HMG-CoA reductase inhibitor-induced L6 myoblast cell death: involvement of the phosphatidylinositol 3-kinase pathway

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Abstract Our previous studies have shown that the HMG-CoA reductase inhibitor (HCRI) causes rhabdomyolysis and electrical myotonia in rabbits and also kills L6 myoblasts in culture. In the present study, we analyzed the intracellular signal transduction pathway of HCRI-induced cell death using L6 myoblasts as a model system. Here, we report that simvastatin, a lipophilic HCRI, efficiently inhibited isoprenylation of Ras proteins and therefore induced translocation of a significant part of Ras proteins from the membrane fraction into the cytosolic fraction within 10 min. With this translocation, PI 3-kinase activity of the Ras-bound form both in total and in the membrane fraction was also decreased profoundly. Furthermore, various PI 3-kinase inhibitors also caused cell death with morphological changes similar to those caused by simvastatin. These results might represent the molecular events of HCRI-induced cell death, and suggest the significance of PI 3-kinase activity of the Ras-bound form in the maintenance of cell viability.

Key words: Hydroxymethylglutaryl coenzyme A reductase inhibitor; Ras protein; Phosphatidylinositol 3-kinase; L6 myoblast; Signal transduction

1. Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (HCRI) is widely used for the treatment of hypercholesterolemia [1,2]. It has been reported that HCRI exerts adverse effects, such as myopathy or rhabdomyolysis [3–5]. In our in vivo studies using rabbits, we found that simvastatin, a lipophilic HCRI, but not a hydrophilic HCRI, induced electrical myotonia and rhabdomyolysis [6]. Because the rat L6 myoblast cell line is an established tool for the investigation of myogenesis and differentiation into myotubes [7], we used this cell line as a model system for simvastatin-induced damage on muscle cells. We found that a lipophilic but not a hydrophilic HCRI kills the cells by a mechanism involving intracellular Ca^{2+} mobilization [8]. Our subsequent study and others demonstrated that HCRI kills L6 myoblasts by an involving apoptotic cell death pathway (manuscript in preparation and [9]).

Isoprenoids are essential for diverse cellular functions. Mevalonate, a product of HMG-CoA reductase action, is a precursor of all isoprenoids. Lovastatin, a lipophilic HCRI, effectively blocks mevalonate synthesis and thereby protein isoprenylation [10]. Lovastatin has also been found to block cell cycling in G_1 and G_2/M phases of cells grown in vitro [11–14], as well as suppressing growth of tumors in vivo [15,16]. These anti-mitogenic effects of lovastatin are believed to be due to the inhibition of isoprenoid synthesis [14–16]. Although the precise mechanism of these anti-mitogenic effects remains unknown, the inhibition of isoprenylation of the oncogene product p21 Ras and thereby the inhibition of the Ras-mediated intracellular signaling pathway have been thought to be responsible for these observed effects [14,16].

Another cardinal intracellular signaling pathway for proliferation and survival is the phosphatidylinositol 3-monosphosphate kinase (PI 3-kinase)-mediated pathway [17–19]. In neuronal cells, this activity plays an important role in the prevention of apoptotic cell death [19].

In this study, we examined the signal transduction pathway of simvastatin-induced cell death in L6 myoblasts. We found that simvastatin disturbed the posttranslational modification of Ras proteins. This altered posttranslational modification of Ras proteins seems to cause the inhibition of PI 3-kinase activity in the Ras-bound form. These decrements in PI 3-kinase activity by HCRI appeared to be well correlated with cell death, because wortmannin and LY294002, specific inhibitors of PI 3-kinase activity [20–22], also induced L6 myoblast cell death with morphological alterations similar to those caused by simvastatin. These results suggest that basal PI 3-kinase activity, especially the Ras-bound form, might serve as a sensor of the initiation of the apoptotic cell death program in the muscle cell system. When this form of PI 3-kinase activity is lowered to below the threshold level, the cells might commit suicide in such a cell system.

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 DMEM containing 7.5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were treated with various concentrations of simvastatin (a generous gift from Sankyo Pharmaceutical Co., Ltd., Tokyo, Japan) for appropriate periods of time at 37°C. For morphological studies, L6 myoblasts were cultured in 12-well culture plates. Cells were then treated with 30 μg/ml simvastatin as well as PI 3-kinase inhibitors (100 nM wortmannin or 2 μM LY294002) for 1 h at 37°C. Moreover, 1 mM mevalonic acid, a metabolite of HMG-CoA reductase, was added to the cells together with simvastatin, and cells were similarly treated for 1 h at 37°C. These cells were examined by phase contrast microscopy and photographs of typical areas of each experiment were taken [23].

2.2. Survival rate of the cells

To examine the effect of the addition of mevalonic acid to the

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; SDS, sodium dodecyl sulfate; PI 3-kinase, phosphatidylinositol 3-monophosphate kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethyl sulfoxide; ATP, adenosine-5′-triphosphate

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culture medium on the survival rate of the cells, cells were treated with 1 mM mevalonic acid together with 30 μg/ml simvastatin. After various times of incubation, cells were stained with 0.1% trypan blue solution for 10 min at 37°C. Photographs of all areas of each sample were taken and the percentage of dead cells (stained) was calculated.

2.3. Immunoprecipitation and immunoblot analysis

After treatment, cells were rinsed with ice-cold PBS (phosphate-buffered saline) and lysed with lysis buffer (1% Nonidet P-40, 25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1 mM sodium vanadate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin) for 20 min on ice as previously described [24]. Insoluble materials were removed by centrifugation at 12 000 rpm for 2 min at 4°C. In each experiment, the amount of cell-free lysates was normalized by protein content [25]. The lysate was incubated with an appropriate amount of anti-pan-Ras antibody (Oncogene Research Products, USA) with constant agitation at 4°C, and Ras proteins were recovered by incubating with protein G-agarose. The beads were washed 3 times with lysis buffer and once with TBS (Tris-buffered saline), and suspended with the kinase reaction buffer (50 mM HEPES, pH 7.4, 20 mM $MnCl_2$, 5 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.1 mM sodium vanadate). For immunoblot analysis, the washed immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis. Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed with an anti-pan-Ras antibody and a horseradish peroxidase-conjugated anti-mouse antibody using an enhanced chemiluminescence (ECL) detection system (Amersham, UK) [24].

2.4. Subcellular fractionation

L6 myoblasts were homogenized using a glass homogenizer with homogenization buffer (40 mM Tris-HCl, pH 7.5 containing 10 mM $MgCl_2$, 2 mM $CaCl_2$, 250 mM sucrose, and 1 mM PMSF). Membrane and cytosolic fractions were obtained by centrifugation at $200\,000 \times g$ for 35 min in a Beckman TLA 100 [26].

2.5. Measurement of PI 3-kinase activity

The total cell-free lysate and the membrane fraction obtained as

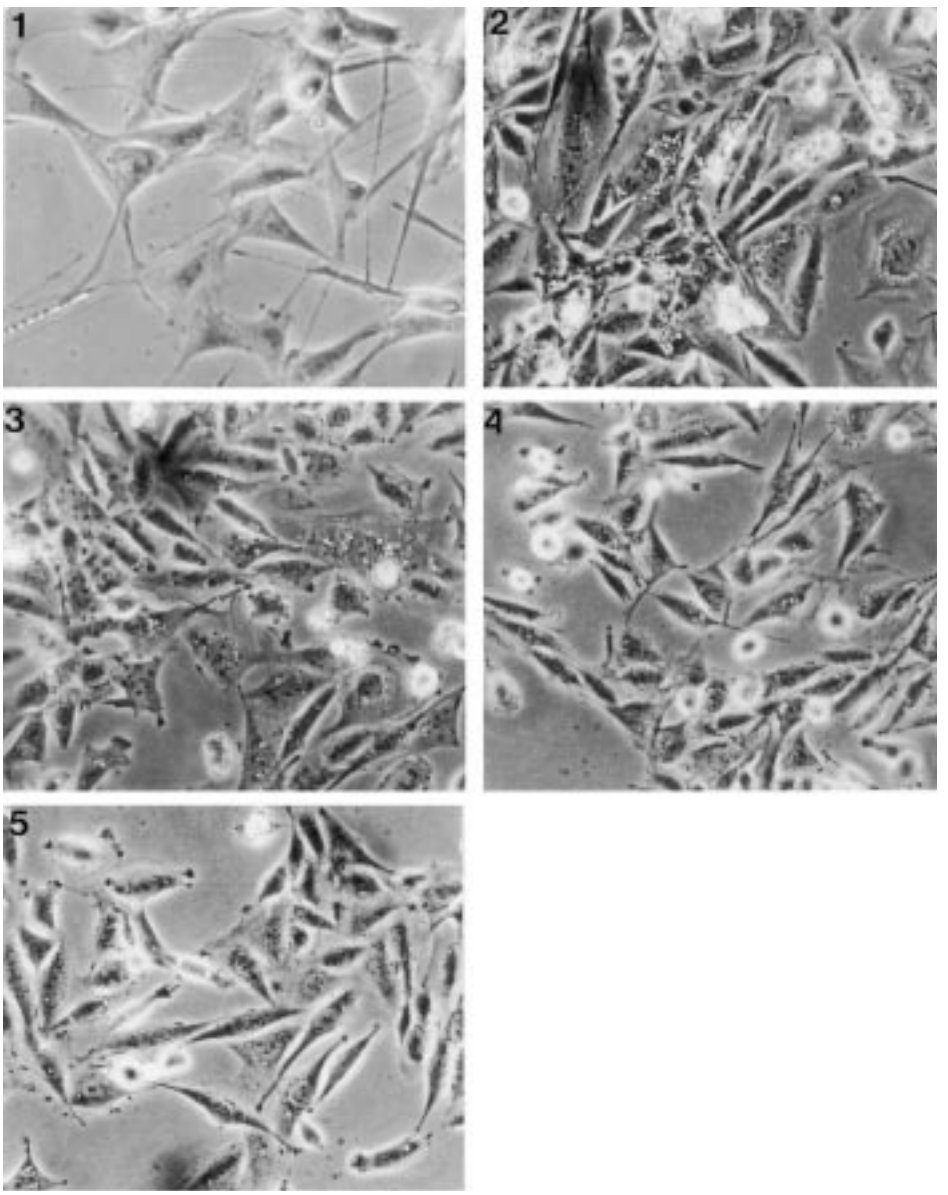

Fig. 1. The morphology of L6 myoblasts treated with simvastatin, as well as with wortmannin or LY294002, two specific inhibitors of PI 3-kinase under phase-contrast microscopy. L6 myoblasts were cultured as described in Section 2 and exposed to 30 μg/ml simvastatin (2), 100 nM wortmannin (3), 2 μM LY294002 (4), or untreated (1) for 1 h at 37°C. Both simvastatin and PI 3-kinase inhibitor-treated cells show cytoplasmic vacuolations and shrinkage of the cytoplasm. We also examined morphological changes in cells treated with 1 mM mevalonic acid together with simvastatin (5). The figure represents typical areas of three independent experiments. Magnification ×400.

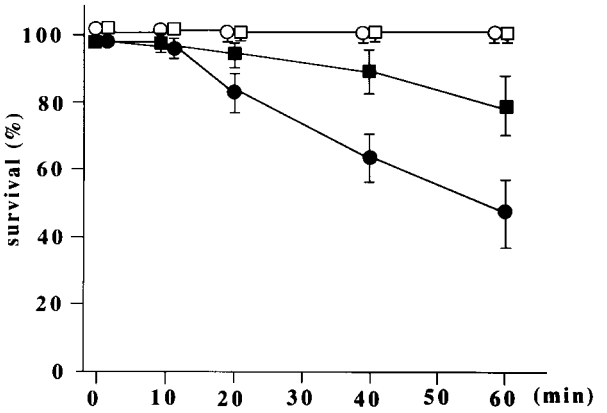

Fig. 2. Effect of simvastatin on the survival of L6 myoblasts. Cells were treated with simvastatin and/or simvastatin plus mevalonic acid and stained with 0.1% trypan blue solution to calculate the cell survival rate. White dots represent control cells, black dots represent cells treated with 30 μg/ml simvastatin, while white squares represent cells treated with 1 mM mevalonic acid alone and black squares represent cells treated with 1 mM mevalonic acid together with simvastatin.

described above were incubated with an anti-pan-Ras antibody. PI 3-kinase activity of these Ras immunoprecipitates was assayed as previously described [27]. Briefly, Ras immunoprecipitates were preincubated in 50 μl of kinase reaction buffer at room temperature for 10 min using phosphatidylinositol (PI) as substrate. The assay was initiated by the addition of 5 μl of ATP solution (80 μM ATP and 2 mCi/ml [γ-^{32}P]ATP) and incubated at 30°C for 20 min. The reaction was stopped by the addition of 100 μl of chloroform-methanol-11.6 N HCl (50:100:1). After centrifugation, the lower organic phase was taken for the analysis by thin layer chromatography (TLC) on silica gel plates (Polygram SIL N-NR, Macherey-Nagel, Germany) and the TLC plates were developed in chloroform-methanol-25% ammonium hydroxide-water (43:38:5:7). The plates were exposed to X-ray film at −70°C with an intensifying screen for 16–24 h.

3. Results and discussion

Simvastatin, as well as PI 3-kinase inhibitors, caused massive vacuolations in the cytoplasm and shrinkage of the cell body at 1 h after its addition to the culture medium, as illustrated in Fig. 1. On the other hand, the addition of mevalonic acid to the culture medium partially blocked these simvastatin-induced morphological changes. These data ware further confirmed by trypan blue dye exclusion experiments. Only 48% of the cells treated with 30 μg/ml simvastatin for 60 min survived, whereas 74% of cells treated with mevalonic acid together with simvastatin survived. Mevalonic acid under the present condition was not toxic to L6 myoblasts (Fig. 1 (5) and Fig. 2). These data suggest the possible involvement of HCRI-impaired mevalonate synthesis in the development of cell death.

We next tried to elucidate the details of the intracellular signaling pathway of the simvastatin-induced cell death. We found that simvastatin rapidly inhibited normal maturation of Ras proteins (Fig. 3). It caused the accumulation of non-prenylated premature Ras in the cytosolic fraction within 10 min after its addition to the culture medium (Fig. 3B). HCRI inhibits the synthesis of isoprenoids, which is important for the isoprenylation of many proteins, including Ras proteins. The isoprenylation of Ras proteins is thought to be an essential

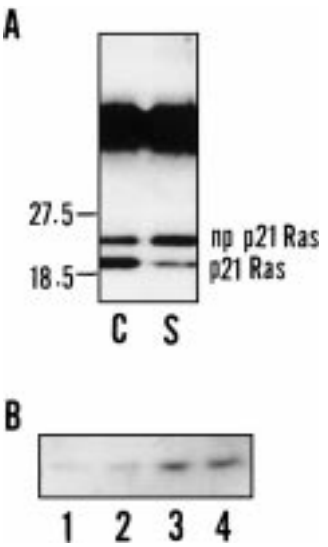

Fig. 3. Effect of simvastatin on the association of Ras proteins with the plasma membranes. A: L6 myoblasts were treated with 30 μg/ml simvastatin for 10 min (S) or untreated (C). Then, Ras proteins were immunoprecipitated with an anti-pan-Ras antibody from total cell-free lysates, and subjected to immunoblot analysis with the same antibody. The positions of p21Ras and non-processed p21Ras (np p21Ras) are indicated on the right, and those of molecular weight standards (in kDa) on the left. B: L6 cells were treated with 30 μg/ml simvastatin for 5 min (2), 10 min (3), 30 min (4), or untreated (1). Then, cells were homogenized with homogenization buffer and fractionated as described in Section 2. 20 μl of cytosolic fraction (700 μl) was boiled with SDS sample buffer and subjected to the immunoblot analysis with an anti-pan-Ras antibody.

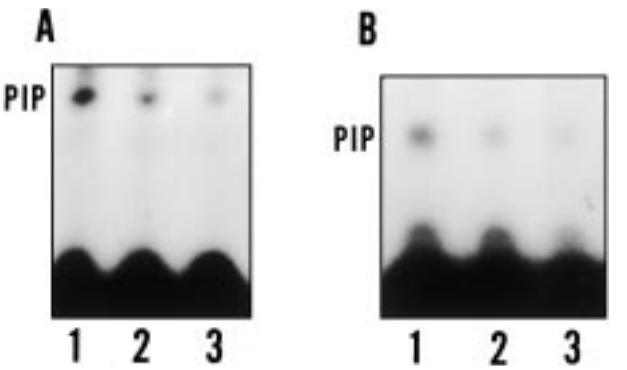

Fig. 4. Effects of simvastatin on PI 3-kinase activity in pan-Ras immunoprecipitates. A: PI 3-kinase activity in pan-Ras immunoprecipitates obtained from total cell-free lysates of the cells cultured in the presence (2: 5 min, 3: 10 min) or absence (1) of 30 μg/ml simvastatin. PI 3-kinase activity was measured as described in Section 2. PIP represents phosphatidylinositol phosphate. B: PI 3-kinase activity in pan-Ras immunoprecipitates prepared from the membrane fraction. L6 cells were treated with 30 μg/ml simvastatin for 5 min (2), 10 min (3), or untreated (1). Then, cells were homogenized with homogenization buffer and fractionated into cytosolic and membrane fractions by ultracentrifugation, as described in Section 2. After subcellular fractionation, pellets (the membrane fraction) were lysed with lysis buffer for 20 min on ice, and Ras proteins were immunoprecipitated with an anti-pan-Ras antibody. The immunoprecipitates from the membrane fractions were assayed for PI 3-kinase activity as described in Section 2. The radioactivity in each PIP spot obtained by Cerenkov counting was follows: A1, 329 ± 26 cpm; A2, 135 ± 17 cpm; A3, 70 ± 13 cpm; B1, 283 ± 28 cpm; B2, 113 ± 14 cpm; B3, 61 ± 7 cpm (triplicate assay; mean ± S.D.).

step in posttranslational modification for targeting Ras proteins to the plasma membrane [28,29].

We next measured PI 3-kinase activity in the Ras immunoprecipitates prepared from total cell-free lysates (Fig. 4A) and from the membrane fraction of the cells (Fig. 4B), because a previous study had shown that PI 3-kinase can be regulated by the direct binding of the p110 catalytic subunit of PI 3-kinase to the active form of Ras proteins [30]. The kinase activity in Ras immunoprecipitates prepared from total cell-free lysates and from the membrane fraction of the cells was decreased drastically with simvastatin treatment (Fig. 4A,B). This decrease in PI 3-kinase activity is not due to the loss of cell viability, since none of the cells was stained with trypan blue at 10 min after the addition of simvastatin. At this point, we should bear in mind that naive L6 myoblasts exhibit an appreciable high basal PI 3-kinase activity presenting as a Ras-bound form under the present experimental conditions. PI 3-kinase activity in Ras immunoprecipitates prepared from the cytosolic fraction of naive cells was hardly detected (data not shown). Therefore, it is reasonable to assume that in normal dividing cells, a part of PI 3-kinase activity might be present in a Ras-bound form, especially localized in the membrane fraction. These results suggest that simvastatin inhibits the normal targeting of Ras proteins and therefore caused a decrement of Ras proteins on the plasma membrane by decreasing isoprenylation. Therefore, PI 3-kinases might lose their chance to bind to Ras proteins at the plasma membrane, which in turn results in a reduction of the kinase activity. Although we cannot exclude the possibility of a direct effect of simvastatin on PI 3-kinase activity in vivo, simvastatin did not affect PI 3-kinase activity in vitro (data not shown). Thus, the present results strongly suggest that at least in L6 myoblasts, basal PI 3-kinase activity of the Ras-bound form, which is localized in the membrane fraction, is important for cell survival. Failure of this pathway might, at least in part, play an important role in the development of cell death.

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