Tyrosine phosphorylation of the catalytic subunit p110 of phosphatidylinositol-3 kinase induced by HMG-CoA reductase inhibitor inhibits its kinase activity in L6 myoblasts

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Abstract Previous studies from this laboratory have shown that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (HCRI) causes apoptotic cell death of a muscle cell-derived cell line, L6 myoblasts, by involving the phosphatidylinositol-3 (PI-3) kinase pathway and tyrosine phosphorylation of several cellular proteins, although the relationship between PI-3 kinase pathway and tyrosine phosphorylation responses remained to be elucidated. Here, we show that HCRI induces tyrosine phosphorylation of catalytic subunit p110 of PI-3 kinase as early as 5 min after addition of HCRI into culture medium. We could not detect the tyrosine phosphorylation of the regulatory subunit p85 of PI-3 kinase under the present experimental conditions. Concomitantly, the kinase activity toward PI in p110 immunoprecipitates was decreased with a similar time course. Furthermore, both herbimycin A and genistein, potent inhibitors of tyrosine kinase activity, inhibited HCRI-induced inhibition of PI-3 kinase activity as well as HCRI-induced apoptotic cell death. Once the catalytic subunit p110 becomes tyrosine-phosphorylated, the regulatory subunit p85 appears to be dissociated from the catalytic subunit, because we observed a decreasing amount of p85 regulatory subunits in p110 immunoprecipitates in response to HCRI treatment. These results strongly suggest the novel function of tyrosine phosphorylation of catalytic subunit p110 of PI-3 kinase in the regulation of its kinase activity. The tyrosine phosphorylation of these catalytic subunits may play an important role in the intracellular signal transduction of apoptotic cell death. To our knowledge, this is the first report that tyrosine phosphorylation of p110 catalytic subunit acts as a negative regulator of its kinase activity.

Key words: apoptosis; tyrosine phosphorylation; PI-3 kinase; p110 subunit; HMG-CoA; reductase inhibitor

1. Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is a rate-limiting enzyme for the production of cholesterol and its inhibitor (HCRI) is now widely used for the treatment of hypercholesterolemia [1,2]. HCRI catalyzes the conversion of HMG-CoA to mevalonate that is an important precursor of all isoprenoids. Isoprenoids serve as an important lipid for the normal translational modification of the membrane-associated proteins such as Ras and Rho. It has been reported that HCRI exerts adverse effects such as myopathy, rhabdomyolysis, suicidal tendency, and neurosis [3–7]. Our previous studies have shown that some HCRIs provoked apoptotic cell death of a muscle cell-derived cell line, L6 myoblasts in culture, which is involved in tyrosine phosphorylation of several cellular proteins and the phosphatidylinositol-3 (PI-3) kinase-dependent pathway [8,9]. HCRI induced tyrosine phosphorylation of several cellular proteins and this tyrosine phosphorylation seemed to act as a positive signal transducer for apoptotic cell death in L6 myoblasts, because tyrosine kinase inhibitors such as herbimycin A and genistein prevent HCRI-induced cell death [8]. In the follow up study, we identified one of these tyrosine-phosphorylated target proteins as phospholipase γl [10]. On the other hand, we also examined the possible involvement of the Ras-dependent pathway in HCRI-induced apoptotic cell death, because as described above HCRI is reported to inhibit the production of isoprenoid derivatives in addition to cholesterol synthesis [11]. Ras protein is known to be isoprenylated and anchored to the plasma membrane to do its job during many cellular events. We found that HCRI indeed prevented normal maturation of Ras protein and it induced the inhibition of PI-3 kinase activity of the Ras-bound form resulting in the translocation of its kinase activity from membrane fraction to cytosolic fraction, which has been reported to be one of the most important anti-apoptotic signal transducers in several cell systems [9].

PI-3 kinase is a cytosolic heterodimerized protein consisting of p110 catalytic subunit (α and β isoforms) and p85 regulatory subunit (α and β isoforms). It catalyzes the formation of the family of phosphoinositides with phosphate at the D-3 position of the inositol ring [12–14]. This kinase contains several motifs by which it can associate with cytoplasmic tyrosine kinase receptors or membrane-bound receptor-type tyrosine kinases forming signaling complexes. Although the entire scheme of the mechanism of the activation of this kinase activity still remained to be extensively studied, several mechanisms of the activation have been reported. One mechanism is a small GTP-binding protein, Ras protein-dependent pathway. PI-3 kinase can be associated with the active form of the Ras protein, which results in the activation of its kinase activity [15]. It could be due to an allosteric activation by its binding to the Ras protein. The other is the association with activated receptor-type tyrosine kinase through SH-2 domain

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of the p85 regulatory subunit. SH-2 domain-mediated binding of PI-3 kinase to multiple receptors has been demonstrated for the platelet-derived growth factor receptor [16], CD28 [17], and interleukin-2 receptor [18]. This type of association is known to help this enzyme to be translocated near its lipid substrates and to result in the activation of PI-3 kinase activity. Furthermore, the primary amino acid sequences of the p85 and p110 subunits also show several putative sites for tyrosine phosphorylation [12,19,20]. A direct relationship between the tyrosine phosphorylation of PI-3 kinase (p85 or p110 subunits) and an activation of its kinase activity, however, has not been clearly demonstrated. A recent report by Mazerolles and Fisher demonstrated that binding of CD4 ligands induces tyrosine phosphorylation of PI-3 kinase p110 subunit, although they did not address the role of tyrosine phosphorylation of p110 subunit of PI-3 kinase in the regulation of the kinase activity [21].

Here, we show that HCRI induces a tyrosine phosphorylation of the p110 catalytic subunit of PI-3 kinase but not p85 regulatory subunit resulting in the inhibition of PI-3 kinase activity. Furthermore, once the p110 subunit of PI-3 kinase gets tyrosine-phosphorylated, p85 subunit seems to become dissociated from p110 subunit. This dissociation of p110 from p85 subunit could result in the abnormal translocation of PI-3 kinase from the membrane to the cytosolic fraction.

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 containing 7.5% fetal calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin [8–10]. Cells were treated with various concentrations of simvastatin (a generous gift from Sankyo Ltd., Tokyo, Japan) for an appropriate period of time at 37°C. Control sister cultures were conducted in a similar way except for the absence of simvastatin. In some case, cells were preincubated with 10 μM herbimycin A (BioMol, USA) or genistein (75 μM) (Extrasynthese, France), potent inhibitors of tyrosine kinase activity for 3 h before the addition of simvastatin into culture medium as described previously [8].

2.2. Immunoprecipitation and immunoblot analysis

L6 myoblasts were exposed to 30 μg/ml simvastatin for indicated times. After stimulation with simvastatin, 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/ml leupeptin) as described previously [22]. Non-specific binding was eliminated by preclearing cell-free lysates with protein A-agarose in the cold room for 1 h. The resultant supernatant (normally 1 mg/ml) was used for the immunoprecipitation either with an anti-p110 catalytic subunit of PI-3 kinase (α-p110, Santa Cruz Laboratories, USA) or with an anti-p85 α regulatory subunit (α-p85, Santa Cruz Laboratories, USA) as described previously [9]. These immunoprecipitates were subjected to Western blotting and probed with an anti-phosphotyrosine antibody (α-PY, Upstate Biotechnology, Lake Placid, NY, USA), α-p110, and α-p85 antibody. Detection of positive bands in the immunoblot was performed using the ECL detection kit (New England Biolab, Inc., MA, USA). Protein concentration of cell-free lysate was determined using the Bradford reagent (Bio-Rad, USA) with γ-globulin as a standard [23].

2.3. Measurement of PI-3 kinase activity in crude homogenates and immunoprecipitates

PI-3 kinase activity was measured in crude homogenates and p110 immunoprecipitates using PI as a substrate as described previously [9]. Briefly, crude cell homogenates or p110 immunoprecipitates were preincubated in 50 μl of the kinase reaction buffer at room temperature for 10 min using PI as substrate. The assay is 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 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, which were developed in chloroform–methanol–2.5% ammonium hydroxide–water (43:38:5:7, v/v/v/v). The plates were exposed to X-ray film at −70°C. We scraped each spot from TLC plates and measured the radioactivity by Cerenkov counting [9].

3. Results and discussion

3.1. PI-3 kinase activity in crude homogenates

L6 myoblasts were incubated with 30 μg/ml simvastatin for 5 or 10 min. Then, the cells were harvested with chilled PBS and were homogenized with 20 mM HEPES, pH 7.4, containing 137 mM NaCl, 0.1 mM sodium vanadate, 1 mM PMSF, 1 mM EDTA, and 1 mM EGTA. PI-3 kinase activity was measured with PI as a substrate as described in Section 2. Simvastatin caused an inhibition of PI-3 kinase activity in crude homogenates as early as 5 min after its addition in the culture medium (Fig. 1-I).

3.2. PI-3 kinase activity in α-p110 immunoprecipitates

Cells were treated with simvastatin (30 μg/ml) and p110 subunit of PI-3 kinase was immunoprecipitated with α-p110. The resultant p110 immunoprecipitates were subjected to PI-3

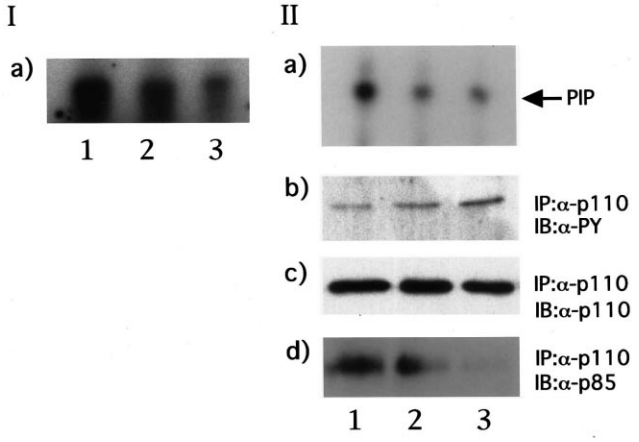

Fig. 1. Effects of simvastatin on PI-3 kinase activity in crude homogenates (I) and p110 immunoprecipitates (II). I: PI-3 kinase activity in crude homogenates as prepared in Section 2 from cells cultured in the presence (2: 5 min, 3: 10 min) or absence (1) of 30 μg/ml simvastatin. PI-3 kinase activity was measured with an equal amount of protein samples as described in Section 2. II: (a) PI-3 kinase activity in p110 immunoprecipitates prepared from cell-free lysates obtained from cells treated with 30 μg/ml simvastatin. PI-3 kinase treated for 5 min (2) and 10 min (3) or untreated (1) was assayed with PI as a substrate as described in Section 2. PIP represents PI phosphate. The radioactivity in each spot corresponding to PIP obtained by Cerenkov counting was as follows: I-1, 887 ± 45 cpm; I-2, 412 ± 25 cpm; I-3; 221 ± 19 cpm; II-1, 359 ± 22 cpm; II-2, 185 ± 27 cpm; II-3, 102 ± 14 cpm (triplicate assay; mean ± S.D.). (b) Immunoblot analysis of the same samples used for II-a probed with α-PY to check tyrosine phosphorylation of p110 subunit. (c) Western blot analysis of p110 immunoprecipitates used for II-b was reprobed after stripping the first antibody (α-PY) with α-p110. Note the equal amount p110 subunit protein present in each immunoprecipitate. (d) Immunoblot analysis of p110 immunoprecipitates prepared from cells cultured in the presence of simvastatin was evident with simvastatin treatment.

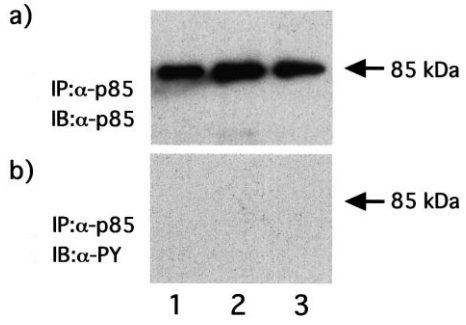

Fig. 2. p85 regulatory subunit was not tyrosine-phosphorylated in response to simvastatin treatment. L6 myoblasts were treated with 30 μg/ml simvastatin for 5 min (2) and 10 min (3) or untreated (1). Then, p85 subunit was immunoprecipitated with α-p85 (a) or α-PY (b). Simvastatin treatment did not induce any tyrosine phosphorylation of p85 subunit of PI-3 kinase.

kinase assay as described above. PI-3 kinase activity recovered in p110 immunoprecipitates was decreased progressively by simvastatin treatment with a similar time course to that in crude homogenates (Fig. 1-IIa).

3.3. *Tyrosine phosphorylation of p110 subunit of PI-3 kinase in response to simvastatin treatment*

p110 immunoprecipitates as prepared above were subjected to immunoblot analysis with α-PY. p110 subunit of PI-3 kinase was time-dependently tyrosine-phosphorylated in response to simvastatin treatment (Fig. 1-IIb). There is no correlation between time-dependent tyrosine phosphorylation and the amount of protein present in each immunoprecipitate (Fig. 1-IIc). We next examined the amount of p85 subunit of PI-3 kinase in these immunoprecipitates. The result showed progressive decrement of p85 protein in these immunoprecipitates with the same time course of the reduction of the kinase activity (Fig. 1-IId).

3.4. *Tyrosine phosphorylation of p85 subunit of PI-3 kinase by simvastatin treatment*

We also examined whether or not p85 subunit of PI-3 kinase is also tyrosine-phosphorylated in response to simvastatin treatment. We immunoprecipitated p85 subunit from cell-free lysates prepared from cells treated with simvastatin as described above and these immunoprecipitates were immunoblotted with α-PY. The results showed that we could not observe any tyrosine phosphorylation response of p85 subunit to simvastatin treatment as shown in Fig. 2a,b.

3.5. *Effects of tyrosine kinase inhibitors on simvastatin-induced PI-3 kinase activity recovered in α-p110 immunoprecipitates*

Then, we examined the effects of tyrosine kinase inhibitors on PI-3 kinase activity in p110 immunoprecipitates (Fig. 3). Herbimycin A pretreatment caused a significant inhibition of simvastatin-induced reduction of PI-3 kinase activity (Fig. 3). Moreover, genistein, another tyrosine kinase inhibitor, also gave the same results (Fig. 3). As reported previously, these tyrosine kinase inhibitors also rescued the cells from apoptotic cell death induced by simvastatin [8].

These results are consistent with the notion that tyrosine phosphorylation of p110 subunit of PI-3 kinase negatively regulates its kinase activity. This tyrosine phosphorylation might also cause some change in the binding affinity of p110 subunits of PI-3 kinase to p85 subunit, resulting in the dissociation of p85 from p110 subunit when p110 subunit is tyrosine-phosphorylated induced by simvastatin treatment. As mentioned earlier, tyrosine phosphorylation induced by PI-3 kinase p110 subunit can be induced by CD4 ligands binding [21]. On the other hand, these authors speculated that tyrosine phosphorylation of p110 subunit activates the kinase activity, although they did not show any direct data on PI-3 kinase activity. Although we do not know the reason for this discrepancy at present, different tyrosine residues of p110 subunit might be phosphorylated in response to different stimuli employed in each experiment. Moreover, they also presented the data that this tyrosine phosphorylation of p110 subunit did not cause any change in the association with p85 subunit regulatory subunit of PI-3 kinase. On the contrary, we clearly observed the dissociation of tyrosine-phosphorylated p110 induced by simvastatin treatment from p85 subunit, although there was no obvious tyrosine phosphorylation of p85 subunit in both reports. Several reports have suggested that the activation of PI-3 kinase involves tyrosine phosphorylation [24–26]. For example, it has been reported that treatment of purified bovine brain PI-3 kinase with tyrosine phosphatase decreases its kinase activity [24]. These reports have suggested the positive role of tyrosine phosphorylation of PI-3 kinase in the regulation of its activity. Therefore, the present data suggest for the first time the negative role of tyrosine phosphorylation of p110 subunit of PI-3 kinase in regulating the activity. The entire scheme of HCRI-induced apoptotic cell death of L6 myoblasts is now apparent. HCRI treatment causes tyrosine phosphorylation of several cellular proteins including PLC-γ1 and PI-3 kinase, the latter of which in turn causes the dissociation of p110 subunit from p85 regulatory subunit. These sequential events would cause the decrement of PI-3 kinase activity in cells, which is well known to be the strong

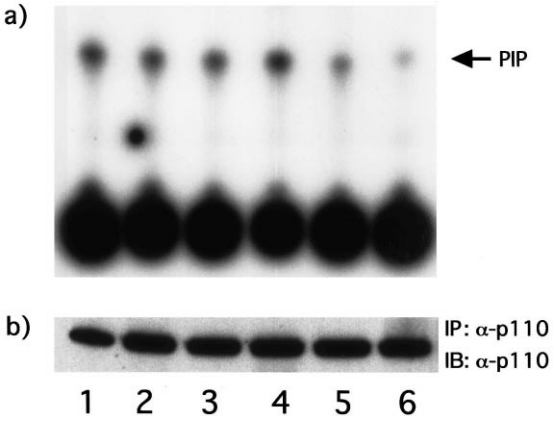

Fig. 3. PI-3 kinase activity in p110 immunoprecipitates prepared from cells pretreated with tyrosine kinase inhibitors herbimycin A (1, 2) and genistein (4, 5) as described in Section 2 and treated with 30 μg/ml simvastatin for 10 min (1, 5, 6) or untreated (3). a: PI-3 kinase activity was measured with p110 immunoprecipitates as described in Section 2. PIP indicates the product of the enzymatic action of PI-3 kinase. The radioactivity in each PIP spot obtained by Cerenkov counting was as follows: 1432 ± 14 cpm; 2428 ± 21 cpm; 3429 ± 25 cpm; 4456 ± 23 cpm; 5302 ± 19 cpm; 684 ± 33 cpm (duplicate assay; mean ± S.D.). b: Western blot analysis of p110 immunoprecipitates used for (a) probed with α-p110. Note the same amount of p110 subunit protein present in each immunoprecipitate.

anti-apoptotic signaling molecule. Further experiments need to be performed to elucidate the exact site(s) of tyrosine phosphorylation in p110 subunit and the mechanism of the dissociation of p85 subunit from the tyrosine-phosphorylated p110 subunit of PI-3 kinase.

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