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Impaired oxidative phosphorylation regulates necroptosis in human lung epithelial cells

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ABSTRACT

Cellular metabolism can impact cell life or death outcomes. While metabolic dysfunction has been linked to cell death, the mechanisms by which metabolic dysfunction regulates the cell death mode called necroptosis remain unclear. Our study demonstrates that mitochondrial oxidative phosphorylation (OXPHOS) activates programmed necrotic cell death (necroptosis) in human lung epithelial cells. Inhibition of mitochondrial respiration and ATP synthesis induced the phosphorylation of mixed lineage kinase domain-like protein (MLKL) and necroptotic cell death. Furthermore, we demonstrate that the activation of AMP-activated protein kinase (AMPK), resulting from impaired mitochondrial OXPHOS, regulates necroptotic cell death. These results suggest that impaired mitochondrial OXPHOS contributes to necroptosis in human lung epithelial cells.

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1. Introduction

Mitochondrial energy metabolism serves as a critical process for the acquisition and utilization of energy required for cell viability, growth, and reproduction. Oxidative phosphorylation (OXPHOS) represents a major metabolic pathway in the mitochondria for the production of energy by oxidation of nutrients to generate ATP [1,2]. The amount of ATP synthesis during OXPHOS exceeds that generated by glycolysis or fatty acid β-oxidation. OXPHOS is driven by five major protein complexes (complexes I–IV) that constitute the electron transport chain (ETC) in the mitochondria [1]. The ATP synthase (complex V) localizes to the inner membrane of the mitochondria [1,2]. Additionally, AMP-activated protein kinase (AMPK) serves as one of the central regulators of cellular energy metabolism [3]. AMPK responds to activation by various types of cellular stress. When nutrients are depleted, AMPK acts as a metabolic checkpoint that inhibits cell growth [4]. Reduced intracellular ATP levels lead to the activation of AMPK *via* Thr-172 phosphorylation [3,4].

Mitochondria are crucial components of cell death pathways, including apoptosis and necrosis. These two major types of cellular death are distinguished on the basis of morphological and biochemical features [5–8]. Apoptosis refers to a programmed cell death requiring the activation of proteases (caspases), and which is characterized by membrane blebbing, cell shrinkage, chromatin fragmentation, nuclear breakdown, and cellular disintegration to form apoptotic bodies [9–11]. In contrast, necrotic death results from exposure to harmful chemical or physical factors, and is characterized by cell swelling, membrane damage, organelle dysfunction, ATP depletion, and extracellular leakage of the cytosol, which may promote inflammation [12–14]. While apoptosis is programmed by molecular events, necrosis was classically defined as a random and unregulated degenerative process [15,16]. Emerging studies have identified a genetically-regulated form of necrotic cell death termed necroptosis [17]. Cells undergoing necroptosis display activation of apoptotic pathways, yet terminate in a necrosis-like cell death [18,19]. Necroptosis is activated by the formation of a necrosome complex consisting of receptor-interacting protein 1 and 3 (RIP1 and RIP3) [20,21]. The RIP1-RIP3 complex recruits the mixed lineage kinase domain-like protein (MLKL) [22,23], a key regulator of necroptosis. The phosphorylation of MLKL by necrosome formation is regarded as the critical step in the initiation of necroptosis [24–27]. The mechanisms by which the

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mitochondrial metabolic pathway regulates the activation of necroptotic cell death remain unclear.

In the current study, we demonstrate that inhibition of OXPHOS can activate MLKL-dependent necroptosis in human lung epithelial cells. We show that pharmacologic inhibition of OXPHOS with mitochondrial uncoupling agents can induce necroptotic cell death. Furthermore, we found that the activation of AMPK resulting from impaired mitochondrial OXPHOS contributes to necroptotic cell death. Our results, taken together, identify inhibition of OXPHOS as an underlying metabolic mechanism for the activation of necroptosis.

2. Materials and methods

2.1. Reagents and antibodies

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (C2759), Oligomycin A (75351) and AICAR (A9978) were from Sigma–Aldrich (St Louis, MO, USA). Recombinant Human TNF-α (210-TA-020, R&D systems), 5-Aminoindole for Smac mimetic (A59654, Sigma–Aldrich), Z-VAD-FMK (2163, Tocris Bioscience) and Necrosulfonamide (480073, EMD Millipore Corporation, Billerica, MA, USA) were used. The following antibodies were used: polyclonal rabbit anti-phospho MLKL antibody (Thr-357) (ABC234, EMD Millipore Corporation, Billerica, MA, USA), polyclonal rabbit anti-MLKL antibody (M6697, Sigma–Aldrich), AMPK and ACC antibody sampler kit for phospho-AMPKα and AMPKα (#9957, Cell Signaling Technology), Total OXPHOS Rodent WB antibody cocktail for mitochondrial electron transport chains (ab110413, Abcam), polyclonal rabbit anti-Tom20 Antibody (sc-11415, Santa Cruz Biotechnology), and monoclonal mouse anti-β-actin (A5316, Sigma–Aldrich).

2.2. Cell culture

Human Beas-2B bronchial epithelial cells (CRL-9609™, ATCC) were cultured in DMEM media (Invitrogen, Life Technologies, Grand Island, NY, USA) containing 10% (*vol/vol*) heat-inactivated FBS, 100 units/ml penicillin, 100 mg/ml streptomycin.

2.3. Immunoblot analysis

Cells were harvested and lysed in RIPA Buffer (R0278, Sigma–Aldrich) and then briefly sonicated. Lysates were centrifuged at 15,300 $\times$ *g* for 10 min at 4 °C, and the supernatants were obtained. The protein concentrations of the supernatants were determined using the Bradford assay (Bio-Rad Laboratories). Proteins were electrophoresed on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and transferred to Protran nitrocellulose membranes (10600001, GE Healthcare Life Science, Piscataway, NJ, USA).

2.4. Glycolytic and mitochondrial function assay

For the glycolytic and mitochondrial function assay, cells (2×10^4 cells/well) were plated on XF96 cell culture microplates (101085-004, Seahorse Bioscience, Billerica, MA, USA). ECAR and OCR, as parameters of glycolytic flux or mitochondrial respiration, respectively, were measured on a Seahorse XF96 Bioanalyzer, using the XF Glycolysis Stress Test Kit according to the manufacturer's instructions (102194-100, Seahorse Bioscience).

2.5. Cell cytotoxicity assay

Cell cytotoxicity was measured from cell culture medium by LDH-Cytotoxicity Colorimetric Assay Kit II (#K313-500, BioVision, Milpitas, CA) according to the manufacturer's instructions.

2.6. Apoptosis/necrosis assay

Apoptosis/Necrosis of cells was measured by GFP CERTIFIED® Apoptosis/Necrosis detection kit for microscopy and flow cytometry (ENZ-51002-100, Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions.

2.7. Statistical analysis

All data are mean ± s.d., combined from three independent experiments. All statistical tests were analyzed by Student's two-tailed t-test for comparison of two groups, and analysis of variance (ANOVA) (with post hoc comparisons using Dunnett's test), using a statistical software package (GraphPad Prism version 4.0) for comparison of multiple groups. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Mitochondrial respiration is suppressed in necroptotic cell death

To investigate whether metabolic stress is linked to necroptotic cell death in human bronchial epithelial cells (Beas-2B), we used a TNF-induced necroptosis model that employs a combination of TNF, Smac mimetic and Z-VAD treatments [24]. We analyzed the change of mitochondrial respiration and glycolysis in Beas-2B cells treated with TNF, Smac mimetic and Z-VAD for 4 h. We measured the mitochondrial oxygen consumption rate (OCR) as an index of mitochondrial respiration, and the extracellular acidification rate (ECAR), as a measure of lactate production. The mitochondrial OCR was significantly suppressed by TNF, Smac mimetic and Z-VAD treatment relative to the vehicle-treated control cells (Fig. 1A). In contrast, the ECAR measured after TNF, Smac mimetic and Z-VAD treatment was not changed compared to the control (Fig. 1B). To confirm that the observed changes in the mitochondrial OCR correspond to cell death, we analyzed cell cytotoxicity after treatment with TNF, Smac mimetic and Z-VAD treatment. Cell cytotoxicity was quantitatively measured by lactate dehydrogenase (LDH) released into the culture media from damaged cells. Treatment with TNF, Smac mimetic and Z-VAD increased cell death (Fig. 1C), as previously described [24]. Next, we analyzed whether the cell death induced by TNF, Smac mimetic and Z-VAD treatment (Fig. 1C) corresponds to necroptotic cell death. The number of cells staining for both apoptosis and necrosis (Annexin V^+/7-AAD^+) was increased 2.3 fold from 0.7% in the vehicle control to 0.3% after TNF, Smac mimetic and Z-VAD treatment for 4 h (Fig. 1C). These results suggest that mitochondrial respiration was suppressed in cells undergoing necroptotic cell death.

3.2. Impaired OXPHOS activates necroptotic cell death

Since mitochondrial respiration is regulated by OXPHOS [1,2], we investigated whether the inhibition of OXPHOS can activate necroptotic cell death in Beas-2B cells. We inhibited OXPHOS activity using carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a chemical inhibitor of OXPHOS [28]. We measured the activation of necroptosis in cells treated with CCCP for 4 h. The phosphorylation of MLKL was dose-dependently increased by CCCP treatment

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