Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochimica et Biophysica Acta 1743 (2005) 130–140

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

http://www.elsevier.com/locate/bba

Clinorotation prevents differentiation of rat myoblastic L6 cells in association with reduced NF-κB signaling

Katsuya Hirasaka[a], Takeshi Nikawa[a,*], Louis Yuge[b], Ibuki Ishihara[a], Akira Higashibata[c], Noriaki Ishioka[c], Atsuko Okubo[b], Takashi Miyashita[b], Naoto Suzue[d], Takayuki Ogawa[d], Motoko Oarada[e], Kyoichi Kishi[a]

[a]*Department of Nutrition, The University of Tokushima School of Medicine, Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan*
[b]*Institute of Health Science, Faculty of Medicine, Hiroshima University, Hiroshima, Japan*
[c]*Department of Space Biology and Microgravity Sciences, Institute of Space and Astronautical Science, JAXA, Japan*
[d]*Department of Orthopaedics, The University of Tokushima School of Medicine, Tokushima, Japan*
[e]*Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan*

Received 28 July 2004; received in revised form 6 September 2004; accepted 8 September 2004
Available online 28 September 2004

Abstract

In this study, we examined effects of the three-dimensional (3D)-clinorotation, a simulated-model of microgravity, on proliferation/differentiation of rat myoblastic L6 cells. Differentiation of L6 cells into myotubes was significantly disturbed in the 3D-clinorotation culture system, although the 3D-clinorotation had no effect on the proliferation. The 3D-clinorotation also suppressed the expression of myogenesis marker proteins, such as myogenin and myosin heavy chain (MHC), at the mRNA level. In association with this reduced differentiation, we found that the 3D-clinorotation prevented accumulation of ubiquitinated proteins, compared with non-rotation control cells. Based on these findings, we focused on the ubiquitin-dependent degradation of IκB, a myogenesis inhibitory protein, to clarify the mechanism of this impaired differentiation. A decline in the amount of IκB protein in L6 cells was significantly prevented by the rotation, while the amount of the protein in the non-rotated cells decreased along with the differentiation. Furthermore, the 3D-clinorotation reduced the NF-κB-binding activity in L6 cells and prevented the ubiquitination of IκB proteins in the IκB- and ubiquitin-expressing Cos7 cells. Other myogenic regulatory factors, such as deubiquitinases, cyclin E and oxygen, were not associated with the differentiation impaired by the clinorotation. Our present results suggest that simulated microgravity such as the 3D-clinorotation may disturb skeletal muscle cell differentiation, at least in part, by inhibiting the NF-κB pathway.
© 2004 Elsevier B.V. All rights reserved.

Keywords: 3D-clinorotation; Rat myoblastic L6 cell; Ubiquitination; NF-κB signaling; IκB

1. Introduction

Microgravity and its simulated conditions preferentially disturb differentiation of skeletal muscle cells [1,2]. Reculturing of the space-flown L8 myoblastic cells after landing failed to stimulate them to fuse and differentiate into myotubes [1]. Mouse satellite cells cultured in a rotating wall vessel bioreactor proliferated normally, but their differentiation was significantly inhibited [2]. These inhibitory effects of microgravity have been suggested to contribute to the retarded recovery from microgravity-induced muscle atrophy, since differentiation of satellite cells and their fusion to myofibers are necessary for the recovery [3]. Therefore, an increased understanding of molecular mechanisms of this impaired muscle cell differentiation may

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; IKK, IκB kinase; MHC, myosin heavy chain; JAXA, Japan Aerospace Exploration Agency; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; 3D-clinorotation, three-dimensional clinorotation; β-TrCP, β-transducin repeat-containing protein

* Corresponding author. Tel.: +81 88 633 9248; fax: +81 88 633 7086.
E-mail address: nikawa@nutr.med.tokushima-u.ac.jp (T. Nikawa).

0167-4889/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2004.09.013

lead to the development of effective therapies for the recovery from muscle atrophy.

Several signal transduction molecules, including myogenin and cyclin E, mediate differentiation of myoblasts into myotubes [4,5]. Recent investigations have shown that proteolytic systems, especially the ubiquitin-proteasome pathway, are involved in activation or inactivation of these myogenic regulatory factors [6–8]. For example, NF-κB activation by 26S proteasome is essential for up-regulating myogenin expression during myotube formation [6]. In contrast, we previously reported that space shuttle flight (STS-90) as well as tail-suspension specifically activated the ubiquitin-dependent proteolysis in rat skeletal muscles in vivo [9]. Based on these findings, we envisaged that the differentiation of skeletal muscle cells under microgravity is modified by the ubiquitin-dependent proteolytic system.

To address this issue, we examined the effects of three-dimensional (3D)-clinorotation on the differentiation and protein-ubiquitination of rat L6 myoblastic cells. The 3D-clinorotation is a microgravity-simulating model that has two independent axes of rotation to disperse the gravity vector [10]. Myotube formation of L6 cells cultured with a 3D-clinorotation apparatus was impaired, and the 3D-clinorotation prevented the expression of myosin heavy chain (MHC) and myogenin in L6 cells. Ubiquitinated proteins in L6 cells were accumulated during myotube formation, whereas the 3D-clinorotation significantly repressed the protein-ubiquitination in L6 cells. We also found that the 3D-clinorotation prevented the ubiquitin-dependent IκB degradation, leading to the reduction of NF-κB signaling in L6 cells. Our results suggest that the reduced NF-κB signaling contributes, at least in part, to skeletal muscle cell differentiation disturbed by the 3D-clinorotation.

2. Materials and methods

2.1. Cell culture

L6 cells were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). Cos7 cells were a kind gift from Dr. Taketani, The University of Tokushima School of Medicine, Tokushima, Japan. L6 or Cos7 cells were maintained and proliferated at 37 °C with 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

2.2. Transfection

We constructed an expression vector for rat IκB using a reverse transcription-polymerase chain reaction (RT-PCR) and cloning techniques as described previously [9,11]. Total RNA was extracted from L6 cells with an acid guanidinium thiocyanate–phenol–chloroform mixture (Isogen™; Nippon Gene, Tokyo, Japan) according to the standard protocol [12]. First-strand cDNAs were reverse-transcribed at 37 °C for 50 min from 1 μg of the extracted total RNA with oligo-dT15 primer and SuperScript II™ reverse transcriptase (Invitrogen, Carlsbad, CA). After initial denaturation at 94 °C for 2 min, second-strand synthesis and DNA amplification with Pfx™ Taq polymerase (Invitrogen) and the IκB primer set (5′-CACCATGTTTCAGCCAGCTGGGCA-3′ and 5′-TAACGTCAGACGCTGGCCTCCAAAC-3′) [13] were accomplished through 30 cycles of the following incubations: 15 s at 94 °C, 30 s at 60 °C, 90 s at 68 °C by using a thermal cycler (MJ Research, Watertown, MA). The PCR products were sequenced and cloned into an expression vector pcDNA3.1/V5-His (Invitrogen). The plasmid containing FLAG-tagged ubiquitin cDNA was kindly provided by Dr. Ishidoh, Juntendo University School of Medicine, Tokyo, Japan. Cos7 cells were transfected with 1 μg/dish of the purified plasmid containing IκB or FLAG-tagged ubiquitin by using FuGene6 (Roche Diagnostics, Mannheim, Germany) according to the method of Hellgren et al. [14]. IκB- and ubiquitin-expressing Cos7 cells were subjected to the 3D-clinorotation 24 h after transfection as described below.

2.3. 3D-clinorotation

We subjected L6 cells or the transfected Cos7 cells to 3D-clinorotation in an apparatus (Mitsubishi Heavy Industries, Kobe, Japan) according to the method of Arase et al. [10]. Flasks containing L6 cells at about 30% and 75% confluence were filled with DMEM in the presence of 10% FCS (proliferation medium) and 2% horse serum (differentiation medium), respectively. They were rotated at 37 °C on the 3D-clinostat apparatus in a 5% CO_2 chamber. The rate and cycle of rotation were controlled by the computer to randomize the gravity vector both in magnitude and in direction, and then the dynamic stimulation of gravity to cells was cancelled in any direction. Reagents and media in the flasks were not changed during rotation (7 days). Control cells were incubated in parallel under the same conditions except for the rotation. Flasks containing the transfected Cos7 cells were rotated in the same manner after being filled with the proliferation medium.

2.4. Immunoblot analysis

Immunoblot analysis was performed as described previously [9]. The whole-cell extracts (40 μg protein/lane) from L6 or Cos7 cells were subjected to SDS-8%, 10% or 12% polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane at 35 mA for 6 h at 4 °C. The membrane was blocked with 3% skim milk and then incubated for 1 h at 25 °C in phosphate-buffered saline (PBS) with a 1:500 dilution of antiserum

Download English Version:

https://daneshyari.com/en/article/9887188

Download Persian Version:

https://daneshyari.com/article/9887188

Daneshyari.com