Role of tyrosine phosphorylation of phospholipase C γ1 in the signaling pathway of HMG-CoA reductase inhibitor-induced cell death of L6 myoblasts

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Received 11 January 1999; received in revised form 3 February 1999

Abstract Our previous studies have shown that the HMG-CoA reductase (HCR) inhibitor (HCRI), simvastatin, kills L6 myoblasts by involving Ca^{2+} mobilization from the Ca^{2+} pool in the cells but not by influx from extracellular space. More recently, we found that HCRI induced tyrosine phosphorylation of several cellular proteins, followed by apoptotic cell death of L6 myoblasts. The present study was aimed to elucidate the molecular target(s) of these tyrosine phosphorylations induced by HCRI and demonstrated that simvastatin induces tyrosine phosphorylation of phospholipase C (PLC) γ1. This tyrosine phosphorylation of PLC-γ1 caused the increment of the intracellular inositol triphosphate (IP3) levels in L6 myoblasts. Pretreatment of the cells with herbimycin A, a specific inhibitor of protein tyrosine kinase, inhibited a simvastatin-induced increase in IP3 level in the cells as well as tyrosine phosphorylation of PLC-γ1. Interestingly, pretreatment of the cells with U-73122, a specific inhibitor of PLC, prevented simvastatin-induced cell death. Thus, these results strongly suggest that simvastatin-induced tyrosine phosphorylation of PLC-γ1 plays, at least in part, an important role for the development of simvastatin-induced cell death.

Key words: HMG-coenzyme A reductase inhibitor; Tyrosine phosphorylation; Phospholipase C γ1; Apoptosis; L6 myoblast

1. Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HCR) is known to be a rate limiting enzyme in the biosynthesis of cholesterol. Therefore its inhibitor (HCRI) is a powerful and widely used medicine for the treatment of hypercholesterolemia [1,2]. HCR catalyzes the conversion of HMG-CoA to mevalonate which is an important precursor of all isoprenoids. Isoprenoids serve as an important lipid for the normal post-translational modification of the membrane-associated proteins such as Ras and Rho. Lovastatin, a lipophilic HCRI, is reported to block cell cycling in G1 and G2/M phase progression and therefore suppresses the growth of tumors in vitro [3,4]. Our previous studies have revealed that simvastatin, another lipophilic form of HCRI, but not hydrophilic forms, caused electrical myotonia and muscle cell degeneration in rabbits [5–7]. Although these previous studies disclosed the possible cytotoxicity of these HCRIs to muscle cells, the detailed molecular mechanism remained to be elucidated.

In order to explore the molecular sequences of simvastatin-induced cytotoxicity to muscle cells, we used L6 myoblasts in culture as a model system, because the L6 myoblasts were originally developed from rat thigh muscle [8] and have retained the characteristics of skeletal muscle [9]. We have shown that simvastatin, a lipophilic HCRI, kills L6 myoblasts by involving intracellular Ca^{2+} mobilization from the Ca^{2+} pool in the cells [5–7]. Our subsequent study disclosed that simvastatin inhibited the normal post-translational modification of the Ras protein, and inhibited phosphatidylinositol 3-kinase (PI 3-kinase) activity followed by the apoptotic cell death of L6 myoblasts [10,11]. We further found that simvastatin induced rapid tyrosine phosphorylation responses of cellular proteins, which was thought to be an active signal transducer of cell death [11], although the identity of the target proteins of tyrosine phosphorylation response remains to be determined.

Protein tyrosine phosphorylation response mediated by either protein kinase(s) or protein tyrosine phosphatase(s) has been known to play a pivotal role in the intracellular signal transduction pathway of many extracellular signals for cellular proliferation and differentiation [12]. It has been suggested that protein tyrosine phosphorylation is also involved in the signal transduction of cell death event in blood cancer cells [13].

In this study, we tried to identify the targets of HCRI-induced tyrosine phosphorylation in L6 myoblasts and found that phospholipase C (PLC) γ1 is phosphorylated on tyrosine residue in response to simvastatin treatment with a concomitant increase of inositol triphosphate (IP3) level in the cells. Moreover, pretreatment of the cells with U-73122, a specific inhibitor of PLC, prevented the occurrence of simvastatin-induced cell death in L6 myoblasts. These results strongly suggest that tyrosine phosphorylation of PLC-γ1 and thereby the activation of PLC-γ1 activity is involved, at least in part, in the signal transduction pathway of simvastatin-induced cell death of L6 myoblasts. These results are the first demonstration of the essential roles of the tyrosine phosphorylation and PLC-γ1 activity in the development of muscle cell death.

2. Materials and methods

2.1. Cell culture and treatment

L6 myoblasts (a generous gift from Dr. K. Nakahara, Kagoshima University School of Medicine, Kagoshima, Japan) were cultured as monolayers in culture dishes with Dulbecco's modified Eagle's medium (DMEM) containing 7.5% fetal calf serum (FCS) and 100 U/ml penicillin and 100 μg/ml streptomycin [7]. Cells were treated with various concentrations of simvastatin (a generous gift from Sankyo Pharmaceutical Co. Ltd.) for appropriate periods of time at 37°C.

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PII: S0014-5793(99)00188-X

Control sister cultures were conducted in a similar way except for the absence of simvastatin. In some case, cells were incubated with 5 μM U-73122, a specific inhibitor of PLC, at 37°C for 1 h and then treated with 30 μg/ml simvastatin. For morphological studies, L6 myoblasts were cultured either in 12-well culture plates or on a cover slip at 37°C [14–17].

2.2. Immunoprecipitation and immunoblot analysis

L6 myoblasts were pretreated with or without herbimycin A (10 μM) for 3 h at 37°C, and then exposed to 30 μg/ml simvastatin for the indicated times. After simvastatin treatment, cells were rinsed briefly with chilled phosphate-buffered saline (PBS) containing 1 mM *p*-nitrophenylphosphate and lysed with a lysis buffer (20 mM HEPES, pH 7.2/1% Nonidet P-40/10% glycerol/50 mM NaF/1 mM Na_3VO_4/10 μg leupeptin per ml) as described previously [16,17]. Tyrosine phosphorylated proteins in these cell-free lysates were recovered with an anti-phosphotyrosine (PY)-conjugated protein A-Sepharose. PLC-γ1 was immunoprecipitated with an anti-PLC-γ1 antibody from cell-free lysates (usually 1 mg of protein) as prepared above and analyzed with 7.5% SDS-polyacrylamide gel electrophoresis followed by the immunoblot analysis with either anti-phosphotyrosine (α-PY) or anti-PLC-γ1 antibody as described previously [16,17]. Detection of positive bands in the immunoblot was performed using the ECL detection system (Amersham, UK). Protein concentration of cell-free lysates was determined using the Bradford reagent (Bio-Rad) with γ-globulin as a standard [18].

2.3. Intracellular IP3 measurement

For quantitation of IP3 level in the cells, cells were incubated in the regular medium containing 10 mM LiCl. After 10 min, the medium was quickly aspirated and replaced with regular medium containing 30 μg/ml simvastatin. The cells were incubated at 37°C for the indicated times. In the case of cells pretreated with herbimycin A, cells were pretreated with 10 μM herbimycin A for 3 h, and processed in the same way as described above, in the continuous presence of herbimycin A in the culture medium. The reaction was terminated by the addition of ice-cold 10% perchloric acid and then neutralized by the addition of 75 mM HEPES containing 1.53 M KOH. The solution was kept on ice for 1 h, transferred from dish to microtube and centrifuged at $2000 \times g$ for 15 min at 4°C. The supernatant was then centrifuged at $12\,000 \times g$ for 15 min at 4°C and the final supernatant was subjected to a IP3 assay using a radioreceptor assay kit (Dupont, USA) as described previously [19]. The amount of IP3 in the test samples was calculated from the standard curve using authentic 1,4,5-IP3 as specified in the manufacturer's protocol.

3. Results

3.1. Morphology

We examined the morphology of simvastatin-treated L6 myoblasts by phase-contrast microscopy. In the absence of simvastatin, the cells showed a spindle-shaped appearance with normal nuclei (Fig. 1b) as reported previously [10]. On the other hand, in the presence of simvastatin, the cells showed some intracytoplasmic vacuolations and shrinkage of the cytoplasm (Fig. 1c) at 45 min and (Fig. 1d) at 1 h after the addition. However, pretreatment of the cells with U-73122, a specific inhibitor of PLC, rescued the cells from simvastatin-induced cell death (Fig. 1a).

3.2. Protein tyrosine phosphorylation and immunoblot analysis

Our previous study has shown that simvastatin kills L6 myoblasts by involving intracellular Ca^{2+} mobilization from intracellular Ca^{2+} pool [7]. Therefore, we hypothesized that PLC-γ1 is involved in the signal transduction of simvastatin-induced cell death. To verify this hypothesis, we immunoprecipitated tyrosine phosphorylated proteins from cell-free lysates of simvastatin-treated cells by use of an anti-PY antibody-conjugated protein A-Sepharose. These immunoprecipitates were analyzed with immunoblot analysis and probed with an anti-PLC-γ1 antibody (Fig. 2A). The increasing amount of PLC-γ1 was present in these immunoprecipitates

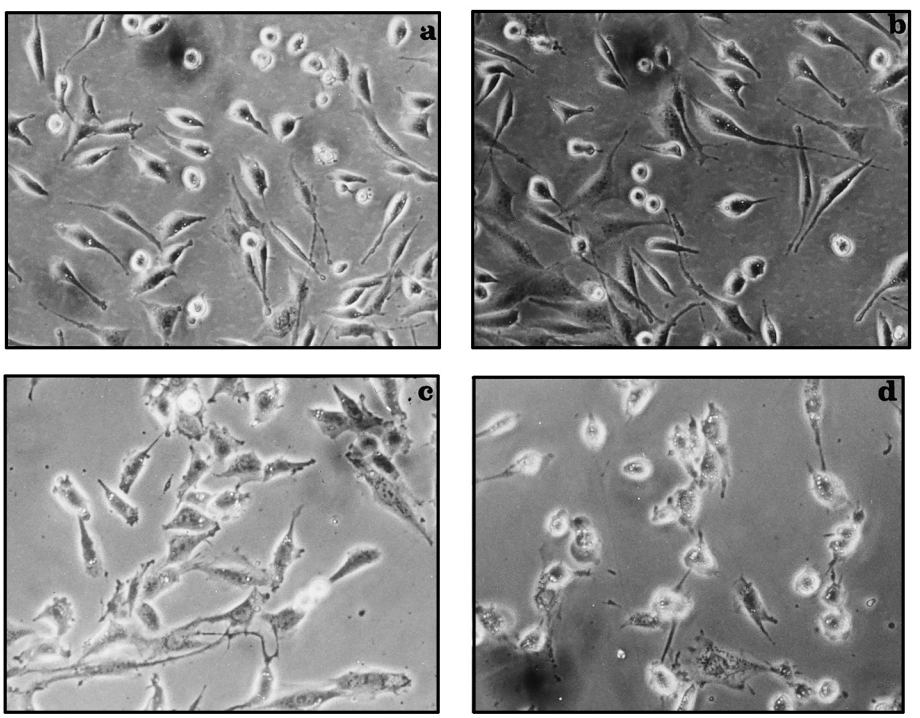

Fig. 1. Effect of U-73122, a specific inhibitor of PLC, on the morphology of simvastatin-treated L6 myoblasts. L6 myoblasts were cultured on a coverslip with regular culture medium and were pre-treated with (a) or without (b–d) 5 μM U-73122 for 1 h at 37°C. Then the cells were incubated with (a, c, d) or without (b) 30 μg/ml simvastatin for 45 min (c) and 60 min (a, d). Phase-contrast micrographs made from typical areas of these cultures. Note that U-73122 shows an inhibitory effect on simvastatin-induced morphological degeneration of the cells.

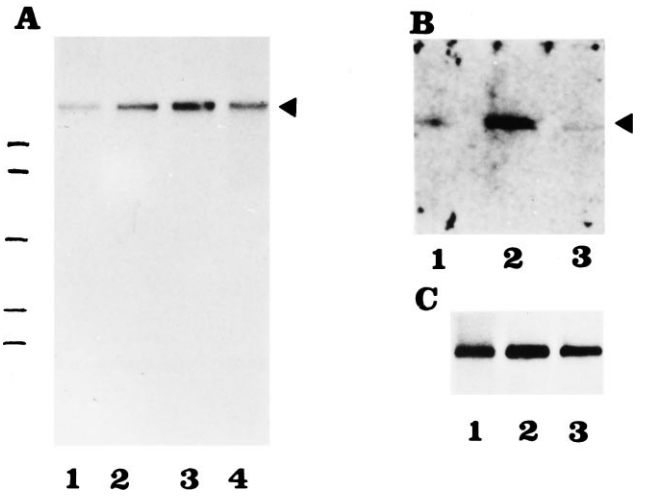

Fig. 2. Simvastatin-induced tyrosine phosphorylation of PLC-γ1. A: L6 myoblasts were treated with (2–4) or without (1) 30 μg/ml simvastatin for 2.5 min (2), 10 min (3), and 30 min (4). Tyrosine phosphorylated proteins were immunoprecipitated with an anti-phosphotyrosine antibody-conjugated protein A-Sepharose (UBI, Lake Placid, NY, USA) from cell-free lysates (normally 1 mg of protein) prepared from the cells as described in Section 2. Then the samples were analyzed with immunoblot, and probed with an anti-PLC-γ1 antibody (Transduction Laboratory, USA). Lines on the left side of the panel represent positions of molecular weight markers (120 kDa, 98 kDa, 56 kDa, 38 kDa, 28 kDa). B: PLC-γ1 was immunoprecipitated with an anti-PLC-γ1 antibody from cell-free lysates (normally 1 mg of protein) of L6 myoblasts pretreated with (3) or without (1, 2) 10 μM herbimycin A for 3 h and then stimulated with (2, 3) or without (1) simvastatin (30 μg/ml) for 10 min. The resultant samples were subjected to the immunoblot probed with an anti-phosphotyrosine antibody. C: The same samples used in B were reprobed with an anti-PLC-γ1 antibody to measure protein amount of PLC-γ1 in the immunoprecipitates.

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in a time-dependent manner. We further verified more directly whether simvastatin treatment induces tyrosine phosphorylation of PLC-γ1. We immunoprecipitated PLC-γ1 from cell-free lysates obtained from L6 myoblasts cultured in the presence or absence of simvastatin and subjected to immunoblot analysis with either an anti-PY antibody or an anti-PLC-γ1 antibody. Simvastatin clearly induced tyrosine phosphorylation of PLC-γ1 as shown in Fig. 2B (compare lanes 1 and 2). Herbimycin A, a specific tyrosine kinase inhibitor, inhibited tyrosine phosphorylation of PLC-γ1 (Fig. 2B, lane 3).

3.3. IP3 formation

To examine whether tyrosine phosphorylation of PLC-γ1 activates its catalytic activity, we measured IP3 level, a prod-

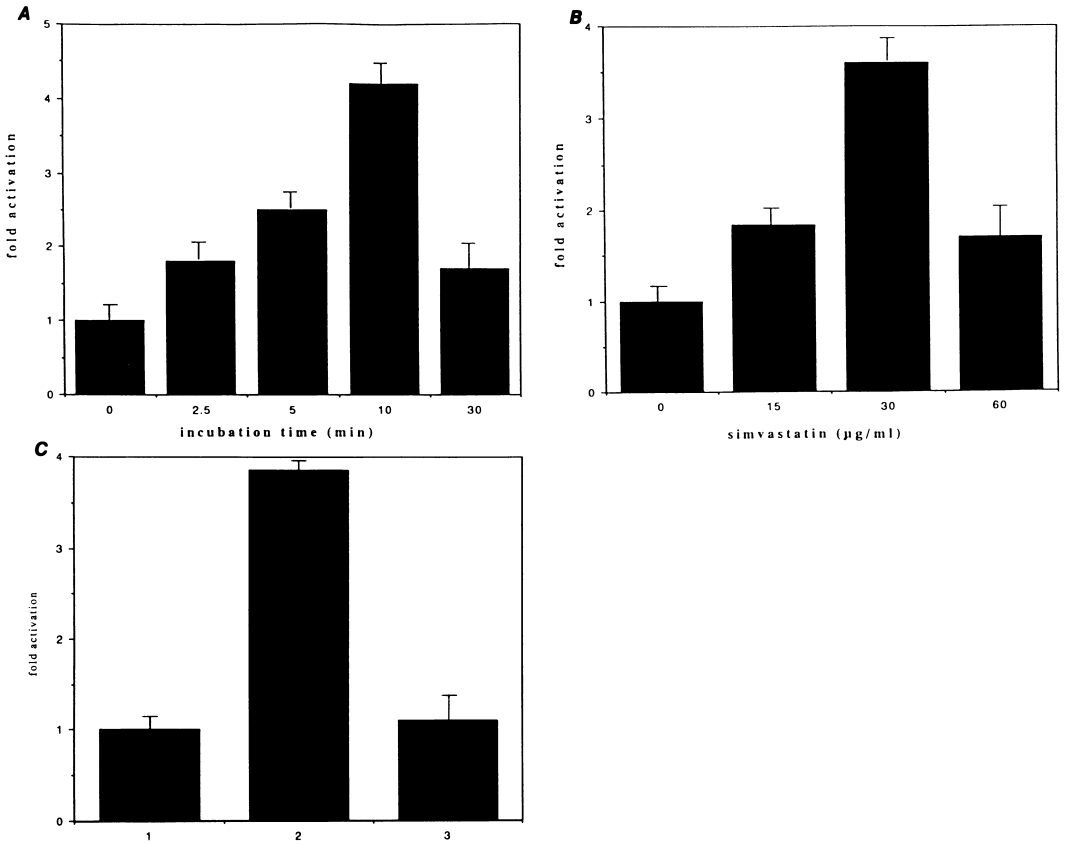

Fig. 3. Simvastatin-induced IP3 formation in L6 myoblasts. L6 myoblasts wre pretreated with 10 mM LiCl for 10 min, then were treated with various concentrations of simvastatin for the indicated times. Intracellular IP3 level was measured as described in Section 2 using radioreceptor assay kit (Dupont New England Nuclear). A: Time course of IP3 production by 30 μg/ml simvastatin. The data were obtained from three different samples with duplicate assay (mean ± S.D.). B: Dose dependence of IP3 production by simvastatin for 10 min incubation. The data were obtained from three different samples with duplicate assay (mean ± S.D.). C: The effect of the herbimycin A pretreatment on simvastatin-induced IP3 production. L6 myoblasts were pretreated with 10 μM herbimycin A for 3 h (3) or without (1, 2) and treated with 30 μg/ml simvastatin (2, 3), then further processed as described in Section 2. The data were obtained from three different samples with duplicate assay (mean ± S.D.). Data represent the percentage against control values (10 pmol/10^6 cells).

uct of the enzymatic reaction of PLC-γ1, in the cells. Intracellular IP3 level was increased with simvastatin-treatment in a dose-dependent and time-dependent manner. Simvastatin clearly upregulated the intracellular IP3 levels up to 4-fold over basal level at 10 min after the addition of simvastatin (Fig. 3A,B). On the other hand, in herbimycin A-pretreated cells, we could not observe any increase in simvastatin-induced IP3 production in the cells (Fig. 3C).

4. Discussion

Our previous studies have shown that lipophilic HCRIs such as simvastatin, but not hydrophilic derivatives, induced muscle cell damage on rabbits and kills L6 myoblasts by involving intracellular Ca^{2+} mobilization [5–7]. The subsequent study has revealed that simvastatin induced tyrosine phosphorylation of cellular proteins and this tyrosine phosphorylation response acts as a signal transducer of simvastatin-induced apoptotic cell death of L6 myoblasts [11]. Such an obligatory role of protein tyrosine phosphorylation in the cell death event has been observed only in blood cells and ovarian tumor cell systems [13,20–22], although the identity of protein tyrosine kinase(s) and the target proteins remains to be elucidated. In this study, we found that PLC-γ1 is a possible target protein of these tyrosine phosphorylation reactions which act as a positive signal transducer for apoptosis. Heretofore, accumulating evidence has suggested that tyrosine phosphorylation of PLC-γ1 resulted in the activation of its enzymatic activity, although this tyrosine phosphorylation of PLC-γ1 is not the only means of activation [23,24]. For example, some kinds of fatty acids and arachidonic acid can activate the enzymatic activity [25]. These activated PLC-γ1 catalyze IP3 formation and thereby induce Ca^{2+} mobilization of the Ca^{2+} pool in the cells. In order to verify that tyrosine phosphorylation of PLC-γ1 actually activates the enzymatic activity, we measured intracellular IP3 level of simvastatin-treated cells. We observed an obvious increase in IP3 production in response to simvastatin treatment correlated with the extent of tyrosine phosphorylation of PLC-γ1. As reported previously, simvastatin induces a rise in the intracellular Ca^{2+} concentration within 10 min of its addition to the culture medium [7]. Tyrosine phosphorylation of PLC-γ1 reached its maximum 10 min after the addition. These time sequences fit well the hypothesis that simvastatin activates PLC-γ1 by its phosphorylation on tyrosine residue(s) and in turn induces Ca^{2+} release from the intracellular Ca^{2+} pool. These results strongly suggest that PLC-γ1 can act as a positive signal transducer of the apoptosis. In fact the pretreatment of the cells with U-73122, a specific inhibitor of PLC, prevented the occurrence of simvastatin-induced apoptotic cell death. Although we cannot exclude a possible involvement of PLC-β in these events, this type of PLC isozyme is reported to be much faster in provoking the Ca^{2+} release from the Ca^{2+} pool in the cells. Recent reports that PLC-γ2 activation by tyrosine phosphorylation is necessary for surface immunoglobulin M-induced B cell apoptosis is consistent with the present results [26].

Thus, these results indicate that a special role of PLC-γ1 in the intracellular signal transducing machinery for apoptotic cell death of muscle cell-derived cell system. We are now exploring the responsible protein tyrosine kinase(s) acting as the initiating signal for apoptosis.

Acknowledgements: We thank Sankyo Pharmaceutical Co. Ltd. for the generous gift of HCRIs, simvastatin and pravastatin. This work was supported in part by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan to T.M.

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