Experimental Cell Research 346 (2016) 9–16

Contents lists available at ScienceDirect

Experimental Cell Research

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

Research article

Requirement of IP_3 receptor 3 (IP_3R3) in nitric oxide induced cardiomyocyte differentiation of mouse embryonic stem cells

Wenjie Wei [a,b], Wei Huang [b], Jianbo Yue [a,*]

[a] Department of Biomedical Sciences, City University of Hong Kong, Hong Kong, China
[b] Department of Biology, South University of Science and Technology of China, Shenzhen 518052, China

ARTICLE INFO

Article history:
Received 2 December 2015
Received in revised form
21 June 2016
Accepted 23 June 2016
Available online 24 June 2016

Keywords:
ES cells
Nitric oxide
Ca^{2+}
IP_3 receptor 3
Cardiomyocyte

ABSTRACT

Nitric oxide (NO) markedly induces cardiomyocyte (CM) differentiation of embryonic stem (ES) cells. Here we examined the role of the Ca^{2+} signaling in the NO-induced CM differentiation of mouse ES cells. We found that NO induced intracellular Ca^{2+} increases in ES cells in a dose-dependent manner, and application of IP_3 pathway antagonists not only significantly inhibited this induced Ca^{2+} increase but also abolished NO-induced CM differentiation of ES cells. Subsequently, all 3 types of inositol 1, 4, 5-trisphosphate (IP_3) receptors (IP_3Rs) in mouse ES cells were individually or triply knocked down. Interestingly, only knockdown of type 3 IP_3R (IP_3R3) or triple-knockdown of three types of IP_3Rs significantly inhibited the NO-induced Ca^{2+} increases. Consistently, IP_3R3 knockdown blocked the NO-induced CM differentiation of ES cells. CMs derived from IP_3R3 knockdown ES cells also showed both structural and functional defects. In summary, our results indicate that the IP_3R3-Ca^{2+} pathway is required for NO-induced CM differentiation of ES cells.

1. Introduction

ES cells are one of the most reliable sources of cardiomyocytes (CMs) for cell replacement therapy to treat heart diseases, such as heart failure. During the past two decades, numerous efforts have been made to increase the efficiency of CM differentiation of ES cells for clinical application, including treatment of ES cells with various factors, e.g. Apelin [1], DKK1 [2], retinoic acid [3], insulin-like growth factor-1 [4], basic fibroblast growth factor-2 (FGF-2), bone morphogenetic protein-2 (BMP-2) [5], nitric oxide (NO) [6,7], and microRNA-1 [8].

Among all the approaches, NO is attractive for its potential clinical application since NO is a nature gas and is easy to be removed after treatment. In vertebrate, NO can either be synthesized from nitrites catalyzed by nitric oxide synthase (NOS), nitrite reductases, or other heme-containing proteins, or can be generated through non-enzymatic pathways such as nitrite photolysis [9]. NO plays a role in a variety of biological processes, e.g. relaxation of vascular smooth muscle [10], contractility of cardiac muscle [11], neurotransmission in the nerve system [12], and antimicrobial activity of macrophage [13]. Ever since the first study showed that NO served as a cardiomyogenic inducer, researchers have been trying to resolve the mechanism underlying this effect. Previous study has indeed found the differential expression of NO signaling components during CM differentiation of both mouse and human ES cells [14]. However, the mechanism of the NO-induced cardiomyogenesis of ES cells still remains unclear.

Ca^{2+} signaling plays important roles in a wide variety of cellular process. Ca^{2+} with its associating binding proteins and various Ca^{2+} channels have been reported to be involved in cardiomyogenesis during embryonic development. For example, calreticulin, the endoplasm reticulum (ER) Ca^{2+} buffering protein, acts upstream of myocyte enhancer factor 2 C (MEF2C) in a Ca^{2+}-dependent way to regulate cardiac protein expression [15]. In addition, the interaction between TRPC channels and L-type voltage-gated Ca^{2+} channel plays a key role in regulating pacemaking, conduction, ventricular activity, and contractility of the developing heart [16]. It has also been reported that activation of the Ca^{2+}-activated potassium channels of small and intermediate conductance (SKCas) can promote pluripotent stem cells to differentiate preferentially into nodal-like CMs [17].

The activation of soluble guanylatecyclase (sGC) is one of the main intracellular effects of NO, and active sGC subsequently induces an increase in cyclic GMP (cGMP) level to modulate the downstream targets [18]. Since the sGC/cGMP pathway can markedly increase intracellular Ca^{2+} concentration [19], the NO induced Ca^{2+} signaling might contribute to various cellular functions mediated by NO. Previous studies have shown that NO

Abbreviations: NO, Nitric oxide; CM, cardiomyocyte; ES cells, embryonic stem cells; IP_3R3, IP_3 receptor 3; SNAP, S-Nitroso-N-acetyl-DL-penicillamine; shRNA, short hairpin RNA; EB, embryoid body; LIF, Leukemia inhibitory factor

* Corresponding author.
E-mail address: jianbyue@cityu.edu.hk (J. Yue).

http://dx.doi.org/10.1016/j.yexcr.2016.06.016
0014-4827/

directly activates the ryanodine receptor 1 (RyR1) to induce Ca^{2+} release from intracellular stores in the neuronal cells, and this Ca^{2+} increase is critical in cerebellar long-term potentiation and cell death regulation [20]. NO also causes Ca^{2+} dependent polarization of the uterus myocytes by increasing the permeability of sarcolemma to Ca^{2+} [21]. In addition, exogenous NO evokes Ca^{2+} release from ER stores via inositol 1, 4, 5-trisphosphate (IP_3) receptors (IP_3Rs) in the respiratory burst neutrophils [22]. Here we studied the role of Ca^{2+} signaling in the NO-induced CM differentiation of mouse ES cells, and found that IP_3R3-Ca^{2+} pathway is involved in NO-induced CM differentiation of ES cells.

2. Materials and methods

2.1. Cell culture

D3 ES cell line was a kind gift from Prof. Tsang SY of Chinese University of Hong Kong. The ES cells were maintained as described previously [23]. Prior to any experimental procedures, ES cells were cultured in ES medium (Dulbecco's Modified Eagle Medium plus 15% FBS (ES qualified, Invitrogen), 1% nonessential amino acids, 1% penicillin-streptomycin, 0.2% 2-mercaptoethanol, 1000 U/mL leukemia inhibitory factor (LIF)) on gelatin-coated plates for two passages. The pluripotency of ES cells were periodically assessed by alkaline phosphatase assay and Oct4 immunostaining.

2.2. Cardiomyocyte differentiation of mouse ES cells

CM differentiation of mouse ES cells was performed as described previously [23]. CMs present in the embryoid bodies (EBs) started to rhythmically contract around day 6. For the NO application, NO donor, S-Nitroso-N-acetyl-DL-penicillamine (SNAP) (250 μM), was added to the culture medium, and the medium was changed every other day.

2.3. IP_3R-shRNA lentivirus production and infection

Three optimal 21-mers were selected for each mouse *IP_3R* genes (Table S1). One 21-mer was selected in the *GFP* gene as a control. These sequences were then cloned into the pLKO.1 vector for shRNA expression. The shRNA lentivirus production was performed in 293 T cells as described previously [24]. For infection, ES cells were plated at a density of 3×10^5 cells/well in the 6-well plates. On the next day, 1 mL of concentrated shRNAs lentivirus was added to the cells in fresh medium containing 8 μg/mL polybrene. 48 h later, the infected cells were selected in fresh medium containing puromycin (2 μg/mL) for 2 days. The puromycin-resistant cells were pooled and the knockdown efficiency was verified by *quantitative real-time RT-PCR* analyses.

2.4. IP_3R1, 2, 3 triple knockdown

The DNA fragment containing three 19nt siRNA targeting IP3R1, IP3R2 and IP3R3, respectively (Table S2), was cloned into PB5-puro-tetON vector. The PB5-puro-tetON-siRNA construct and pN1-CMV-piggybac transpoase vector were co-transfected into mouse ES D3 by Lipofectamine® 3000 reagent, and cells co-transfected PB5-puro-tetON vector and pN1-CMV-piggybac transpoase vector were served as control. After 48 h, the transfection medium was removed and cells were selected in fresh medium containing puromycin (2 μg/mL) for 2 days. 2 μg/mL tetracycline was then applied to puromycin resistant ES cells for 4 days before real-time q-RT PCR analysis of knockdown efficiency or Ca^{2+} measurement.

2.5. Western blot analysis

Cells were lyzed in cold EBC lysis buffer and passed through a 21-gauge needle several times. Protein concentrations were determined by Bradford protein assay (Bio-rad). 30 μg of protein per lane was diluted in the standard SDS-sample buffer and subjected to electrophoresis on 10% SDS polyacrylamide gels. Proteins were then transferred to an Immobilon PVDF membrane (*Millipore, Billerica, MA*), blocked with 5% milk in TBST (20 mM Tris, 150 mM NaCl, pH 7.6), and incubated with the primary antibody (Cardiac Troponin I, ab19615, Abcam, 1:1000 dilution; myosin light chain 3, ab680, Abcam, 1:500 dilution; Raf-B, SC-9002, Santa Cruz Biotechnology, 1:1000 dilution; IP_3R3, 610313, BD biosciences, 1:1000 dilution) at 4 °C overnight. After washing with TBST for three times, the blots were probed with a secondary antibody (1:5000 dilution) for detection by chemiluminescence.

2.6. RNA isolation, RT-PCR, and quantitative real-time RT-PCR

Total RNA of ES cells and EBs at specified differentiated days was extracted using RNA extraction kit (Life Technologies). RT-PCR of *IP_3R1*, *2* or *3* using SuperScript® One-Step RT-PCR kit (Life Technologies) was performed with Takara PCR Thermal Cycler Dice (Takara). The quantitative real-time RT-PCR using the SuperScript® III Platinum®One-Step Q-RT PCR Kit (Invitrogen Life Technologies) was performed in MiniOpticon™ Real-time PCR Detection System (Bio-RAD) according to the manufacturer's instructions. The primers for detecting *IP_3R1*, *IP_3R2*, *IP_3R3*, *mef2c*, *NKX2.5*, *α-MHC*, and *β-actin* mRNAs were listed in Table S3. Relative gene expression was normalized to *β-actin* expression.

2.7. Immunohistochemistry

Cells seeded on glass coverslips were fixed for 15 min with 4% paraformaldehyde at room temperature (RT), washed twice with PBS, and permeabilized with PBST (PBS with 0.1% Triton X-100) for 30 min. Thereafter, the cover glasses were blocked PBST containing 1% normal donkey serum and 1% BSA for 1 h, and incubated with primary antibodies (cardiac Troponin T, MS-295-P, thermo, 1:500 dilution; OCT4, SC-5279, Santa Cruz Biotechnology 1:1000 dilution; SSEA-1, sc-21702, Santa Cruz Biotechnology 1:1000 dilution) for 2 h at RT, followed by secondary antibody (Alexa Fluor® 488 Goat Anti-Mouse IgG, A11008, Life Technologies, 1:500 dilution) incubation for 1 h at RT. DAPI was used to stain the nuclei. The inverted Olympus IX81 fluorescence microscope with a CellR image system was used to obtain and analyze images.

2.8. Flow cytometry analysis

EBs were digested into single cells by incubating with 1 mg/mL collagenase II (Invitrogen) at 37 °C for 30 min. The isolated single cells were then immunostained with cardiac Troponin T as described above and filtered through 40 μm cell strainer (BD Bioscience) to discard cell aggregates. Analyses were subsequently performed by the BD FACS Canto II analytic flow cytometer.

2.9. Ca^{2+} measurement

Cytosolic Ca^{2+} in ES cells were measured as described previously [25]. Briefly, ES cells were cultured in 24-well Corning™ flat bottom (corning 3524) plates at the density of 7×10^4 cells/well in regular medium overnight and were labeled with 2 μM Fura-2 AM (*Invitrogen*) in Hanks' balanced salt solution (HBSS) at room temperature for 30 min. The cells were then washed with HBSS three times and incubated at room temperature for another 10 min. Cells were put on the stage of an Olympus inverted