Biochemical and Biophysical Research Communications xxx (2015) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Mitochondrial Ca^{2+} uniporter is critical for store-operated Ca^{2+} entry-dependent breast cancer cell migration

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ARTICLE INFO

Article history:
Received 11 January 2015
Available online xxx

Keywords:
MCU
Breast tumor
Migration
SOCE

ABSTRACT

Metastasis of cancer cells is a complicated multistep process requiring extensive and continuous cytosolic calcium modulation. Mitochondrial Ca^{2+} uniporter (MCU), a regulator of mitochondrial Ca^{2+} uptake, has been implicated in energy metabolism and various cellular signaling processes. However, whether MCU contributes to cancer cell migration has not been established. Here we examined the expression of MCU mRNA in the Oncomine database and found that MCU is correlated to metastasis and invasive breast cancer. MCU inhibition by ruthenium red (RuR) or MCU silencing by siRNA abolished serum-induced migration in MDA-MB-231 breast cancer cells and reduced serum- or thapsigargin (TG)-induced store-operated Ca2+ entry (SOCE). Serum-induced migrations in MDA-MB-231 cells were blocked by SOCE inhibitors. Our results demonstrate that MCU plays a critical role in breast cancer cell migration by regulating SOCE.

1. Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths worldwide [1]. Despite the significant improvement in both diagnostic and therapeutic modalities for breast cancer patients, metastasis still represents as the major cause of mortality. Therefore, a better understanding of molecular mechanisms involved in cancer metastasis will contribute to develop more effective and rational therapies for breast cancer.

Extracellular Ca^{2+} entry and subsequent elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) play a critical role in many aspects of cancer traits [2–4]. Previous researches have shown that Ca^{2+} influx is essential for migration of various cell types, including cancer cells [5,6]. Mitochondria are known not only to be a passive Ca^{2+} sink to store Ca^{2+} ions, but also regulates cellular Ca^{2+} homeostasis. Mitochondrial Ca^{2+} homeostasis plays a key role in the regulation of aerobic metabolism and cell survival [7]. Four categories of molecules have been identified to be related to mitochondrial Ca^{2+} influx mechanisms, including mitochondrial ryanodine receptor, mitochondrial uncoupling proteins, LETM1(Ca^{2+}/H^{+} exchanger), and Mitochondrial Ca^{2+} uniporter (MCU) [8]. MCU is a main Ca^{2+} channel existing in mitochondrial inner membrane, which is highly selective for Ca^{2+} and sensitive to a commonly used inhibitor, ruthenium red (RuR). Until recently, the molecular identity of MCU was just unveiled [9,10].

Increasing evidence has been showing the participation of mitochondria in the control of global cellular Ca^{2+} homeostasis by regulating the store-operated calcium entry (SOCE) [11–13]. In nonexcitable cells, SOCE is the predominant Ca^{2+} entry mechanism, which is activated when intracellular stores, such as endoplasmic reticulum (ER), release their stored Ca^{2+} [14]. ER Ca^{2+} stores could become depleted physiologically as a consequence of signaling mechanisms from activated G-protein-coupled or tyrosine kinase receptors [15]. Respiring mitochondria rapidly take up Ca^{2+} released from the stores, resulting in more extensive store depletion and thus robust activation of SOCE. Furthermore, by buffering

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http://dx.doi.org/10.1016/j.bbrc.2015.01.092
0006-291X/

some of the incoming Ca^{2+}, mitochondria reduce Ca^{2+}-dependent inactivation of the calcium release-activated calcium (CRAC) channels, resulting in more prolonged Ca^{2+} influx [11]. Recently, SOCE was found to be critical for breast cancer cell migration and metastasis [5]. Although MCU is implicated in the regulation of SOCE and intracellular Ca^{2+} homeostasis, a role of MCU in the context of cancer cell migration has not been defined.

Here, we determined whether MCU expression is increased in metastatic human breast cancer and investigated the contribution of MCU in regulating breast cancer cell migration. MDA-MB-231 cells were employed in our research because they are more invasive than other breast cancer cells. By using MCU inhibitor RuR and down-regulating of the expression of MCU protein by specific siRNAs, we demonstrated that MCU inhibition and silencing abrogate serum-induced SOCE and cell migration in MDA-MB-231 cells. This is the first report demonstrating that MCU protein is critically involved in cancer cell migration via regulating SOCE.

2. Materials and methods

2.1. Reagents and antibodies

$GdCl_3$, $CaCl_2$, RuR, SKF96365 and 2-APB were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 culture medium, fetal bovine serum (FBS), Lipo2000 transfection Kit and endotoxin-free PBS (pH 7.4) without calcium and magnesium were acquired from Life Technologies (Carlsbad, CA, USA). Fluo-4 acetoxymethyl ester (Fluo-4/AM) and rhod-2—acetoxymethyl ester (rhod-2/AM) were purchased from Dojindo Laboratories (Kumamoto, Japan). All working solutions were prepared on the day of experiment. Rabbit polyclonal antibody to MCU and mouse polyclonal antibody to GAPDH were from Sigma and Abcam (Cambridge, MA, USA) respectively.

2.2. Oncomine data mining

We analyzed the gene expression datasets of the cancer tissue microarray database in Oncomine™ (Compendia Bioscience, Ann Arbor, MI, http://www.oncomine.org [16,17]. The Oncomine is a database bank in that gene expression profile data can be queried and analyzed for selected genes across the databases available to the public. These datasets provide fold-change values of gene expression and statistical significance as determined by P values, involving comparison between cancer and normal samples, and subtypes of breast cancer in relation to clinical-pathological significance of the patients. We extracted and compared MCU expression in normal vs. breast cancer tissues, ductal carcinoma in situ vs. invasive ductal breast cancer.

2.3. Cell culture and cell viability assays

MDA-MB-231 cells (ATCC® HTB-26™) were maintained in Dulbecco's modified Eagle's medium (DMEM) with penicillin G, streptomycin sulfate, 10% fetal bovine serum, and 4 mmol/L L-glutamine. Cell viability was evaluated by the MTT assay. The experiment was repeated three times.

2.4. $[Ca^{2+}]_c$ and $[Ca^{2+}]_{mt}$ measurements

Intracellular Ca^{2+} was monitored using Ca^{2+} sensitive fluorescent indicator, Fluo-4/AM by an inverted laser scanning confocal microscope (Olympus, FV1000-IX71, Japan). Cells were loaded in PBS with 2 μM Fluo-4/AM at 37 °C for 30 min in dark, rinsed twice with PBS and kept at room temperature for 20 min to allow de-esterification of Fluo-4 ester. Before experiment, Ca^{2+}-free PBS with 0.3 mM EGTA was added. Green fluorescence of Fluo-4 was excited by a 10 mW multi-tune argon laser at 488 nm, and emitted fluorescence was recorded through a 525 nm channel. For imaging using Fluo-4, $[Ca^{2+}]_i$ changes are presented as F/F_0 after background subtraction, where F is the change in fluorescence signal intensity and F_0 is the baseline as calculated by averaging 20 frames before stimulus application. To determine mitochondrial Ca^{2+} $[Ca^{2+}]_{mt}$, MDA-MB-231 cells were incubated with the cell-permeable Ca^{2+}-indicator rhod-2 AM (3 μmol/L; dissolved in DMSO) in DMEM for 1 h at 37 °C, and then washed for 0.5 h in rhod-2-free DMEM to allow de-esterification. To determine $[Ca^{2+}]_{mt}$, rhod-2 was excited at λexc = 540 nm, and fluorescence was recorded at λem = 605 nm [18].

2.5. In vitro wound-healing assay

Cells in medium containing 3% FBS were seeded into wells of 24-multiwell plates (Becton Dickinson). After the cells grew to confluence, wounds were made by sterile pipette tips. Cells were washed with PBS and refreshed with medium with or without 3% FBS. After 48 h incubation at 37 °C, the cells were fixed and photographed as described previously [19].

2.6. Transwell cell-migration assay

Transwell migration assays were performed as described previously [20]. The migration of MDA-MB-231 cells was assessed using 24-well transwell with an 8-μm pore size (Millipore). A total of 4×10^4 cells in serum-free DMEM (100 μl) were added to the upper chamber, and 500 μl of DMEM with 10% FBS were added to the lower chamber. Transwells were incubated for 18 h at 37 °C. Cells on the inside of the transwell inserts were removed with a cotton swab, and cells on the underside of the insert were fixed and stained. Photographs of three random fields were taken, and the cells were counted to calculate the average number of cells that had transmigrated.

2.7. Cell transfection

RNAi against MCU was performed in MDA-MB-231 cells using Lipo2000 transfection Kit. To silence MCU specific siRNA were designed: siRNA-MCU#1: nucleotides 899—917 of the corresponding mRNA (5′-GCCAGAGACAGACAAUACUtt-3′ and 3′-ttCGGUCUCUGUCUGUUAUGA-5'. siRNA-MCU#2: nucleotides 360—378 of the corresponding mRNA (5′-GGGAAUUGACAGAGUUGCUtt-3′ and 3'-ttCCCUUAACUGUCUCAACGA-5′). The non-targeting siRNA (scrambled) is the following: 5′-GCCUAAGAACGACAAAUCAtt-3′ and 3'-ttCGGAUUCUUGCUGUUUAGU-5' [10]. These were transfected into cells in the absence of antibiotics using Lipofectamine 2000 according to the manufacturer's instructions. The scrambled siRNA were used as controls.

2.8. Western blotting

Cell lysates in RIPA buffer (50 μg of proteins in denaturing conditions) were subjected to SDS-PAGE (10%) and electro-transferred onto polyvinylidene fluoride membranes. After blocking with 5% nonfat dry milk dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 h at room temperature, Western blots were probed overnight at 4 °C, with specific primary antibodies in TBST containing 5% BSA. The primary antibody used was rabbit polyclonal MCU antibody (Cat# HPA016480, 1:200, Sigma, USA). GAPDH (Cat# ab8245, 1:5000, abcam, USA) was used for Western blot loading control. Detection was performed with the enhanced chemiluminescence reagent (KeyGEN).

Please cite this article in press as: S. Tang, et al., Mitochondrial Ca^{2+} uniporter is critical for store-operated Ca^{2+} entry-dependent breast cancer cell migration, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.01.092