EXPERIMENTAL CELL RESEARCH 333 (2015) 228–237

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/yexcr

Research Article

Zinc promotes proliferation and activation of myogenic cells via the PI3K/Akt and ERK signaling cascade

Kazuya Ohashi[a], *Yosuke Nagata*[a], *Eiji Wada*[a], *Peter S. Zammit*[b], *Masataka Shiozuka*[a], *Ryoichi Matsuda*[a,*]

[a]*Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Japan*
[b]*Randall Division of Cell and Molecular Biophysics, King's College London, London SE1 1UL, UK*

ARTICLE INFORMATION

Article Chronology:
Received 30 July 2014
Received in revised form
2 March 2015
Accepted 4 March 2015
Available online 13 March 2015

Keywords:
Skeletal muscle
C2C12
Reserve cells
Zinc
Insulin

ABSTRACT

Skeletal muscle stem cells named muscle satellite cells are normally quiescent but are activated in response to various stimuli, such as injury and overload. Activated satellite cells enter the cell cycle and proliferate to produce a large number of myogenic progenitor cells, and these cells then differentiate and fuse to form myofibers.

Zinc is one of the essential elements in the human body, and has multiple roles, including cell growth and DNA synthesis. However, the role of zinc in myogenic cells is not well understood, and is the focus of this study. We first examined the effects of zinc on differentiation of murine C2C12 myoblasts and found that zinc promoted proliferation, with an increased number of cells incorporating EdU, but inhibited differentiation with reduced myogenin expression and myotube formation. Furthermore, we used the C2C12 reserve cell model of myogenic quiescence to investigate the role of zinc on activation of myogenic cells. The number of reserve cells incorporating BrdU was increased by zinc in a dose dependent manner, with the number dramatically further increased using a combination of zinc and insulin. Akt and extracellular signal-regulated kinase (ERK) are downstream of insulin signaling, and both were phosphorylated after zinc treatment. The zinc/insulin combination-induced activation involved the phosphoinositide 3-kinase (PI3K)/Akt and ERK cascade. We conclude that zinc promotes activation and proliferation of myogenic cells, and this activation requires phosphorylation of PI3K/Akt and ERK as part of the signaling cascade.

Abbreviations: IR, insulin receptor; IGFR, insulin-like growth factor receptor; IRS-1, insulin receptor substrate-1; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; Grb2, growth factor receptor-bound protein 2; FGF2, fibroblast growth factor 2; DTPA, diethylenetriamine pentaacetic acid; GM, growth medium; DMEM, Dulbecco's modified Eagle medium; BSA, bovine serum albumin; PBS, phosphate buffered saline; BrdU, 5-bromo-2′-deoxyuridine; EdU, 5-ethynyl-2′-deoxyuridine; HS, horse serum; SDS, sodium dodecyl sulfate; MyHC, myosin heavy chain; ERK, extracellular signal-regulated kinase

*Correspondence to: *Department of Life Sciences, Graduate School of Arts and Sciences, the University of Tokyo, 3-8-1 Komaba, Meguro, Tokyo, 153-8902, Japan.* Fax: +81 354544306.

E-mail addresses: asuno10k@yahoo.co.jp (K. Ohashi), cynagata@mail.ecc.u-tokyo.ac.jp (Y. Nagata), gacchu1@yahoo.co.jp (E. Wada), peter.zammit@kcl.ac.uk (P.S. Zammit), cmuscle@mail.ecc.u-tokyo.ac.jp (M. Shiozuka), cmatsuda@mail.ecc.u-tokyo.ac.jp (R. Matsuda).

http://dx.doi.org/10.1016/j.yexcr.2015.03.003
0014-4827/

Introduction

Muscle satellite cells play an indispensable role in regeneration of skeletal muscle [1]. Satellite cells are located between the basal lamina and the plasma membrane, and usually exist in a mitotically quiescent state, until activated by injury or overload [2]. Once activated, satellite cells enter the cell cycle and proliferate to produce a large number of myoblast progeny expressing Myf5 and MyoD, which then commit to differentiation and express myogenin. Myoblasts then fuse to existing myofibers to provide new myonuclei, or fuse together to form myotubes de novo, which subsequently mature to muscle fibers [3].

Insulin is a ligand for the insulin receptor (IR) or the insulin-like growth factor receptor (IGFR). When insulin binds to these receptors, tyrosine kinase in the β subunit of the receptor is activated, resulting in autophosphorylation of the kinase regulatory domain. The phospho-tyrosine of IR allows activation of insulin receptor substrate-1 (IRS-1), which operates through several signaling pathways including the phosphoinositide 3-kinase (PI3K)/Akt cascade [4,5]. The PI3K/Akt cascade has various roles, such as controlling proliferation and differentiation, and being anti-apoptotic. Especially in skeletal muscle, PI3K/Akt signaling plays a role in hypertrophy [6,7], and IGF-1-induced PI3K/Akt signaling contributes to increase in size of C2C12 myotubes [8]. In satellite cells, the PI3K/Akt cascade is involved in G1/S cell cycle progression in vitro [9]. Akt induces phosphorylation of mammalian target of rapamycin (mTOR), which is a master regulator of cell growth and cell cycle progression [10,11]. In myogenic cells, mTOR is involved in muscle differentiation [12], and is known to serve as a signal for muscle hypertrophy [7,13]. IR and IGFR also activate extracellular signal-regulated kinase (ERK) through Shc and growth factor receptor-bound protein 2 (Grb2) [4]. ERK is known to regulate cell proliferation, differentiation and cell survival. We previously reported that fibroblast growth factor 2 (FGF2)-induced phosphorylation of ERK through Grb2 has a role in the activation of quiescent myogenic cells [14].

Although the physiological roles of growth factors or hormones on skeletal muscle plasticity have been extensively investigated, the effects of minerals on skeletal muscle function have not been studied in detail. Zinc is an important element for skeletal muscle biology, for example increasing isometric twitch tension and endurance [15,16]. Zinc is an essential element and mainly distributed in skeletal muscle and bone in human. The concentration of zinc in human serum is around 15 μM [17]. More than 300 enzymes require zinc for their function and so zinc is involved in many biological activities including cell growth and DNA synthesis [18]. Addition of zinc into diethylenetriamine pentaacetic acid (DTPA)-treated medium rescues the inhibitory effects of metal ion chelation on muscle differentiation [19]. Surprisingly, zinc also induces phosphorylation of IR and IRS-1 in myoblasts, even in the absence of insulin [20], and increases phosphorylation of Akt in skeletal muscle of mice fed with zinc supplemented diet [21]. Furthermore, the zinc-containing drug, Z-103, improves muscle function in the mdx murine model of Duchenne muscular dystrophy [22,23]. Although zinc has been shown to be important for proliferation of several types of cells and function of skeletal muscle, the role of zinc in myoblast or muscle satellite cell biology remains largely unknown.

Here, we investigated the effects of zinc on activation, proliferation and differentiation of myogenic cells. We found that zinc promotes activation and proliferation of myoblasts, partly via the phosphorylation of PI3K/Akt and ERK.

Materials and methods

Cell culture

C2C12 myogenic cells (ATCC, Manassas, VA) were cultured in growth medium (GM) (Dulbecco's Modified Eagle Medium [DMEM], Gibco, Grand Island, NY) containing 20% fetal bovine serum (JRH Bioscience, St. Lenexa, KC), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) at 37 °C in 5% CO_2. It should be noted that DMEM does not contain zinc. To induce differentiation, 1×10^5 cells were seeded in a 35 mm tissue culture dish with GM and were incubated for 24 h. The medium was replaced with serum-free differentiation medium containing insulin–transferrin–sodium selenite media supplement (Sigma-Aldrich, St. Louis, MO) and 1 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich). To isolate reserve cells, C2C12 cells were cultured for 5 days and then treated with 0.05% trypsin (Gibco) with phosphate buffered saline (PBS) containing $CaCl_2$ and $MgCl_2$ [PBS(+)], for 5 min at 37 °C as reported previously [14].

Proliferation analysis

We used 10 μM 5-ethynyl-2′-deoxyuridine (EdU) or 10 μM 5-bromo-2′-deoxyuridine (BrdU) to assess cell proliferation. 5×10^3 cells were seeded in an 8 well chamber slide (Nunc, UK) or 1×10^4 cells were seeded in a gelatin-coated-24 well culture plate with GM and incubated for 24 h. Then, the medium was changed to 2% horse serum (HS) or 1 mg/ml BSA/DMEM with zinc chloride ($ZnCl_2$) or zinc sulfate ($ZnSO_4$), and EdU or BrdU was added for the last 2 h of the 48 h incubation. Following that, cells were fixed with 10% formalin for 15 min. To visualize BrdU, cells permeabilized with 0.5% Triton X-100. After 1 N hydrochloric acid treatment for 30 min, incorporated BrdU was detected with the anti-BrdU rat monoclonal antibody BU1/75 (Abcam, Cambridge, UK) and Alexa Fluor 594-conjugated anti-rat IgG antibody (Molecular Probes, Eugene, OR) and 100 ng/ml Hoechst 33258. Finally, cells were mounted in Mowiol mounting medium. Approximately 800 cells were counted in each experiment. To visualize EdU, we used the Click-it EdU imaging kit (Invitrogen, Life Technologies, Paisley, UK).

Activation analysis

To induce activation of reserve cells, cells were stimulated with $ZnCl_2$ (the concentration was from 25 to 100 μM) and 10 μg/ml insulin (Sigma-Aldrich). When the effects of the inhibitors were assessed, cells were treated with each inhibitor for 30 min prior to reserve cell stimulation. LY294002 (Cayman Chemical, Ann Arbor, MI), Triciribine (Merck, Whitehouse Station,NJ), rapamycin (Cayman Chemical) and U0126 (Merck) stock solutions were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1% in a culture medium and the same dose of DMSO was added to control conditions. To evaluate activation of reserve cells, cells were incubated with the medium containing 10 μM BrdU for 24 h. Following incubation, cells were fixed with 10% formalin in PBS for 15 min, and then immunostained to detect BrdU.