Molecular Immunology 87 (2017) 23–32

Contents lists available at ScienceDirect

Molecular Immunology

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

Phosphorylation of dynamin-related protein 1 at Ser616 regulates mitochondrial fission and is involved in mitochondrial calcium uniporter-mediated neutrophil polarization and chemotaxis

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ARTICLE INFO

Article history:
Received 18 January 2017
Received in revised form 14 March 2017
Accepted 28 March 2017

Keywords:
Neutrophil polarization
Inflammation
Mitochondrial fission
Mitochondrial calcium uniporter (MCU)
Dynamin-related protein 1 (Drp1)

ABSTRACT

During an inflammatory response, polarization of neutrophils is necessary for effective chemotaxis and bacterial endocytosis. Ca^{2+} uptake into mitochondria through the mitochondrial calcium uniporter (MCU) is crucial for cell metabolism, signaling and survival; however, the physiological role of MCU in human neutrophils remains unclear. Here we show that MCU is vital for the polarization and chemotaxis of neutrophils. Activation of MCU by spermine promotes neutrophil polarization and chemotaxis, whereas inhibition of MCU by Ru360 blunts both processes. We also provide evidence that this role of the MCU in neutrophils may result from modulation of mitochondrial fission by increased levels of pDrp1 S616 via accumulation of Ca^{2+} into the mitochondrial matrix. Thus, our study identifies the dependence of neutrophil polarization and chemotaxis on the MCU and highlights the importance of regulating mitochondrial fission during the anti-inflammatory cascade in human neutrophils.

1. Introduction

Neutrophils are the most abundant circulating leukocytes and the fastest moving cells in humans. They are key players in innate immunity and are rapidly recruited to infection and injury sites (Mollinedo et al., 1999; Nathan, 2006). At the very start of the process, neutrophils undergo asymmetric changes in cell morphology (polarization) and migrate (chemotaxis) in response to an extracellular directional chemoattractant (Lauffenburger and Horwitz, 1996; Weiner et al., 1999). Neutrophils can be activated by a variety of chemoattractants, such as complement fragment C5a, formylated peptides like *N*-formyl-Met-Leu-Phe (fMLP), platelet activating factor and interleukin 8 (IL-8) (Bickel, 1993; Henson, 1981; Shuster et al., 1997; Zhang et al., 1995). Of these, fMLP is a prototypic chemotactic factor (Seely et al., 2003). To achieve polarization and chemotaxis, neutrophils organize a defined anterior (leading edge) and posterior (uropod) to form spatial and functional asymmetry (Lauffenburger and Horwitz, 1996; Sanchez-Madrid and Del, 1999). However, abnormal activation of neutrophils may result in autoimmune diseases (Kaplan, 2013). Therefore, elucidating the mechanisms regulating neutrophil polarization and chemotaxis could potentially lead to novel therapeutic strategies for counteracting chronic activation of neutrophils which can cause tissue damage.

The mitochondrial calcium uniporter (MCU) channel is responsible for Ru360 and spermine-sensitive mitochondrial calcium uptake (Kirichok et al., 2004; Zhang et al., 2006). In 2011, Rizzuto and Mootha identified the key protein of the MCU, known as CCDC109A, as necessary and sufficient for mitochondrial Ca^{2+} uptake (Baughman et al., 2011; De Stefani et al., 2011). Later, investigations affirmed that the MCU was a multimer and the components have since been identified. They coordinately regulate mitochondrial Ca^{2+} uptake to balance mitochondrial function (De Stefani et al., 2015). However, how the MCU contributes to neutrophil polarization and chemotaxis remains unclear.

Dynamin-related protein 1 (Drp1) is one of the major regulatory proteins required to maintain mitochondrial morphology, maintaining the balance between continuous fusion and fission (Chen and Chan, 2004; Praefcke and McMahon, 2004). Drp1 requires activation by multiple post-translation modifications and receptor interactions before this GTPase can regulate mitochondrial fission (Cho et al., 2013). Phosphorylation of serine 637 (S637) of human Drp1 suppresses mitochondrial translocation and GTPase activity (Chang and Blackstone, 2007; Cribbs and Strack, 2007). However, phosphorylation at serine 616 (S616) enhances the activity of Drp1 and results in mitochondrial fragmentation (Cho et al., 2013). As a selective chemical inhibitor of Drp1, mdivi-1 disrupts the mitochondrial network by blunting mitochondrial

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http://dx.doi.org/10.1016/j.molimm.2017.03.019
0161-5890/

fusion and fission (Park et al., 2011). Recent reports have suggested that mitochondrial fission mediated by the MCU may occur in the cerebral cortexes of rats and in the hippocampus during ischemia/reperfusion injury (Liang et al., 2014; Zhao et al., 2015).

It remains unknown whether mitochondrial fission is affected by the MCU in neutrophils and whether mitochondrial fission participates in human neutrophil polarization and chemotaxis. We incubated purified human neutrophils with spermine and Ru360 and observed changes to neutrophil motility and mitochondrial morphological alterations. We also used mdivi-1 to inhibit Drp1 to further investigate the hypothesis.

2. Material and methods

2.1. Reagents and antibodies

Spermine (S3256), Mdivi-1 (M0199) and fMLP (F3506) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ru360 (557440) was purchased from Calbiochem (Bad Soden, Germany). IL-8 was purchased from PeproTech (London, UK). Phosphate-buffered saline (PBS), Hank's buffered salt solution (HBSS) and d-Hank's buffered salt solution (dHBSS) were purchased from Genom (Hangzhou, China). Dextran T500 was purchased from Amersham Biosciences (Piscataway, NJ, USA). Rhodamine phalloidin (R415), Fluo 4-AM (F14217), Rhod 2-AM (R1244), DAPI (D1306) and MitoTrackerR probe (M7512) were purchased from Invitrogen (MA, USA). All protease inhibitors were obtained from Roche (Basel, Switzerland). Drp1, Opa1 and GAPDH antibodies were purchased from Abcam (Cambridge, MA, USA). MCU, phospho-DRP1 (Ser616) and phospho-DRP1 (Ser637) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Fis1 antibody was purchased from ProteinTech (Wuhan, China). Mfn1 and Mfn2 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). IRDye 800CW goat anti-rabbit IgG (H + L) and IRDye 680RD goat anti-mouse IgG (H + L) were purchased from LI-COR Biosciences (Lincoln, NE, USA).

2.2. Human neutrophil isolation

Human neutrophils were isolated from EDTA-K_2 anticoagulated venous blood (Nauseef, 2007) from healthy consenting adults following ethical guidelines at Southern Medical University. Briefly, erythrocytes were removed by dextran sedimentation (4.5% dextran/0.9% NaCl) followed by two rounds of hypotonic lysis using sterile double distilled water. With the exception of dextran sedimentation, the entire isolation procedure was performed at 4 °C. Purified neutrophils were resuspended in dHBSS without Ca^{2+} at a concentration of 1.0×10^7 cells/mL prior to use. More than 95% of the cells isolated were neutrophils, as assessed by Wright–Giemsa staining. Viability, determined by trypan blue exclusion, was >98%.

2.3. Zigmond polarization assay

Neutrophil polarization was quantified in Zigmond chambers (Neuro probe, MD), as described previously (Zigmond, 1977). Briefly, a total of 1.0×10^6 cells were allowed to adhere to coverslips for 5 min at room temperature. The coverslips were then immediately inverted onto the chamber, assembled loosely. One channel of the chamber was filled with HBSS (vehicle) and the other was with 100 nM fMLP or IL-8 with indicated concentrations in HBSS to build chemoattractant gradient. After cells were exposed to the chemoattractant for 15 min, images were taken by using an inverted microscope with a 20 × objective (Olympus IX-71). Polarized cells in each image were counted. Polarized cells were defined as cells with morphological alterations and with length of at least twice that of the width, as well as movement in either any direction or in the direction of the chemoattractant (Heit et al., 2008). Cells with a leading edge and a trailing tail were scored as polarized. Analysis was performed in triplicate (at least), with a minimum of 150 cells in each of five random fields counted for all sets of conditions.

2.4. Transwell chemotaxis assay

Neutrophil chemotaxis was evaluated using a transwell chamber with a 3 μm pore size for neutrophils (Corning, USA). IL8 or fMLP at indicated concentrations were used as chemoattractants in the lower chamber and aliquots of neutrophils (1×10^6 cells/well) were added in the upper chamber. PBS served as a negative control. After incubation for 2 h at 37 °C, the filters were removed and fixed with ethanol before being stained with crystal violet. Neutrophils migrating through the polycarbonate membrane to the lower face were counted in five views per membrane using a phase contrast microscope, and expressed relative to the control.

2.5. F-actin polymerization detection

Preincubated cells were adhered to a confocal dish and fixed in freshly prepared 2% paraformaldehyde in PBS, followed by permeabilization with 0.5% Triton X-100 in PBS and conjugated to 0.3 μM rhodamine phalloidin to detect F-actin polymerization and DAPI to stain nuclei. Images were acquired on an Olympus FV1000 laser scanning confocal microscope (Olympus, FV1000-IX71, Japan) with a × 63/1.4 NA plan apochromat objective and processed using Olympus Fluoview software.

2.6. Cytosolic and mitochondrial Ca^{2+} measurement

Stimulated cells were incubated with 2 μM Fluo 4-AM and 2 μM Rhod 2-AM in HBSS for 30 min in the dark at 37 °C to label cytosolic Ca^{2+} and mitochondrial Ca^{2+}, respectively, followed by gently rinsing the cells with dHBSS three times to remove extracellular Ca^{2+}. Neutrophils were then maintained in dHBSS for mitochondrial Ca^{2+} measurements. Real-time fluorescence intensity of mitochondrial Ca^{2+} was monitored using a live plot recording platform under an inverted laser scanning confocal microscope. Ca^{2+} changes were presented as △F/F0 ratios based on baseline fluorescence subtraction.

2.7. Western blotting

Cell lysates in RIPA buffer were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) and electro-transferred onto a polyvinylidene fluoride membrane. After blocking with 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, Western blots were probed overnight at 4 °C, with specific primary antibodies in TBST containing 3% BSA. Detection was performed using a Li-COR odyssey infrared imaging system (LI-COR Biosciences).

2.8. Mitochondrial morphology

Pretreated neutrophils were conjugated to MitoTracker Red for 30 min in the dark at 37 °C, followed by washing twice in PBS. Cells were centrifuged and resuspended in PBS, then transferred into confocal dishes. fMLP was added to the dish and a uniform chemoattractant concentration was formed. After 15 min, cells were fixed in freshly prepared 2% paraformaldehyde in PBS and mitochondrial morphology was recorded using an Olympus FV1000 laser scanning confocal microscope with a × 100 oil