Biochemical and Biophysical Research Communications 464 (2015) 875–880

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

# Impaired oxidative phosphorylation regulates necroptosis in human lung epithelial cells

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ARTICLE INFO

*Article history:*
Received 1 July 2015
Accepted 9 July 2015
Available online 14 July 2015

*Keywords:*
Mitochondria
Oxidative phosphorylation
AMPK
Necroptosis

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.



## 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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http://dx.doi.org/10.1016/j.bbrc.2015.07.054
0006-291X/

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 × *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 ($2 \times 10^4$ 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

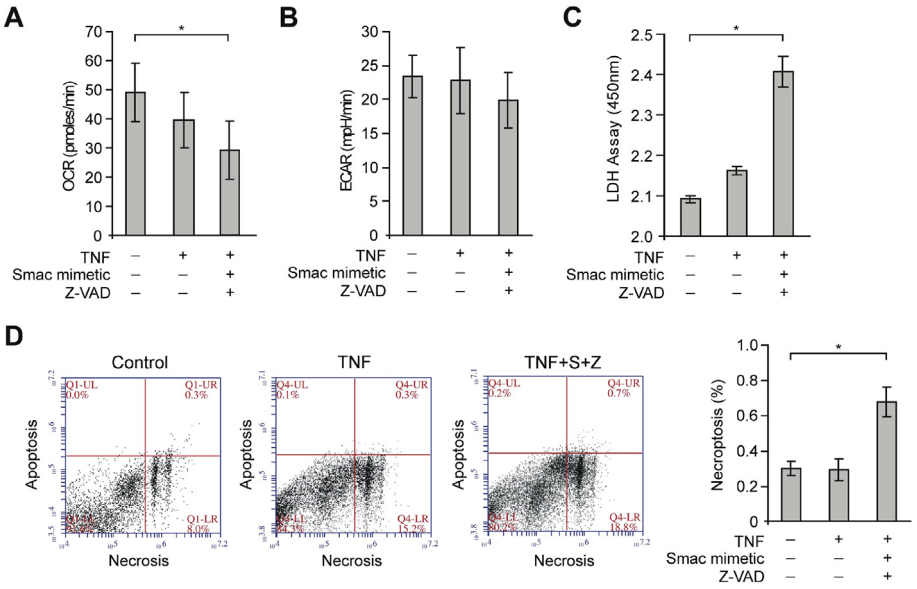


**Fig. 1.** Mitochondrial respiration was suppressed in necroptotic cell death. (A) OCR was measured in Beas-2B cells treated with TNF (10 ng/mL), Smac mimetic (10 μM), and Z-VAD (10 μM), or vehicle (DMSO) for 4 h. Data are mean ± s.d. $^*P < 0.05$ by ANOVA. (B) ECAR was measured in Beas-2B cells treated with TNF (10 ng/mL), Smac mimetic (10 μM), and Z-VAD (10 μM), or vehicle (DMSO) for 4 h. Data are mean ± s.d. (C) Cytotoxicity assay of Beas-2B cells treated with TNF (10 ng/mL), Smac mimetic (10 μM), and Z-VAD (10 μM) or vehicle (DMSO) for 4 h using LDH levels in the culture medium. $^*P < 0.05$ by ANOVA. (D) Flow cytometry analysis for apoptosis/necrosis detection of Beas-2B cells treated with TNF (10 ng/mL), S, Smac mimetic (10 μM), and Z, Z-VAD (10 μM) or vehicle (DMSO) for 4 h and then stained with Annexin V and red-emitting dye 7-AAD. Data are mean ± s.d. $^*P < 0.05$ by ANOVA.

(0–20 μM) relative to the vehicle control (Fig. 2A). To confirm that the observed changes in the phosphorylation of MLKL correspond to cell death, we analyzed cell cytotoxicity after CCCP treatment. Similar to induction of MLKL phosphorylation, CCCP dose-dependently increased cell death (Fig. 2B). Next, we analyzed whether CCCP-induced cell death is dependent on necroptotic cell death, by measuring cell death in cells pre-treated with necrosulfonamide, a MLKL inhibitor, prior to CCCP treatment. Necrosulfonamide significantly suppressed CCCP-induced cell death (Fig. 2C). These results suggest that impaired OXPHOS activated necroptotic cell death.

To confirm that CCCP treatment inhibits OXPHOS leading to changes in mitochondrial energy metabolism, we analyzed mitochondrial respiration and ATP synthesis in Beas-2B cells treated with CCCP. First, we measured the mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), as a

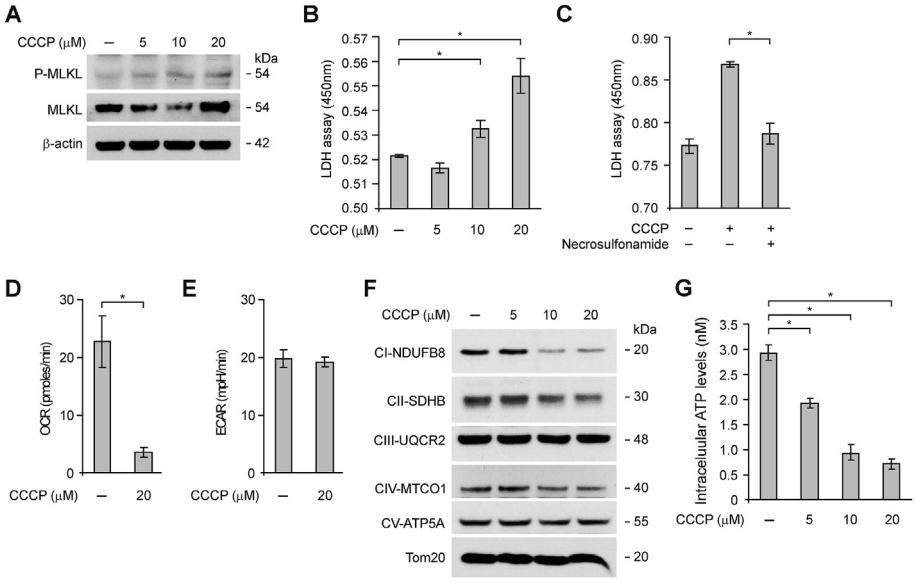


**Fig. 2.** Inhibition of oxidative phosphorylation induced mitochondrial dysfunction. (A) Immunoblot analysis for phosphorylated MLKL and MLKL in cell lysates from Beas-2B cells treated with CCCP (5–20 μM) or vehicle (DMSO) for 4 h. β-actin served as the standard. (B) Cytotoxicity assay of Beas-2B cells treated with CCCP (5–20 μM) or vehicle (DMSO) for 4 h using LDH levels in the culture medium. $^*P < 0.05$ by ANOVA. (C) Cytotoxicity assay of Beas-2B cells pre-treated with necrosulfonamide (1 μM) before CCCP (20 μM, 4 h) treatment using LDH levels in the culture medium. $^*P < 0.05$ by ANOVA. (D) OCR was measured in Beas-2B cells treated with CCCP (10 μM) or vehicle (DMSO) for 4 h. Data are mean ± s.d. $^*P < 0.05$ by ANOVA. (E) ECAR was measured in Beas-2B cells treated with CCCP (10 μM) or vehicle (DMSO) for 4 h. Data are mean ± s.d. (F) Immunoblot analysis for OXPHOS enzymes of cell lysates from Beas-2B cells treated with CCCP (0–20 μM) for 4 h. Tom20 served as the standard. (G) Intracellular ATP production in Beas-2B cells treated with CCCP (0–20 μM) for 4 h. $^*P < 0.05$ by ANOVA.

measure of lactate production in Beas-2B cells treated with CCCP (10 μM) for 4 h. The mitochondrial OCR was significantly suppressed by CCCP treatment relative to the vehicle treated control cells (Fig. 2D). In contrast, the ECAR measured after CCCP treatment was not changed compared to the control (Fig. 2E). Since mitochondrial OCR was suppressed by CCCP (Fig. 2D), we also examined the regulation of the protein expression of ETC complexes, which drive OXPHOS, in mitochondria treated with CCCP (0–20 μM) for 4 h. Importantly, CCCP suppressed the expression of the CI subunit ((NADH dehydrogenase (ubiquinone) 1 beta subcomplex-8, 19 kDa (NDUFB8)) and the CII subunit ((succinate dehydrogenase (ubiquinone) iron-sulfur subunit (SDHB)) among the ETC proteins tested. The CIV subunit ((mitochondrial cytochrome c oxidase 1, (MTCO1)), was mildly inhibited, whereas the expressions of the CIII subunit ((ubiquinol-cytochrome *c* reductase complex core protein-2, (UQCRC2)), and the CV subunit ((ATP synthase, $H^+$ transporting, mitochondrial F1 complex, α-subunit (ATP5A)) were relatively unchanged (Fig. 2F). Consistent with the downregulated expression of ETC proteins, the production of intracellular ATP was significantly inhibited by CCCP treatment compared to the control (Fig. 2G). These results suggest that impaired OXPHOS attenuated mitochondrial respiration and ATP synthesis.

### 3.3. Inhibition of ATP synthesis activated necroptotic cell death

Since ATP synthesis was suppressed by the inhibition of OXPHOS (Fig. 2G), we investigated whether ATP depletion can activate necroptotic cell death. We inhibited ATP synthesis using oligomycin A, a selective ATP synthase (complex V) inhibitor, which inhibits ATP synthase by blocking proton flow through the Fo subunit [29]. Consistent with CCCP treatment, oligomycin A induced MLKL phosphorylation and cell death compared to the vehicle control (Fig. 3A and B).

Next, we analyzed whether the activation of MLKL phosphorylation by CCCP or oligomycin A treatment (Figs. 2A and 3A) corresponds to necroptotic cell death. The number of cells staining for both apoptosis and necrosis (Annexin $V^+$/7-$AAD^+$) were increased 1.7 fold from 9.6% in the vehicle control to 16.7% after CCCP treatment (20 μM) for 4 h. Similarly, oligomycin A (4 μM) induced the number of necroptotic cells (Annexin $V^+$/7-$AAD^+$) by 1.9 fold (from 9.6% in the vehicle control to 18.3% in cells treated with oligomycin A for 4 h) (Fig. 3C). These results suggest that ATP depletion by inhibition of OXPHOS induced the activation of necroptotic cell death.

### 3.4. The activation of AMPK mediates impaired OXPHOS-driven necroptosis

Next, we investigated the molecular mechanism by which impaired OXPHOS regulates the activation of MLKL phosphorylation during necroptotic cell death. Since suppression of OXPHOS leads to ATP depletion, we analyzed the activation of AMPK, which plays a role in cellular ATP homeostasis as a key metabolic regulator, in cells treated with CCCP (20 μM) for 0–6 h. The phosphorylation of AMPK at Thr-172 was significantly increased after CCCP treatment relative to vehicle control (Fig. 4A). Since impaired OXPHOS activated the activation of AMPK, we examined whether the activation of AMPK can induce the phosphorylation of MLKL and necroptotic cell death. We activated the AMPK signaling pathway using AICAR, an activator of AMP-activated protein kinase [30]. First, we measured the activation of MLKL phosphorylation in cells treated with AICAR (20 μM) for 0–6 h. The phosphorylation of AMPK was induced significantly by AICAR treatment compared to the vehicle control (Fig. 4B). Importantly, the phosphorylation of MLKL was activated by AICAR treatment compared to vehicle control (Fig. 4B). Consistent with MLKL phosphorylation, AICAR significantly increased cell death (Fig. 4C). Moreover, AICAR-induced cell death was suppressed by necrosulfonamide treatment (Fig. 4D). Next, we analyzed whether the activation of MLKL phosphorylation by AICAR treatment corresponds to necroptotic cell death. The number of cells staining for both apoptosis and necrosis (Annexin $V^+$/7-$AAD^+$) were increased 2.5 fold from 6.1% in the vehicle control to 15.6% after AICAR treatment (20 μM) for 4 h (Fig. 4E). These results suggest that the activation of AMPK contributed to necroptotic cell death caused by impaired OXPHOS.

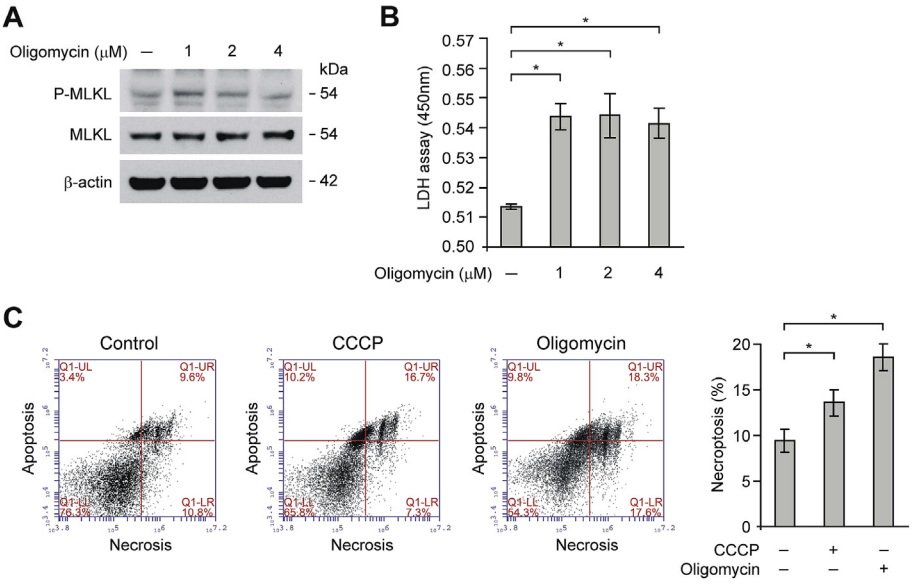


**Fig. 3.** Suppression of ATP synthesis induces necroptotic cell death. (A) Immunoblot analysis for phosphorylated MLKL and MLKL of cell lysates from Beas-2B cells treated with oligomycin (1–4 μM) for 4 h. β-actin served as the standard. (B) Cell cytotoxicity assay of Beas-2B cells treated with oligomycin (1–4 μM) or vehicle (DMSO) for 4 h using LDH levels in culture medium. *$P < 0.05$ by ANOVA. (C) Flow cytometry analysis for apoptosis/necrosis detection of Beas-2B cells treated with either CCCP (20 μM) or oligomycin (4 μM) for 4 h and then stained with Annexin V and red-emitting dye 7-AAD. Data are mean ± s.d. *$P < 0.05$ by ANOVA.

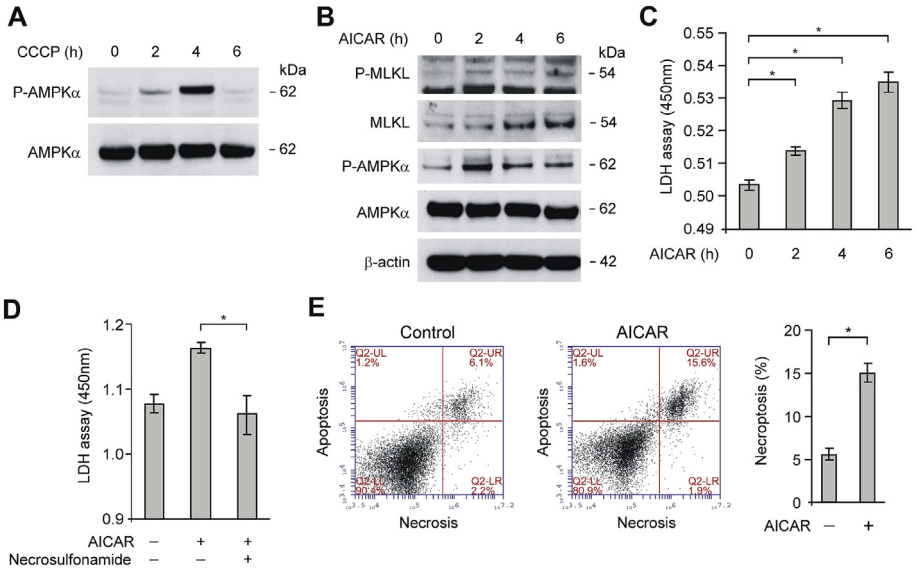


**Fig. 4.** The activation of AMPK by impaired OXPHOS contributes to necroptotic cell death. (A) Immunoblot analysis for phosphorylated AMPK in cell lysates from Beas-2B cells treated CCCP (20 μM) for 2–6 h. AMPK served as the standard. (B) Immunoblot analysis for phosphorylated MLKL, MLKL, phosphorylated AMPK and AMPK of cell lysates from Beas-2B cells treated AICAR (20 μM) for 2–6 h. β-actin served as the standard. (C) Cell cytotoxicity assay of Beas-2B cells treated with AICAR (20 μM) for 2–6 h using LDH levels in the culture medium. $^{*}P < 0.05$ by ANOVA. (D) Cytotoxicity assay of Beas-2B cells pre-treated with necrosulfonamide (1 μM) before AICAR (20 μM, 4 h) treatment using LDH levels in the culture medium. $^{*}P < 0.05$ by ANOVA. (E) Flow cytometry analysis for apoptosis/necrosis detection of Beas-2B cells treated with AICAR (20 μM) or vehicle (DMSO) for 2–6 h and then stained with Annexin V and red-emitting dye 7-AAD. Data are mean ± s.d. $^{*}P < 0.05$ by ANOVA.

## 4. Discussion

In this report, we demonstrate that impaired OXPHOS provides a critical metabolic mechanism for the activation of necroptosis in human lung epithelial cells. Moreover, we demonstrate that the activation of AMPK regulates necroptotic death driven by impaired OXPHOS.

Mitochondria are crucial organelles that regulate cellular energy metabolism, signaling and cell death [31]. The most prominent role of mitochondria is the production of ATP through OXPHOS, as well as the regulation of other cellular metabolic pathways [32,33]. However, it has remained unclear whether mitochondrial energy metabolism can regulate necroptotic death. Recent studies have revealed that the phosphorylation of MLKL at Thr-357 and Ser-358 represents a marker of cells undergoing necroptosis [34]. We found that inhibition of OXPHOS by the chemical inhibitor CCCP activated the phosphorylation of MLKL and necroptotic cell death in lung epithelial cells. Consistent with CCCP treatment, the suppression of ATP synthesis by oligomycin A also activated the phosphorylation of MLKL and necroptotic cell death.

Previous studies suggest that necrosis may occur during metabolic stress conditions such as ischemia/reperfusion, in which tissues are subjected to nutrient deprivation during ischemia involving restriction of oxygen supply and glucose needed for cellular metabolism [35]. While the role of necrosome complex proteins such as RIP1, RIP3 and MLKL have been identified as regulators of necroptosis, it has remained unclear whether necroptosis can be activated by cellular metabolic stress. Importantly, we found, using the uncoupling agent CCCP, that the energy sensing kinase AMPK is activated by ATP depletion subsequent to impaired OXPHOS. Consistent with necroptosis activation by CCCP, the activation of AMPK by the agonist AICAR also induced MLKL phosphorylation and necroptotic death. Our results demonstrate that the activation of AMPK acts as a signaling mediator for necroptosis resulting from impaired OXPHOS. Among the various functions of AMPK as a key signaling molecule in cellular energy signaling pathway, our results suggest that AMPK is also involved in the activation of necroptotic death as an upstream regulator of MLKL phosphorylation.

For several decades, critical roles of mitochondria have been demonstrated in controlling cell death through apoptotic or non-apoptotic mechanisms [36,37]. Apoptosis is driven by activation of caspase proteins *via* the release of cytochrome *c* or caspase-activating proteins from the mitochondria through the opening of channels in the outer membrane or the intermembrane spaces [38]. Non-apoptotic cell death such as necrosis is caused by loss of inner transmembrane potential, production of mtROS, or reduction of ATP production [39,40]. While we demonstrated that the inhibition of OXPHOS leads to necroptotic death, inhibition of OXPHOS also increased the numbers of cells undergoing either apoptotic or necrotic death. Although the mechanism for necroptosis initiation is considered independent of necrosis or apoptosis, necroptosis remains difficult to define solely on the basis of morphological features [41]. Recent studies indicate that necroptosis can also be activated independently of mitochondria [42]. Thus, the roles of mitochondria in necroptosis regulation may be clarified by further studies.

In conclusion, we demonstrate that inhibition of mitochondrial OXPHOS regulates necroptosis in human lung epithelial cells. We also demonstrate that the inhibition of OXPHOS is linked to the activation of AMPK which contributes to necroptotic death. Given the observed relationship between necroptosis and mitochondrial OXPHOS, our findings may have broad implications for therapeutic targeting in human diseases.

## Conflict of interest

The authors declare that there is no conflict of interest.

## Author contributions

M.J.K., A.M.K.C. and J.S.M. conceived of the study with assistance from S.W.R.; M.J.K and K.R. performed the in vitro experiments;

M.J.K., S.W.R., A.M.K.C., and J.S.M. wrote the paper; A.M.K.C., M.E.C., and J.S.M. supervised the entire project.

## Acknowledgments

This work was supported by NIH grants P01 HL114501-01 and P01 HL105339 (to A.M.K. Choi).

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.07.054.

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