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Research paper

The F0F1 ATP synthase regulates human neutrophil migration through cytoplasmic proton extrusion coupled with ATP generation

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ABSTRACT

Cytoplasmic alkalinization and extracellular adenosine triphosphate (ATP) signals are required for migration of chemokineactivated neutrophils, but the precise functions remain unclear. In this work, the effect of the plasma membrane-expressed F0F1-ATP synthase (FATPase) on human neutrophils was examined. We found F-ATPase to be involved in cytoplasm proton extrusion and extracellular ATP generation. Oligomycin A, an F-ATPase inhibitor that blocks proton transfer, inhibited cytoplasmic alkalinization, extracellular ATP generation, adhesion and chemotaxis in *N*-formyl-Met-Leu-Phe (fMLP)-stimulated neutrophils; however, adenosine diphosphate (ADP), a substrate and activator of F-ATPase, had the opposite effect. Further analysis revealed that cell surface F-ATPase can translocate to the leading edge of directional fMLP-stimulated neutrophils toward ADP hydrolyzed from pannexin 1 channel-released ATP, followed by F-ATPase-catalyzed ATP regeneration using ADP and protons transferred from the cytoplasm. Therefore, the membrane-expressed F-ATPase regulates human neutrophil migration via cytoplasm proton extrusion and extracellular ATP generation.

1. Introduction

F-ATPase is one of the most thoroughly studied complexes in the mitochondrial inner membrane (Boyer, 1997). Interestingly, F-ATPase is also expressed in the plasma membrane of different cell types, including human umbilical vein endothelial cells (Moser et al., 2001; Moser et al., 1999), hepatocyte HepG2 cells (Martinez et al., 2003) and adipocytes (Kim et al., 2004), and is involved in diverse processes, such as lipid metabolism regulation, immune recognition and cell differentiation and death (Chi and Pizzo, 2006). Previous studies have reported that F-ATPase expressed on neutrophils may act as a receptor of angiostatin, a molecule that regulates cell migration (Benelli et al., 2002). The detailed mechanisms underlying this process remain unclear.

Neutrophil activation and migration is accompanied by metabolic acid generation, which is predominantly due to the respiratory burst and energy produced from glycolysis (El-Benna et al., 2016; Maianski et al., 2004). Cytoplasmic alkalinization has been unequivocally demonstrated in activated neutrophils, and hindering cytoplasm alkalization inhibits cell chemotaxis (Lardner, 2001), suggesting that activated neutrophils possess a proton-extrusion pathway. In addition, neutrophil activation and migration are accompanied by the release of ATP, which can amplify chemotactic signals and direct cell orientation via feedback through P2Y2 nucleotide receptors (Chen et al., 2006). Abolishing extracellular ATP with apyrase, an ATP hydrolytic enzyme, inhibits neutrophil chemotaxis. F-ATPase performs both proton transport and ATP generation, but its function on neutrophils has not been resolved.

The F-ATPase complex consists of two major components: the proton transport domain F0 and the ATP synthesis catalytic domain F1. The key role of F-ATPase in mitochondria is to transfer protons from the intermembrane space to the matrix for ATP synthesis; F-ATPase also exhibits this ability for proton transport and ATP generation on endothelial cells and adipocytes (Kim et al., 2004; Moser et al., 2001; Moser et al., 1999). Therefore, it is tempting to hypothesize that neutrophil-surface F-ATPase is required for chemotaxis regulation. The purpose of this study was to test this hypothesis and further explore the function of surface-localized F-ATPase on primary neutrophils. The

Abbreviations: ATP, adenosine triphosphate; F-ATPase, F0F1-ATP synthase; fMLP, *N*-formyl-Met-Leu-Phe; ADP, adenosine diphosphate; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PMSF, phenylmethane sulfonyl fluoride; TTFA, thenoyltrifluoroacetone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HBSS, Hank's balanced salt solution; BCECF-AM, (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; DAPI, 4′,6- diamidino-2-phenylindole

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findings will contribute to a better understanding of chemotaxis regulation in activated neutrophils.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), sodium dodecyl sulfate (SDS) and phenylmethane sulfonyl fluoride (PMSF) were purchased from Amresco (Solon, OH, USA). The SDS-PAGE kit was obtained from Beyotime (Shanghai, China). Tween 20, thenoyltrifluoroacetone (TTFA), polyformaldehyde, adenosine diphosphate (ADP), ATP, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), 4′,6-diamidino-2-phenylindole (DAPI), *N*-formyl-Met-Leu-Phe (fMLP), *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide, Sodium chloride (NaCl) and TRIS hydrochloride (Tris-HCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). (2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was obtained from AAT Bioquest (Sunnyvale, CA, USA). Nigericin was obtained from Molecular Probes (Eugene, OR, USA). Unless otherwise stated, all concentrations shown are the final concentrations.

2.2. Isolation of neutrophils

After approval from the Guangzhou Medical University Institutional Review Board, neutrophils were isolated from heparinized blood of healthy donors aged 25–35 years by density gradient centrifugation using a Human Peripheral Blood Neutrophil Isolation Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. After centrifugation, the neutrophil-containing band was carefully isolated and washed twice. Residual erythrocytes were removed by lysis in cold NH_4Cl buffer, followed by two careful washes in HBSS. The purity was > 98%, as determined by cell counting under a microscope, and the cell viability was > 95%, as assessed by trypan blue dye exclusion. After isolation, the cells were resuspended in HBSS at 1×10^7/ml on ice until use (< 2 h).

2.3. Real-time monitoring of intracellular pH

To measure intracellular pH, neutrophils were labeled with BCECF-AM as previously described (Hayashi et al., 2008). Briefly, purified neutrophils were resuspended in HBSS-buffered RPMI 1640 (1:1) in the presence of 5 μM BCECF-AM at 37 °C for 30 min. After incubation, the cells were washed once with HBSS to remove unbound dye. The BCECF-labeled cells (1×10^6/ml) were treated with different concentrations of oligomycin A, ADP in PBS (all ADP mentioned in this work contained 1:1 inorganic phosphate) or 200 μM ^{10}panx (ApexBio, Boston, MA, USA) for 10 min and stimulated with 100 nM fMLP for 3 min. Neutrophils treated with 0.1% DMSO with or without fMLP activation were used as the activated control and resting control, respectively. For monitoring average intracellular pH changes, the ratio of relative fluorescence intensity was measured at an emission of 525 nm and excitation of 490 and 440 nm using an LS55 fluorescence spectrometer (PerkinElmer, Waltham, MA, USA). For preparation of a calibration curve, cells were equilibrated in K^+ HBSS to various pH values (between 6.8 and 7.8) in the presence of nigericin (3 μM). The calibration values were fit to a standard curve, which was used to calculate unknown intracellular pH values (Chien et al., 2007). All cells were incubated and detected at 37 °C.

2.4. Extracellular ATP analysis

Freshly isolated neutrophils were resuspended in HBSS (1×10^6/ml), treated with 50 μg/ml oligomycin A, 100 μM ADP, 300 μM TTFA or 200 μM ^{10}panx for 10 min, and stimulated with 100 nM fMLP for 3 min. Neutrophils treated with 0.1% DMSO with or without fMLP were used as the activated control and resting control, respectively. All neutrophils were maintained at 37 °C and rotated at 5 rpm using a VSMR-XA vertical rotating mixer machine (Woxin, Jiangsu, China). The samples were then transferred to ice-cold centrifuge tubes at the indicated time points to stop the reaction and were immediately pelleted by centrifugation at 400*g* at 4 °C for 1 min. ATP in the supernatants was detected using a Luciferin-Luciferase-ATP Detection Kit (Beyotime, Shanghai, China) according the manufacturer's instructions, and chemiluminescence values were measured using a GloMax 20/20 Luminometer.

2.5. Mitochondrial membrane potential ($\Delta\Psi m$) assay

The fluorescent indicator rhodamine 123 (Molecular Probes, Invitrogen) was used to monitor$\Delta\psi m$ during cell stimulation. Freshly isolated neutrophils were resuspended in HBSS, incubated with rhodamine 123 (5 μg/ml) at 37 °C for 30 min, washed, and resuspended in HBSS (1×10^6/ml). Rhodamine 123-labeled cells were treated with 0.1% DMSO, 50 μg/ml oligomycin A, 100 μM ADP, 300 μM TTFA and 10 μM CCCP for 10 min respectively, followed by stimulating with 100 nM fMLP for 3 min. Immediately after removal of aggregated cells by filtration through a 35-μm cell strainer snap cap on a flow cytometer tube (Falcon, Corning, NY, USA), rhodamine 123 fluorescence was analyzed using a BD Accuri C6 Flow Cytometer (BD Biosciences). Green (FL1) fluorescence channels were used to detect rhodamine 123 fluorescence. After flow cytometry, cell viability was assessed in each group using trypan blue exclusion assays to excluded interference of dead cells.

2.6. Neutrophil chemotaxis assays

Transwell assays were performed in 24-well Corning Costar plates containing filters with a 3.0-μm pore-size polycarbonate membrane. fMLP (10 nM) and human interleukin-8 (0.5 ng/ml) in HBSS were added to the bottom chamber as chemoattractants (500 μl/well). A suspension of neutrophils (200 μl; 5×10^6/ml) in HBSS containing 50 μg/ml oligomycin A or 100 μM ADP was added to each well of the upper chamber; a suspension of neutrophils in HBSS containing 0.1% DMSO was used as a control. After incubation for 1 h at 37 °C, the chamber was removed, and the neutrophils in the bottom chamber were collected and centrifuged at 15,000xg for 10 s at room temperature. The elastase activity of the lysed cell suspension in the bottom chamber was used as an indicator of the number of migrated cells as previously described (Chen et al., 2006). Briefly, after careful removal of the supernatant, the cells were mixed with lysis buffer consisting of 100 mM NaCl, 50 mM Tris-HCl and 0.05% (v/v) Triton X-100 (pH 7.4). Then, the elastase-specific chromogenic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide was added to the mixture at a final concentration of 1 mM. The mixture was then incubated for 30 min at room temperature, and the change in optical density was measured at a wavelength of 405 nm after each treatment.

2.7. Neutrophil adhesion assay

To investigate the effects of surface-expressed F-ATPase on neutrophil adhesion, freshly isolated neutrophils were activated by the addition of 10 nM fMLP-containing HBSS, and aliquots were seeded into 20 μg/ml fibronectin-coated 24-well plates (5×10^5 cells/well) in the presence of 50 μg/ml oligomycin A or 100 μM ADP. Suspensions of neutrophils in 0.1% DMSO-containing HBSS were used as controls. The cells were incubated for 30 min at 37 °C and gently washed three times with HBSS to remove non-adherent cells. The number of adherent neutrophils in each well was measured in the same manner as described above for the neutrophil chemotaxis assay.