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Research Article

Sphingosine-1-phosphate mediates epidermal growth factor-induced muscle satellite cell activation

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ABSTRACT

Skeletal muscle can regenerate repeatedly due to the presence of resident stem cells, called satellite cells. Because satellite cells are usually quiescent, they must be activated before participating in muscle regeneration in response to stimuli such as injury, overloading, and stretch. Although satellite cell activation is a crucial step in muscle regeneration, little is known of the molecular mechanisms controlling this process. Recent work showed that the bioactive lipid sphingosine-1-phosphate (S1P) plays crucial roles in the activation, proliferation, and differentiation of muscle satellite cells. We investigated the role of growth factors in S1P-mediated satellite cell activation. We found that epidermal growth factor (EGF) in combination with insulin induced proliferation of quiescent undifferentiated mouse myoblast C2C12 cells, which are also known as reserve cells, in serum-free conditions. Sphingosine kinase activity increased when reserve cells were stimulated with EGF. Treatment of reserve cells with the D-erythro-N,N-dimethylsphingosine, Sphingosine Kinase Inhibitor, or siRNA duplexes specific for sphingosine kinase 1, suppressed EGF-induced C2C12 activation. We also present the evidence showing the S1P receptor S1P2 is involved in EGF-induced reserve cell activation. Moreover, we demonstrated a combination of insulin and EGF promoted activation of satellite cells on single myofibers in a manner dependent on SPHK and S1P2. Taken together, our observations show that EGF-induced satellite cell activation is mediated by S1P and its receptor.

Abbreviations: BrdU, 5-boromo-2'-deoxyuridine; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; DMS, D-erythro-N,N-dimethylsphingosine; DMSO, dimethyl sulfoxide; EDL, extensor digitorum longus; EDG, endothelial differentiation gene; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GPCR, G-protein coupled receptor; IGF, insulin-like growth factor; JNK, c-Jun N-terminal kinase1; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PDVF, polyvinylidene fluoride; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; S1P, sphingosine-1phosphate; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; SPHK, sphingosine kinase; TBST, Tris-buffered saline plus 0.05% Tween 20

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Introduction

Skeletal muscle can regenerate repeatedly [1]. Skeletal muscle is not regarded as regenerative tissue because of low tissue turnover in the normal state; however, injury by trauma, overload, or exercise induces rapid muscle regeneration. The remarkable ability of skeletal muscle regeneration is due to the presence of muscle-resident stem cells called satellite cells [2].

Quiescent satellite cells must be activated before participating in muscle regeneration. Satellite cell activation is the initial step of muscle regeneration. Dramatic changes occur during the transition from a quiescent to an activated state, including onset of transcription, cytoplasmic enlargement, and cell proliferation [3]. Although satellite cell activation occurs in response to several physiological stimuli, the molecules responsible for the process have not been identified. Skeletal muscle tissue contains fibroblasts, pericytes, and peripheral nerves in addition to satellite cells; however, only satellite cells are activated to proliferate during muscle regeneration. Therefore, satellite cells must be selectively or specifically activated by unknown mechanisms in response to the regenerative stimuli [4]. Stimuli that induce skeletal muscle regeneration must be rapid and involve both intercellular and intracellular signaling. The regenerative ability of skeletal muscle is maintained throughout life in healthy individuals; satellite cell activation must be tightly regulated by several signaling pathways. Insufficient satellite cell activation would result in insufficient muscle regeneration, whereas excessive activation would disrupt muscle regeneration and lead to muscle degenerative diseases such as Duchenne muscular dystrophy [2].

Sphingosine-1-phosphate (S1P) is emerging as a new class of signaling molecule [5]. S1P participates in many cell processes, such as proliferation, migration, differentiation, and survival [6]. S1P was originally identified as a second messenger [7] and then shown to be a ligand for S1P receptors [8]. There are five G-protein coupled cell-surface S1P receptors, designated as S1P1−5 [9]. S1P receptors are involved in various cellular processes such as cell proliferation, differentiation, migration, and cytoskeletal rearrangement [9]. Identification of S1P receptors greatly advanced the understanding of S1P-mediated cellular functions. Specific agonists and antagonists of S1P receptors have been developed [10]. Recent studies reveal the importance of S1P in skeletal muscle biology [11]. Exogenous S1P was shown to reduce DNA synthesis of proliferating C2C12 myoblasts but enhance mitogenic effects of serum to reserve cells [12]. It was also demonstrated that exogenous S1P inhibited migration of C2C12 myoblasts [13]. S1P mediates insulin-like growth factor (IGF)-induced differentiation of mouse myoblast C2C12 cells while the mitogenic effect of the growth factor was negatively mediated by S1P via S1P1/S1P3 [14]. It was reported that platelet-derived growth factor (PDGF)-induced myoblast proliferation was negatively regulated by endogenous S1P metabolism [15]. Recent work shows that S1P3 plays a crucial role in maintaining satellite cell function by suppressing cell-cycle progression [16]. S1P is a promising target for work to ameliorate muscular dystrophy [17,18].

Our previous work showed that extracellular S1P induced quiescent satellite cells to enter the cell cycle, whereas inhibition of S1P biosynthesis significantly repressed satellite cell activation and caused significant loss of muscle regeneration in vivo [19]. In that report, satellite cell activation was induced with serum, which contains multiple growth factors and cytokines. The specific growth factor(s) or cytokine(s) responsible for S1P-dependent satellite cell activation were not identified in the previous report, and the precise mechanisms of S1P-dependent signaling during satellite cell activation and muscle regeneration were unknown.

In this study, we identify the growth factor responsible for S1P-dependent activation of quiescent myogenic cells, and investigate the molecular mechanisms that regulate the signaling pathway. We use mouse myoblast C2C12 reserve cells as a model for quiescent satellite cells [20]. The results show that epidermal growth factor (EGF) activates sphingosine kinase 1 (SPHK1) and induces S1P-dependent activation of reserve cells. EGF-induced activation of reserve cells was dependent on the S1P receptor S1P2 and extracellular signal-regulated kinase (ERK). Taken together, our observations suggest that S1P mediates EGF-induced satellite cell activation via the S1P2 receptor by promoting ERK signaling.

Materials and methods

Cell culture

C2C12 myogenic cells [21,22] were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco, Tokyo, Japan) containing 20% fetal bovine serum (FBS, JRH biosciences, Lenexa, KN), 25 mM HEPES (pH7.4), 2.0 g/l sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37.5 °C in 5% CO_2. To induce differentiation and formation of reserve cells, 5×10^4 cells were seeded on a 35-mm tissue culture dish in growth medium, incubated for 24 h, and then the medium was replaced with serum-free differentiation medium [DMEM supplemented with insulin–transferrin–sodium selenite supplement (Sigma-Aldrich, Tokyo, Japan), 1 mg/ml bovine serum albumin (BSA, Sigma-Aldrich), and 25 mM HEPES (pH7.4)]. To isolate reserve cells from myotubes, C2C12 cells were cultured until fully differentiated, and then the myotubes were detached with 0.05% trypsin (Gibco) in phosphate-buffered saline (PBS) containing 0.9 mM $CaCl_2$ and 0.9 mM $MgCl_2$ [PBS(+) buffer] for 5 min at 37 °C.

To induce activation of reserve cells, cells were stimulated with 10% FBS, 1.7 μM (10 μg/ml) insulin (Sigma-Aldrich), 4.2 nM (25 ng/ml) EGF, 13.3 nM (100 ng/ml) IGF-I, or 2.0 nM (25 ng/ml) PDGF-BB (R&D Systems, Minneapolis, MN) in the presence of 10 μM 5-bromo-2'-deoxyuridine (BrdU), for 24 h. For treatment with inhibitor or antagonist, cells were pre-incubated with the drug for 30 min before stimulation of reserve cells. D-erythro-N, N-dimethylsphingosine (DMS, Biomol, Plymouth, PA), Sphingosine Kinase Inhibitor (SKI, CAS 1177741-83-1), U0126, SB239063, and SP600125 (Merck, Tokyo, Japan) stock solutions were prepared in dimethyl sulfoxide (DMSO). W123, JTE-013, and CAY10444 (Cayman Chemicals, Ann Arbor, MI) stock solutions were prepared in dimethylformamide. Stock solutions of S1P (Biomol) were prepared according to the manufacturer's instructions using 4 mg/ml fatty acid-free BSA (Sigma-Aldrich) in DMEM.

Detection of bromodeoxyuridine incorporation

Cells were incubated in the presence of 10 μM BrdU for 24 h, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton