_1 (16.7 μg/ml)+Actinomycin D (5 μg/ml).

cycloheximide (25 μg/ml), a protein synthesis inhibitor canceled the stimulatory effect of G-Rg_1 on $pp60^{c\text{-}src}$ kinase to the untreated control level. Fig. 4 also shows the G-Rg_1-induced activation of c-src kinase was blocked when cells are simultaneously treated with actinomycin D (5 μg/ml), a RNA synthesis inhibitor. These results suggest that the G-Rg_1-induced activation of c-src kinase is due to an increase in the amount of protein of the kinase by elevated RNA synthesis. To confirm the stimulatory action mechanism occured in transcriptional level, cells were simultaneously treated with sodium vanadate (100 μM), a tyrosine phosphatase inhibitor, which activates the $pp60^{c\text{-}src}$ kinase by three times with increasing the degree of phosphorylation of Tyr-416 (Shalloway and Shenoy, 1991). When cells were treated with sod. vanadate and G-Rg_1 at the same time, the kinase activity of $pp60^{c\text{-}src}$ was increased synergistically (Fig. 5). This result confirms that the stimulatory effect of G-Rg_1 on c-src protein tyrosine kinase is caused by an increase in the amount of protein of $pp60^{c\text{-}src}$ not by a post-translational fashion.

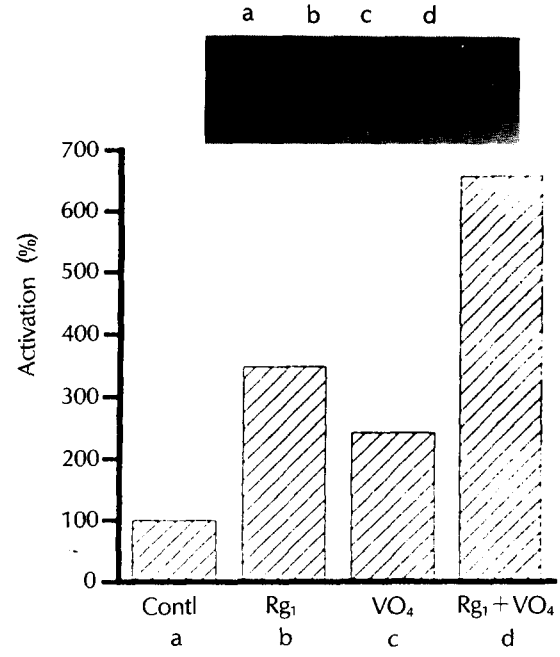

Fig. 5. Synergistic effect of sod. vanadate on the G-Rg_1-induced activation of $pp60^{c\text{-}src}$. (a) Untreated control; (b) G-Rg_1 (16.7 μg/ml); (c) 100 μM Sod. vanadate; (d) G-Rg_1 (16.7 μg/ml) +100 μM Sod. vanadate.

In conclusion, G-Rg_1 shows the most stimulatory effect (2-4 times) on $pp60^{c\text{-}src}$ protein tyrosine kinase at 16.7 μg/ml among 4 ginsenosides tested. The stimulatory effect on $pp60^{c\text{-}src}$ protein tyrosine kinase with G-Rg_1 involves an increase in the amount of protein of the kinase rather than an increase in the kinase activity.

ACKNOWLEDGEMENTS

We thank Dr. D. Shalloway for his generous gift of biological materials and also thank Korea Ginseng and Tobacco Research Institute for providing ginsenosides.

This work was supported by grants from KOSEF and the Research Center for New Drug Development in 1992.

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