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Lipid products of phosphoinositide 3-kinase abrogate genistein-induced fusion inhibition in myoblasts

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Abstract

Genistein (4′,5,7-trihydroxyisoflavone) is a tyrosine kinase inhibitor. Although the agent has shown to inhibit myoblast differentiation, neither intracellular target(s) as a tyrosine kinase inhibitor nor action mechanism of the agent is well known. Here we studied the effect of genistein on the differentiation of myoblasts. Genistein strongly but reversibly blocked both myoblast fusion and synthesis of the muscle-specific proteins. The agent also reversibly reduced the phosphorylation level of focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase, and its interaction with p85, the regulatory subunit of phosphoinositide 3-kinase (PI3-kinase). In addition, genistein indirectly inhibited PI3-kinase activity and blocked calcium influx which is required for myoblast fusion. However, both genistein-induced inhibition of cell fusion and calcium influx were abrogated by the lipid products of PI3-kinase. These results demonstrate that genistein can exert their effect on the signaling pathway from FAK to calcium influx via PI3-kinase in the differentiation of myoblasts.

Keywords: Genistein; PI-3,4,5-P_3 (dipalmitoyl L-α-phosphatidyl-D-myo-inositol 3,4,5-triphosphate); Myoblast differentiation; FAK (focal adhesion kinase); PI3-kinase (phosphoinositide 3-kinase); Ca^{2+} influx

1. Introduction

Myoblasts are a very useful model system to study cell growth and differentiation because embryonic myogenic cells can be easily cultivated and they show a variety of biochemical as well as morphological characteristics. Differentiation of skeletal muscle cells is characterized by a well-defined sequence of events, including irreversible withdrawal of proliferating myoblasts from the cell cycle, fusion of plasma membranes to form multinucleated myotubes, and the induction of muscle-specific gene expression (Wakelam, 1985; Fishman, 1986). The probability that myoblasts will withdraw from the cell cycle and initiate terminal differentiation is highly sensitive to environmental cues, including growth factors and extracellular matrix components (Sastry et al., 1999).

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that localizes to focal adhesions and that may play an important role in the integration of signals from integrins and growth factors (Zachary and Rozengurt, 1992; Hanks and Polte, 1997; Parsons et al., 2000; Schaller, 2001). One of the early events during integrin signaling is the tyrosine autophosphorylation of FAK (Schaller et al., 1994; Parsons, 1996), which represents a key step in the assembly of focal adhesion (Craig and Johnson, 1996). The phosphorylation of FAK is believed to initiate a cascade of subsequent phosphorylation events and new protein interactions required for adhesion-dependent signaling complexes.

The major site of FAK phosphorylation, Tyr397, serves as a binding site for the SH2 domains of Src kinase (Schaller et al., 1994; Eide et al., 1995) and phosphoinositide 3-kinase (PI3-kinase) (Chen et al., 1996). In platelet-derived growth factor (PDGF) signaling of NIH 3T3 cells, PI3-kinase is recruited to the plasma membrane and associates with FAK (Chen and Guan, 1994). Activation of PI3-kinase is involved in mitogenesis, inhibition of apoptosis, intracellular vesicle trafficking/secretion, and regulation of actin and integrin function (Carpenter and Cantley, 1996). Accumulating evidence points a pivotal role for PI3-kinase in the differentiation of myoblasts.

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Interfering with PI3-kinase activity with inhibitors such as 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) or wortmannin or using a dominant negative mutant of p85α, the regulatory subunit of PI3-kinase, blocks myoblast fusion and the expression of muscle-specific proteins (Coolican et al., 1997; Kaliman et al., 1998; Jiang et al., 1999). Although tyrosine phosphorylation of FAK and the activation of PI3-kinase have been reported to be important events in the differentiation of skeletal myoblasts, relatively little is known about their relationship in the regulation of myogenesis.

Genistein, an isoflavone, is well known to inhibit several protein tyrosine kinases and DNA topoisomerase II in several cell types (Akiyama et al., 1987; Linassier et al., 1990; McCabe and Orrenius, 1993). In addition, the agent has been reported as an inhibitor of proliferation and fusion of myoblasts through inhibiting protein synthesis in myofiber (Ji et al., 1999). However, cellular target(s) of the agent as a tyrosine kinase inhibitor, as well action mechanism of the agent in the differentiation of myoblasts is still unclear. Here we have shown that FAK can be a cellular target of genistein and that the inhibition of FAK by the agent results in inhibition of myogenesis. In addition, lipid products of PI3-kinase abrogated the genistein-induced inhibitions of myoblast fusion. These results demonstrate that genistein can exert its effect on the signaling pathway from FAK to PI3-kinase which may play an important role in the differentiation of myoblasts.

2. Materials and methods

Rabbit anti-p85, anti-FAK, anti-phospho-FAK on Tyr397, mouse anti-FAK, and anti-phosphotyrosine antibodies (4G10) were from Upstate Biotechnology. Anti-myogenin antibody was obtained from Santa Cruz Biotechnology. Anti-Akt and anti-phospho-Akt (Ser473) antibodies were purchased from Cell Signaling Technology. Anti-creatine kinase antibody was prepared as previously described (Kim et al., 2000). Anti-β-actin antibody and secondary antibodies (anti-rabbit immunoglobulins and anti-mouse immunoglobulins coupled with horseradish peroxidase) were purchased from Sigma. Thin layer chromatography silica gel-60 plates were from Merck. Fluo3-acetoxymethyl ester (fluo-3) was from Molecular Probes. L-α-phosphatidylinositol, L-α-phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylserine were purchased from Sigma. Dipalmitoyl L-α-phosphatidyl-D-myo-inositol-3-phosphate (PI-3-P), dipalmitoyl L-α-phosphatidyl-D-myo-inositol-3,4-diphosphate (PI-3,4-P_2), and dipalmitoyl L-α-phosphatidyl-D-myo-inositol 3,4,5-triphosphate (PI-3,4,5-P_3) were from Calbiochem. [γ-^{32}P] ATP was from New England Nuclear or Amersham. Other materials were obtained from Sigma.

2.1. Cell culture and fusion index

L6 rat skeletal myoblasts were obtained from the American Type Culture Collection (ATCC). Cells cultured in 10% fetal bovine serum-containing medium (growth medium) for 3 days were induced to differentiate by changing to 5% horse serum-containing medium (differentiation medium). The time of media change is designated as 0 h. Approximately 24 h or 30 h after change to differentiation medium, the myoblasts become bipolar, align with each other, and initiate membrane fusion. The percentage of cell fusion dramatically increases thereafter. For the determination of fusion, cells were washed with ice-cold phosphate buffered saline (PBS) and were immediately fixed using a mixture of 95% ethanol, 40% formaldehyde, and acetic acid (20:2:1 by volume). The fixed cells were stained with hematoxylin solution and observed under an Olympus IMT2 microscope. Cells were considered to be fused only if there was clear cytoplasmic continuity and at least three nuclei were present within the myotubes (Shin et al., 2000).

2.2. Immunoprecipitation and immunoblotting

At the indicated culture times, cells were washed three times with ice-cold PBS and disrupted by ultrasonication in lysis buffer (20 mM HEPES, pH 7.5, containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, and 0.1 mM Na_3VO_4). The cells were subsequently centrifuged at 15,000×*g* to remove cell debris. Protein concentrations were determined by the Bradford procedure using the Bio-Rad dye reagent, with bovine serum albumin (BSA) as a standard. Equal amounts (50 μg) of cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels (Laemmli, 1970). Gel proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and were incubated with the indicated antibodies. After incubation with anti-rabbit or anti-mouse IgGs conjugated with horseradish peroxidase, the immunoreactive protein bands were visualized by enhanced chemiluminescence detection.

For immunoprecipitation analysis, equal amounts (500 μg) of cell lysates (1 mg/ml) were incubated with the indicated antibodies (1 μg) overnight at 4 °C. The immunocomplexes were collected by incubation with Protein A for 2 h at 4 °C and centrifugation at 5000×*g* for 2 min. The pellets were serially washed three times with wash I (PBS containing 1% Nonidet P-40 and 100 μM Na_3VO_4), wash II (100 mM Tris, pH 7.5, containing 500 mM LiCl and 100 μM Na_3VO_4), and wash III (10 mM Tris, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4), respectively. The resulting immunocomplexes were resuspended in Laemmli sample buffer and resolved by SDS-PAGE on 10% gels.

2.3. PI3-kinase activity assay

PI3-kinase activity was determined by the incorporation of [^{32}P] from [γ-^{32}P] ATP into exogenous phosphatidylinositol, resulting in the production of radio-labeled phosphatidylinositol as described by Tsakiridis et al. (1995), with some modification. The p85 was immunoprecipitated with anti-p85 antibody or anti-FAK antibody. The precipitated enzymes in wash III buffer (50 μl) were incubated with 2.5 μg of