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# Studies on the Activation Mechanism of *c-src* Protein Tyrosine Kinase by Ginsenoside-$Rg_1$

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**Abstract :** We have studied an activation mechanism of $pp60^{c\text{-}src}$ protein tyrosine kinase (PTK) by ginsenoside-$Rg_1$ (G-$Rg_1$) in NIH(pMcsrc/foc)B *c-src* overexpressor cells. It was previously reported that G-$Rg_1$ stimulated the activation of *c-src* kinase at 20 μM with a 18 hr-incubation, increasing the activity by 2-4-fold over that of untreated control, and this effect was blocked by treatments of inhibitors of either protein synthesis (cycloheximide) or RNA synthesis (actinomycin D) (Hong, H.Y. *et al. Arch. Pharm. Res.* **16**, 114 (1993)). However, an amount of *c-src* protein itself in wild-type cells was not changed by G-$Rg_1$. When the cells mutated at one or two tyrosine residue(s) (Y416/527) that are important sites to regulate the kinase activity were treated with G-$Rg_1$, increases both in the activity of *c-src* kinase and in the expression of the protein were not observed. In addition, removal of extracellular calcium ion by EGTA or inhibition of PKC by H-7 canceled the G-$Rg_1$-induced activation of the kinase. Although the activation was little affected by G-$Rg_1$ with a calcium ionophore A23187, it was synergistically stimulated by treatment of G-$Rg_1$ and PMA, a PKC activator. Taken together, these results suggest that the activation of *c-src* kinase by G-$Rg_1$ is caused by an increase in the specific activity of the kinase, but not in amount of it, and is involved with both cellular calcium ion and PKC. Further the increase in the specific activity of *c-src* kinase may result from altered phosphorylation at tyr-416 and -527.

**Key words :** *Panax ginseng* C. A. Meyer, ginsenoside-$Rg_1$, $pp60^{c\text{-}src}$ protein tyrosine kinase.

## Introduction

G-$Rg_1$ is a dammarane-type protopanaxatriol saponin presents most abundantly in ginseng and shares many pharmacological effects of this plant. It has been shown to possess DNA, RNA, and protein anabolic effects[1,2] and anti-thrombotic effect.[3] Kenarova *et al.* showed that the ginseng saponin had an immunomodulating activity in mice by increasing the number of spleen plaque-forming cells, the titer of sera hemagglutinins as well as the number of antigen-reactive T cells.[4] Himi *et al.* reported that G-$Rg_1$ exerted a survival-promoting effect on both chick and rat cerebral cortex neurons in cell cultures.[5] Deng and Zhang found that G-$Rg_1$ could inhibit lipid peroxidation followed by generation of free radicals in rat liver and brain microsomes.[6] Recently Lee *et al.* described that G-$Rg_1$ stimulates the proliferation of cultured human hepatoma cells by inducing the levels of cyclin E and cdk2 proteins.[7] There have been many evidences to support that G-$Rg_1$ may participate in the activation of cellular signal transduction pathway.

$pp60^{c\text{-}src}$ is a membrane-associated nonreceptor-type protein tyrosine kinase (PTK), which acts upstream of *ras* and *raf*.[8] The kinase is non-transforming, even when expressed at high levels.[9,10] It is generally believed that phosphorylation by *c-src* kinase is a functionally important component of the cellular signaling mechanisms in normal cells and transformed cells. A number of evidences suggest that $pp60^{c\text{-}src}$ plays some fundermental roles in the normal regulation of cell growth, many of which involve membrane and cytoskeletal alterations that are associated with chromosome condensation, mitotic

spindle assembly, and nuclear envelope breakdown, occurring at mitosis. The kinase activity is mainly regulated by phosphorylation and dephosphorylation at two tyrosine residues, Tyr-416 and -527. The site of $pp60^{c\text{-}src}$ autophosphorylation, Tyr-416 is highly conserved in tyrosine kinases, upregulates *c-src* kinase activity by phosphorylation, and is the major phosphorylation site in *v-src*. In normal cells Tyr-527 in regulatory domain of *c-src* is phosphorylated to almost stoichiometric levels which inhibits its tyrosine kinase activity.[11] The substitution of Tyr-527 of *c-src* by Phe produces an oncogenic mutant that has high kinase activity throughout the cell cycle comparable to that of *v-src*.[12]

We previously reported that G-$Rg_1$ showed stimulatory effects on two *src*-family PTKs, $pp60^{c\text{-}src}$ in NIH 3T3 mouse fibroblasts and $p56^{lck}$ in Jurkat human T cells.[13,14] Based on these reports, further we have pursued the activation mechanism of $pp60^{c\text{-}src}$ kinase, a prototype of *src*-family PTKs, by G-$Rg_1$.

## Materials and Methods

### 1. Cell lines and cell culture

All *src* proteins were immunoprecipitated from NIH 3T3 cells that had been transfected with *src*-expression plasmids. Wild-type *c-src* was from NIH(pMcsrc/foc)B *c-src* overexpressor cells.[15] Sources of mutant *src* proteins were as follows: Tyr 416→Phe, NIH(pcsrc416/pSV2neo/MC)A cells; Tyr 527→Phe, NIH(pcsrc527/foc/EP)B1 cells; Tyr 416→Phe/Tyr 527→Phe, NIH(pc 416527/foc)A cells. All cell lines used were provided from Dr. Shalloway, D. at Cornell University (NY, USA). Cells were plated at $1.0\times10^6$ cells per 100 mm plate 16-24 hr before the initiation of all experiments and were grown at 37 ℃, 10% $CO_2$, in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) with 10% calf serum.

### 2. Drug treatment

Cells prepared as above were treated with specified drugs. The treatments consist of additions of G-$Rg_1$ that was kindly provided from Korea Ginseng and Tobacco Research Institute (Taejeon, Korea) and/or each of specified drugs. After treatments of drugs, cells were incubated for the designated times and then collected for further experiments.

### 3. Immunoprecipitation of $pp60^{c\text{-}src}$

Cells were washed twice in STE buffer [0.15M NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 7.2)] and lysed in 0.5 m*l* of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 20 mM $NaH_2PO_4$) supplemented with 1mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 50 mM NaF, 0.2 mM $Na_3VO_4$, and 100KIU of aprotinin (Sigma Chemical Co., USA) before use. Lysates were clarified at 25,000×g for 30 min. The amount of total cell protein (TCP) was determined by Bradford's method[16] and normalized to equal amount of proteins. $pp60^{c\text{-}src}$ was immunoprecipitated from a volume of lysate containing 100 μg of TCP with 1 μ*l* of monoclonal antibody 327[17] for 45 min at 0℃. Immune complexes were collected on 30 μ*l* of 10% *S. aureus* suspension that had been precoated with 1 μg of anti-mouse IgG (heavy plus light chains) by a 20 min-incubation at 0℃. It was washed once with high-salt buffer [1M NaCl, 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.2)] and twice with RIPA buffer.

### 4. *In vitro* kinase assay of $pp60^{c\text{-}src}$

For kinase reactions, $pp60^{c\text{-}src}$ immunoprecipitates were resuspended in phosphorylation buffer [5 mM $MnCl_2$, 20 mM HEPES (pH 7.0), 2 mM β-mercaptoethanol]. Aliquots equivalent to 6 μg of TCP for each sample were used for the kinase reactions at room temperature for 5 min in 40 μ*l* of reaction mixture, consisting of phosphorylation buffer, acid-denatured rabbit muscle enolase (4 μg/sample), and 1 μM [$\gamma$-$^{32}$P] ATP (400 Ci/mmol, Amersham, England). At the end of 5 min the reactions were stopped by adding sample buffer for SDS-PAGE [210 mM Tris-HCl (pH 6.8), 34.2% glycerol, 9% SDS, 0.03% bromphenol blue, 15% β-mercaptoethanol] and were analysed on 10% SDS-PAGE followed by autoradiography. Band intensity was quantified by densitometry of autoradiograms using a Model

CS-9000 densitometer (Shimadzu Co., Japan).

**5. Western analysis**

Cell lysate containing indicated amount of TCP was directly separated by 7.5% SDS-PAGE without immunoprecipitation. PVDF membrane, Immobilon P (Millipore, USA), was used for electroblotting in Trans-Blot electrophoretic transfer cell (Bio-rad, USA). Immobilon is to be wetted first in 95% of methanol for 20~30 sec, washed four times with distilled water, and then soaked in transfer buffer [25 mM Tris-HCl (pH 7.4), 192 mM glycine]. To block non-specific binding of antibody, transferred membrane was incubated for 2hr in 3%(w/v) BSA in TBS solution [50 mM Tris-HCl, 150 mM NaCl (pH 7.5)]. The membrane was probed with anti-src MAb 327, subsequently labelled with peroxidase (POD)-conjugated secondary antibody, and finally detected with the substrate, luminol by chemiluminescence reaction (Boehringer Mannheim GmbH, Germany).

## Results and Discussion

Previously we reported that G-$Rg_1$ showed the marked stimulatory effect on the activity of *c-src* PTK at 20 μM (100 μg/6 m*l*) with a 18 hr-incubation in NIH(pMcsrc/foc)B *c-src* overexpressor cells, increasing the activity by 2-4 fold over that of untreated control.[13] The effect of G-$Rg_1$ on *c-src* kinase was canceled by treatment of either transcriptional (actinomycin D) or translational (cycloheximide) inhibitor to the untreated control level.[13] These findings suggested that the G-$Rg_1$-induced activation of *c-src* kinase results from an increase in the amount of the kinase or other protein(s) involved in the regulation of the kinase activity through its elevated RNA synthesis. To investigate that the activation occurs in transcriptional level of the kinase, western analysis of *c-src* expression was performed after NIH(pMcsrc/focus)B cells were treated with G-$Rg_1$ (20 μM) for 18 hr. However, there was no increase in the amount of *c-src* protein (Fig. 1). Most likely, the stimulatory effect

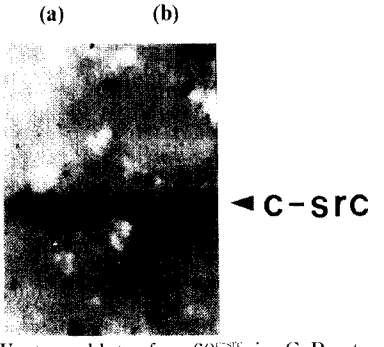


**Fig. 1.** Western blot of $pp60^{c\text{-}src}$ in G-$Rg_1$-treated *c-src* overexpressed NIH(pMcsrc/foc)B cells. Cell lysates equal to 20 μg of total cell protein were electrophoresed in 7.5% SDS-polyacrylamide gel and transferred to PVDF membrane. The membrane was probed with MAb 327 *c-src* antibody and then detected by chemiluminescence reaction. (a) Untreated control; (b) G-$Rg_1$ (20 μM).

involves an increase in the specific activity of *c-src* kinase, which can be regulated by phosphorylation and dephosphorylation of tyrosine residues of the kinase or by binding to its SH2-containing cellular ligands, but not in the amount of the kinase. That is, inhibitory effects of both cycloheximide and actinomycin D on the G-$Rg_1$-induced activation of *c-src* kinase reflect inhibition of the expression of other protein(s) responsible for regulating the activity of *c-src* kinase, rather than inhibition of that of the kinase itself.

In an earlier paper we also reported that the activity of $pp60^{c\text{-}src}$ kinase was increased synergistically, when the cells were treated with sodium vanadate (100 μM) and G-$Rg_1$ together.[13] Vanadate is a tyrosine phosphatase inhibitor, which activates *c-src* kinase by three times with increasing the degree of phosphorylation of Tyr-416.[18] The synergistic effect of two drugs indicates that either vanadate stabilizes a phosphorylation of Tyr-416 that is increased by G-$Rg_1$ or G-$Rg_1$ enhances dephosphorylation of Tyr-527 on *c-src* kinase, which upregulates the kinase activity, while vanadate acts on its own way. Taken together, it is necessary to explain our earlier suggestion[13] in a different way that the stimu-

latory effect of G-$Rg_1$ on *c-src* kinase involves an increase in the specific activity of the kinase through an increase in phosphorylation of Tyr-416 and/or in dephosphorylation of Tyr-527.

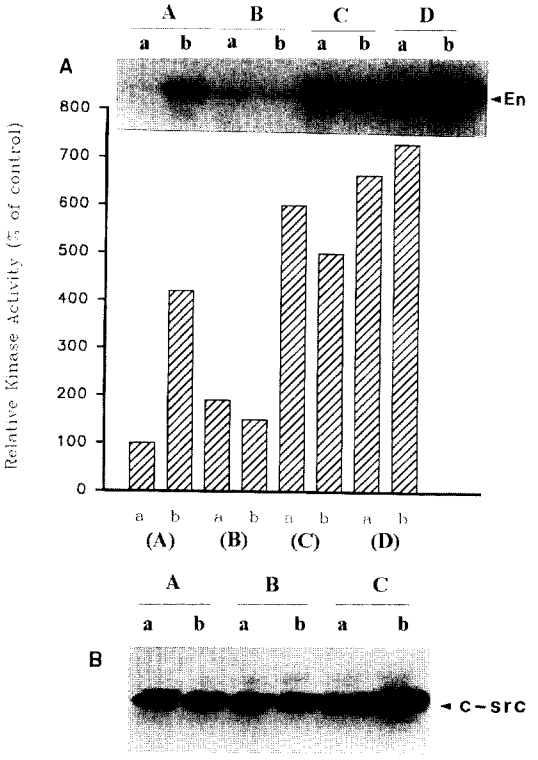


**Fig. 2.** A. Effect of G-$Rg_1$ on $pp60^{c\text{-}src}$ kinase activity in NIH 3T3 cells mutated at tyrosine residues. Cells were treated with 20 μM of G-$Rg_1$ for 18 hr and collected. $pp60^{c\text{-}src}$ was immunoprecipitated with MAb 327 from cell lysate, incubated with [$\gamma$-$^{32}$p] ATP and rabbit muscle enolase (En) as a substrate, and analysed by 10 % SDS-PAGE and autoradiography. Band intensity was quantified by densitometry as described in Materials and Methods. (A) NIH (pMcsrc/foc)B; (B) NIH(pcsrc416/pSV2neo/MC)A; (C) NIH(pMcsrc527/foc/EP)B1; (D) NIH(pc416527/foc)A. (a) Untreated control; (b) G-$Rg_1$ (20 μM). B. Western blot of $pp60^{c\text{-}src}$ in G-$Rg_1$-treated mutant cells. Cell lysates equal to 25 μg of total cell protein were electrophoresed in 7.5% SDS-polyacrylamide gel and transferred to PVDF membrane. The membrane was probed with MAb 327 *c-src* antibody and then detected by chemiluminescence reaction. (A) NIH(pcsrc416/pSV2neo/MC) A; (B) NIH(pMcsrc527/foc/EP)B1; (C) NIH (pc416527/foc)A. (a) Untreated control; (b) G-$Rg_1$ (20 μM).

To investigate the mechanism of an increase in the specific activity of *c-src* kinase, *in vitro* kinase assay was performed using mutant cell lines that have Tyr→Phe mutation at two important tyrosine residues (Y416 and 527) of *c-src* kinase; F416, F527, and F416/527. When mutant cells were treated with G-$Rg_1$ (20 μM) for 18 hr, any significant changes in *c-src* kinase activity between G-$Rg_1$-treated and -untreated groups were not observed in all of the mutated cell lines except wild-type cells (Fig. 2A). The amount of *c-src* protein was also not changed in all mutant cell lines (Fig. 2B). If there is no change in *c-src* kinase activity in F416 mutant cells after treatment of G-$Rg_1$, this would imply that the G-$Rg_1$-induced activation of *c-src* kinase depends on a change in phosphorylation of Tyr-416 residue. Therefore, our results suggest that phosphorylation states of two tyrosine residues on $pp60^{c\text{-}src}$ are responsible for the G-$Rg_1$-induced activation of the kinase. The activation of the kinase via altered phosphorylation at two tyrosine residues could be achieved by possible mechanisms as follows; one is that G-$Rg_1$ sensitizes Tyr-527 of *c-src* to a phosphatase or desensitizes it to a kinase. The other is that G-$Rg_1$ conversely acts on two enzymes for Tyr-416.

It has become clear that activations of most of cellular signal transduction pathways include the changes in the concentration of intracellular calcium ion and/or in the activity of protein kinase C (PKC). Thus we examined whether both G-$Rg_1$ bring about the increase of intracellular calcium ion concentration ($[Ca^{2+}]i$) and cellular calcium ion mediates the G-$Rg_1$-induced activation of *c-src* kinase. G-$Rg_1$ did not give an increase of $[Ca^{2+}]_i$ in the range of 0.25 to 25 mg/m*l* of concentration (data not shown). As shown in Fig. 3A, however, when cells were simultaneously treated with a calcium chelator, EGTA (2.5 mM), the stimulatory effect of G-$Rg_1$ on *c-src* kinase reduced to the level of EGTA-treated cells. In addition, treatment of G-$Rg_1$ along with calcium ionophore A23187 (0.1 μM) resulted in no significant increase in the ac-

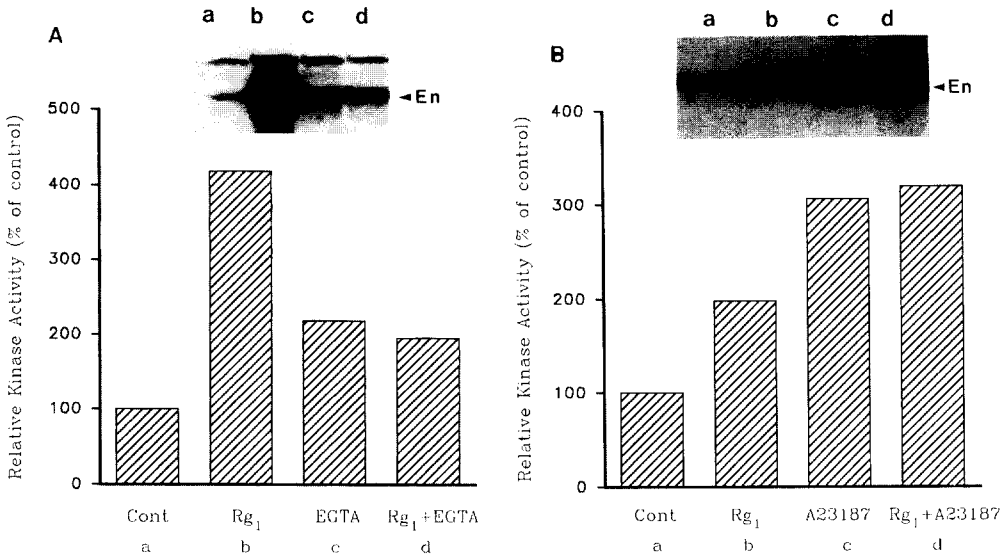


**Fig. 3.** A. Inhibitory effect of EGTA on the G-$Rg_1$-induced activation of $pp60^{c\text{-}src}$. (a) Untreated control; (b) G-$Rg_1$ (20 μM); (c) EGTA (2.5 mM); (d) G-$Rg_1$ (20 μM) and EGTA (2.5 mM). B. Effect of A23187 on the G-$Rg_1$-induced activation of $pp60^{c\text{-}src}$. (a) Untreated control; (b) G-$Rg_1$ (20 μM); (c) A23187 (0.1 μM); (d) G-$Rg_1$ (20 μM) and A23187 (0.1 μM). *In vitro* kinase assay was done like in Fig. 2A.

tivity of *c-src* kinase comparing with that of A 23187-treated cells (Fig. 3B). A23187 alone activated *c-src* kinase by three times over untreated control. These results indicate that extracellular calcium ion is essential for the G-$Rg_1$-induced activation of *c-src* kinase and G-$Rg_1$ activates *c-src* kinase through the pathway of increasing $[Ca^{2+}]_i$ like A23187, inferred from little increase in the G-$Rg_1$-induced activation of *c-src* kinase by adding A23187.

Both Ser-12 and -48 in a unique domain of $pp60^{c\text{-}src}$ are phosphorylated by PKC and they usually remain unphosphorylated. Although deletions or insertions within this region have little effect on transformation potential,[19] the significance of the sites in regulating the activity of the kinase is still unclear. By only PMA (phorbol-12-myristate-13-acetate; PKC activator, 20 ng/ml), the activity of *c-src* kinase was increased to 2-fold over untreated control, and by G-$Rg_1$ and PMA together, it was increased to more than 4-fold over untreated control (Fig. 4A). In addition, H-7 ((1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; PKC inhibitor, 30 μM) blocked the G-$Rg_1$-induced activation of $pp60^{c\text{-}src}$ to the untreated control level, although it alone did not inhibit the kinase activity (Fig. 4B). Our results suggest that the G-$Rg_1$-induced activation of *c-src* kinase is mediated through primarily other pathway rather than PKC-involved one. We do not exclude a regulatory function of PKC on the G-$Rg_1$-induced activation of *c-src* kinase.

Dent *et al.* reported that Triton X-100, a non-ionic detergent, alters membrane organization and modulates the ability of $p21^{ras}$ to mediate MAP kinase activation.[20] Alder *et al.* also demonstrated that Triton X-100 interferes with cellular signaling cascade through blocking JNK (*jun*-$NH_2$-terminal kinase) activation by both UV and vanadate.[21] Similar detergents were shown to alter the ability of the epidermal growth factor receptor (EGF) to dimerize and autophosphorylate.[22] These reports show that saponin generally considered to possess a detergent effect might be involved in cellular signal transduction. Consequently, we suggest that the stimulatory effect of G-$Rg_1$ on *c-src* kinase results from an increase in the specific activity of *c-src* kinase, due to the expression of regulatory protein(s) that is responsible for altered tyrosine phosphorylation of the kinase, rather than an increase in the amount of protein of the kinase. The

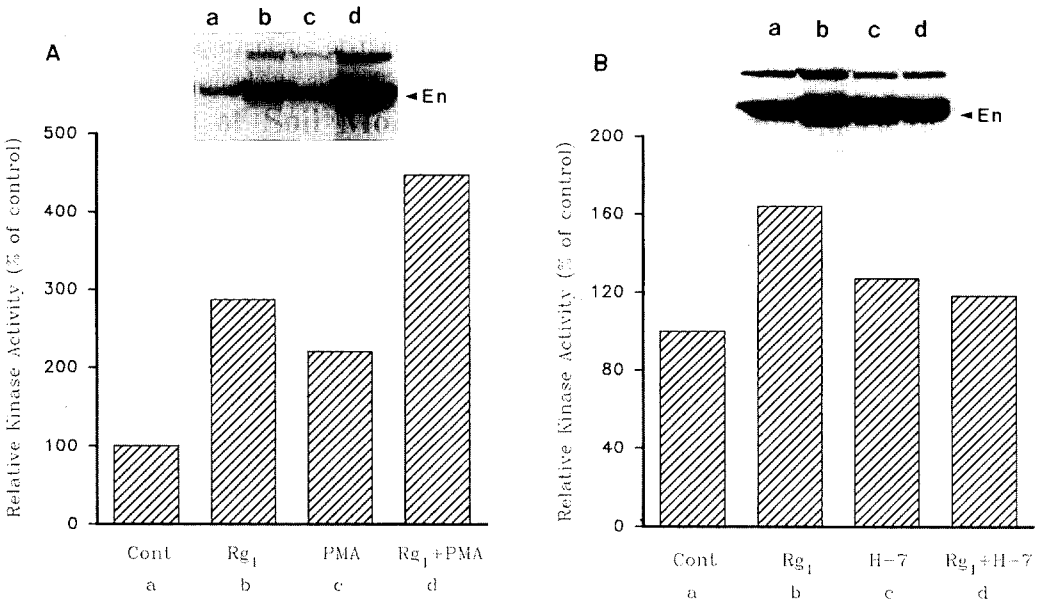


**Fig. 4.** A. Synergistic effect of PMA on the G-$Rg_1$-induced activation of $pp60^{c\text{-}src}$. (a) Untreated control; (b) G-$Rg_1$ (20 μM); (c) PMA (20 ng/m*l*); (d) G-$Rg_1$ (20 μM) and PMA (20 ng/m*l*). B. Inhibitory effect of H-7 on the G-$Rg_1$-induced activation of $pp60^{c\text{-}src}$. (a) Untreated control; (b) G-$Rg_1$ (20 μM); (c) H-7 (30 μM); (d) G-$Rg_1$ (20 μM) and H-7 (30 μM). *In vitro* kinase assay was done like in Fig. 2A.

activation of *c-src* kinase is more sensitive to cellular calcium ion than PKC. Further investigation of the expression of protein(s) involved in regulating the specific activity of *c-src* kinase would be valuable.

## 요 약

인삼사포닌 $Rg_1$은 20 μM에서 18시간 처리시 *c-src* kinase 활성을 2~4배 촉진시켰으며, 이 효과는 RNA 합성억제제(actinomycin D)와 단백질 합성억제제(cycloheximide) 처리에 의해 억제되었음을 보고한 바 있다.[13] 본 연구에서는 $Rg_1$에 의한 *c-src* kinase의 활성화 기전을 분자수준에서 보다 구체적으로 밝히고자 하였다. Wild형 세포의 Western 분석을 통해 인삼사포닌 $Rg_1$에 의한 *c-src* kinase 활성화는 효소의 단백질 양의 증가에 기인한 것이 아님을 확인하였으며, 이 효소의 활성 조절부위인 Tyr-416, 527 를 변형시킨 세포에서는 *c-src* kinase 활성 및 그 효소 단백질의 양적 변화가 없었다. 이 결과로부터 *c-src* kinase의 tyr-416, -527의 인산화 상태변화가 G-$Rg_1$에 의한 효소활성화를 좌우하는 것임을 알 수 있었다. 세포내 신호전달 체계의 주요 매개체로 알려진 세포내 칼슘이온 농도와 PKC 활성 변화가 *c-src* kinase 활성에 미치는 영향을 알아본 결과, EGTA(칼슘 chelator)와 H-7(PKC 억제제) 처리시 G-$Rg_1$에 의한 효소 활성화 효과는 억제되었으며, A23187 (calcium ionophore)과 병행처리시 효소활성은 A23187 단독처리한 세포에서와 유사한 정도로 나타났고 PMA(PKC 활성화제)에 의해서는 PMA와 G-$Rg_1$ 각각에 의한 효소활성 증가분 만큼의 상승작용이 나타났다. 이것은 G-$Rg_1$에 의한 *c-src* kinase 활성화는 세포내 칼슘이온 농도 및 PKC 활성과 관련이 있으며, 특히, PKC- 보다는 칼슘이온-관련 신호전달 경로가 G-$Rg_1$에 의한 *c-src* kinase 활성화에 영향이 크다는 것을 시사해 준다. 따라서 G-$Rg_1$에 의한 *c-src* kinase 의 활성화는 이 효소의 단백질양의 증가에 의해서라기 보다는 다른 활성조절 단백질(들)(예, tyrosine phosphatase, C-terminal *src* kinase)의 작용에 의한 효소의 특이적 활성증가에 기인하는 것으로 추측된다.

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