Cell Calcium 56 (2014) 257–268

Contents lists available at ScienceDirect

Cell Calcium

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

CREB modulates calcium signaling in cAMP-induced bone marrow stromal cells (BMSCs)

Linxia Zhang[a], Li Liu[b], Ryan Thompson[c], Christina Chan[a,d,*]

[a] Department of Chemical Engineering and Materials Science, Michigan State University, East Lansing, MI 48824, United States
[b] Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, United States
[c] Cellular and Molecular Biology Program, Michigan State University, East Lansing, MI 48824, United States
[d] Department of Computer Science and Engineering, Michigan State University, East Lansing, MI 48824, United States

ARTICLE INFO

Article history:
Received 23 January 2014
Received in revised form 28 July 2014
Accepted 29 July 2014
Available online 6 August 2014

Keywords:
CREB
Bone marrow stromal cells
Calcium signal
Morphology

ABSTRACT

Calcium signaling has a versatile role in many important cellular functions. Despite its importance, regulation of calcium signaling in bone marrow stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells) has not been explored extensively. Our previous study revealed that cyclic adenosine monophosphate (cAMP) enabled BMSCs to generate calcium signal upon stimulation by dopamine, KCl and glutamate. Concurrently, cAMP transiently activated the transcription factor cAMP response element binding protein (CREB) in BMSCs. Activity of CREB can be modulated by the calcium/calmodulin-dependent kinase signaling pathway, however, whether the calcium signaling observed in cAMP-induced BMSCs requires CREB has not been investigated. In an effort to uncover the role of CREB in the generation of calcium signaling in response to modulators such as dopamine and KCl, we knocked down CREB activity in BMSCs. Our study indicated that BMSCs, but not its close relative fibroblasts, are responsive to dopamine and KCl after cAMP treatment. Calcium signal elicited by dopamine depends, in part, on calcium influx whereas that elicited by KCl depends completely on calcium influx. Knock-down of CREB activity significantly reduced or abolished the cAMP-induced calcium response, and reintroducing a constitutively active CREB partially restored the calcium response.

1. Introduction

Bone marrow stromal cells (BMSCs), also known as bone marrow-derived mesenchymal stem cells, have the potential to undergo osteogenic, chondrogenic and adipogenic differentiation [1]. Calcium is one of the many important players that regulates differentiation [2], such as osteogenic [3,4], chondrogenic [5,6] and adipogenic differentiation [7–9]. Regulation of calcium signaling in BMSCs has been explored to some extent. Human BMSCs has exhibited spontaneous calcium oscillations without agonist stimulation [10–12]. Inhibition of calcium influx through L-type voltage-gated calcium channel reduced osteogenic differentiation of BMSCs [4], while stimulating cytosolic calcium oscillation facilitated osteogenic differentiation in human BMSC [3]. Tropel et al. demonstrated that mouse BMSCs induced by basic fibroblast growth factor (bFGF) are able to generate calcium signal upon stimulation by dopamine, glutamate and veratridine [13]. While these studies demonstrated the importance of calcium signaling in BMSCs, studies regarding calcium signaling in BMSCs are still at an early stage.

Rise in cytosolic calcium concentration mainly results from calcium release from internal stores or calcium influx from external medium [2,14]. Endoplasmic reticulum (ER) is generally recognized as the intracellular calcium stores. Release of calcium from ER is mediated by second messengers, such as inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) and calcium itself [14,15]. It has been shown that spontaneous calcium oscillations observed in BMSCs mainly occurred through calcium release from internal stores, mediated by the inositol-1,4,5-trisphosphate receptor (InsP3R) [10]. External medium is another source of cytosolic calcium. Calcium influx through ion channels on the cell membrane, including voltage-gated channels and receptor-gated channels, contribute to the dynamical changes in cytosolic calcium concentrations [2,14]. Voltage-gated ion channels or receptor-gated channels are predominantly expressed in excitable cells [15]. However, a previous study showed that many ion channels, such

* Corresponding author at: Michigan State University, Department of Chemical Engineering and Materials Science, 2527 EB, East Lansing, MI 48824, United States. Tel.: +1 517 432 4530; fax: +1 517 432 1105.
E-mail address: krischan@egr.msu.edu (C. Chan).

http://dx.doi.org/10.1016/j.ceca.2014.07.013
0143-4160/

as L-type voltage-gated Ca^{2+} channels, voltage-gated potassium channels, Ca^{2+}-activated potassium channels and voltage-gated sodium channels, are expressed in undifferentiated rat BMSCs [16]. In addition to voltage-gated channels, influx of calcium can also be mediated by receptor-gated channels, i.e., G-protein coupled dopamine receptors. Dopamine induces calcium rise mainly through binding to G-protein coupled dopamine receptors, the D1-like (including D1 and D5) and the D2-like (including D2, D3 and D4) dopamine receptors [17,18].

Cyclic AMP is another important intracellular second messenger that can crosstalk with calcium signaling and regulates cell function. cAMP activates the downstream transcription factor cAMP response element binding protein (CREB), which serves as a hub for many cellular processes including metabolism, survival, immune response as well as learning and memory [19,20]. Phosphorylation of CREB at the key serine 133 site by kinases such as protein kinase A (PKA), Ca^{2+}/calmodulin-dependent kinases (CaMKs), and mitogen-activated protein kinases (MAPKs) is required for its transcriptional activity [21,22]. Previously, we observed that after cAMP treatment, BMSCs exhibited robust calcium response upon stimulation by dopamine and KCl [23]. Dopamine not only plays an important role in the central nervous system, but also functions in the peripheral system. Many conditions, including stress, hypovolemia and exercise, can increase the dopamine level in the plasma [24]. In mouse bone marrow, evidence of dopamine uptake was observed, suggestive of the presence of dopamine receptors in the bone marrow [25]. In further support, dopamine receptor D1 protein was detected in uninduced human BMSCs which further increased with brain-derived neurotrophic factor (BDNF) treatment [26].

Although it is well known that the cAMP downstream signaling component CREB can be modulated by the Ca^{2+}/calmodulin dependent kinases (CaMKs) [21], little is known regarding the role of the cAMP-CREB pathway on calcium signaling. In particular, the impact of cAMP-CREB pathway on calcium signaling in BMSCs has not been explored. cAMP is increased in many pathophysiological conditions, such as fibrous dysplasia of the bone [27,28], hyperparathyroidism [29,30] and myocardial infarction [31,32]. In other cases, elevation of cAMP has been beneficial for treating a number of ailments, such as spinal cord injury [33,34] and autoimmune diseases [35,36]. Reagents that are able to boost cAMP levels are also used widely in modulating osteogenic- and adipogenic-differentiation of BMSCs [37–40]. BMSCs are excellent sources for cell-based therapies, due to their differentiation potential, secretion of a plethora of trophic factors, and immunomodulatory capability [41]. Currently, clinical trials using BMSCs are being studied for a variety of diseases, such as Graft-versus-Host disease, myocardial infarction, spinal cord injury, stroke, and amyotrophic lateral sclerosis (ALS) [41,42]. Accordingly, it is important to understand how BMSCs would respond to calcium modulators in an environment with elevated cAMP levels.

Previous studies showed that certain dopamine receptors and voltage-gated calcium channels are expressed on BMSCs [16,26], therefore, we set out to determine how the cAMP-CREB pathway might modulate calcium signaling through receptor-gated channels stimulated by dopamine and through voltage-gated channels stimulated by KCl. In this study, we examined intracellular calcium signaling in BMSCs through fluorescence imaging. CREB activity was modulated by siRNA silencing and the generation of a dominant negative CREB cell line. Our results suggested that the calcium response to dopamine and KCl in cAMP-induced BMSCs requires calcium influx from an external source. Long term knock-down of CREB activity significantly reduced the calcium response to dopamine and KCl, along with dramatic morphological changes in cAMP-induced BMSCs.

2. Materials and methods

2.1. Cell culture and materials

All procedures in the cell isolation were approved by the Institutional Animal Care and Use Committee at Michigan State University. Bone marrow stromal cells (BMSCs) were isolated from 6 to 8 week old Sprague-Dawley female rat as previously described [43]. In brief, femurs and tibias were taken from the hind legs of 6–8 week old rat. The marrow was flushed out with Dulbecco's modified Eagle medium (DMEM) and filtered through a 65 μm nylon mesh to remove bone debris and blood aggregates. Cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 μg/mL streptomycin (Invitrogen) and 100 U/mL penicillin (Invitrogen) and placed in an incubator with a humidified atmosphere containing 5% CO_2 at 37 °C. Non-adherent cells were removed on the second day after plating. After culturing for two passages, magnetic cell sorting using markers CD54 and CD90 was applied to enrich BMSCs. Cell sorting results were verified by flow cytometry as shown in [43].

NIH3T3 fibroblasts were purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM (high glucose (4.5 g/L); Invitrogen) supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin and placed in an incubator with a humidified atmosphere containing 10% CO_2 at 37 °C.

Forskolin (Sigma, St. Louis, MO, USA) and isobutylmethylxanthine (IBMX) (Sigma) were used to increase intracellular cAMP levels at concentrations of 10 and 100 μM, respectively.

2.2. Cell proliferation assay

Cell proliferation was measured by the CyQUANT NF cell proliferation assay kit from Invitrogen. This assay is based on the binding of a fluorescent dye to DNA and therefore enabled the quantification of cellular DNA. Briefly, cells were cultured in 96-well culture plate and assayed at the desired time. After washing with Hank's balanced salt solution (HBSS), the cells were incubated in the dye-binding solution for 45 min at 37 °C. Fluorescence was measured by Spectra MAX GEMINI EM plate reader at excitation of 485 nm and emission of 530 nm.

2.3. Western blot

Whole cell extracts lysed with CelLytic (Sigma) were assayed for protein concentrations by Bradford assay (Bio-Rad). 15–30 μg protein samples were separated by 10% Tris–HCl gel and transferred to nitrocellulose membrane. Membranes were then blocked with 5% milk in 0.05% Tween 20-Tris buffered saline (T-TBS) (USB corporation, Cleveland, OH, USA) for one hour and incubated with primary antibodies, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell signaling, Danvers, MA, USA), CREB (Cell signaling), dopamine receptor D1 (Novus Biologicals), c-fos (Cell Signaling), TATA-binding protein (TBP) (Sigma) and inducible cAMP early repressor (ICER) (a kind gift from Dr. Carlos Molina) overnight at 4 °C. After removing excessive primary antibodies, anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Thermo Scientific) were added and the blots were incubated for one hour at room temperature. The blots were then washed three times with 0.05% T-TBS and visualized by SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific).

2.4. Immunocytochemistry

Triple staining for actin filaments, microtubules and nucleus was performed as previously described [44]. In brief, actin filaments were stained with Texas Red-X phalloidin (Invitrogen),