Title: Inhibition of mitoNEET induces Pink1-Parkin-mediated mitophagy

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Running title: Role of mitoNEET in mitophagy

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Expanded Material and Method

Cell culture

RAW264.7 cells were cultured in Dulbecco Modified Eagle Medium (Life Technologies, Grand Island, NY, USA), 5 % fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin under an atmosphere of 95 % air and 5 % CO_2 at 37 ˚C. 100 mg/mL streptomycin under an atmosphere of 95 % air and 5 % CO_2 at 37 ˚C. mitoNEET Inhibitor, NL-1, was purchased from (Merck Millipore, Billerica, MA, USA, 475825). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was purchased from (Sigma-Aldrich, St Louis, MO, C2759).

Construction of mitoNEET shRNA-expressing cells

The mitoNEET shRNA and the nonspecific control shRNA (Sigma-Aldrich, St Louis, MO) were transfected into RAW264.7 cells using transfection reagents (Promega, Madison, WI, USA) according to the manufacturer's protocol. The sequences of mouse mitoNEET shRNA were as follow: 5'-CCG GCG TAG GAC CTC TGA TCA TCA ACT CGA GTT GAT GAT CAG AGG TCC TAC GTT TTT TG-3'. The expression of mitoNEET and -actin in stable cells was measured.

Western Immunoblotting

Western immunoblotting was performed as previously described (1). The blots were then incubated with antibody an anti-CISD1 (1:2000) (Proteintech, 16006-1-AP), an anti-β-actin (1:5000), an anti-LC3-II (1:2000) (Sigma-Aldrich, L7543), an anti-p62/SQSTM1 (1:1000) (Sigma-Aldrich, P0067) and an anti-AMPKα (23A3) (1:1000) (Cell signaling technology, #2603), an anti-Phospho-AMPKα (Thr172) (1:1000) (Cell signaling technology, 2535S) and an anti-Pink1 (1:1000) (Proteintech, 23274-1-AP), an anti-Parkin (H-300) (1:1000) (Santa Cruz Biotechnology, sc-30130), an anti-SOD2 (1:1000) (Santa Cruz Biotechnology, sc-30080) and an anti-Caspase-9 (1:1000) (Cell signaling technology, #9504), an anti-Cleaved Caspase-9 (Asp353) (1:1000) (Cell signaling technology, #9509) and an anti-Caspase-3 (1:1000) (Cell signaling technology, #9662), an anti-Cleaved

Caspase-3 (Asp175) (1:1000) (Cell signaling technology, #9664) and an anti-PARP-1 (H-250) (1:1000) (Santa Cruz Biotechnology, sc-7150) in TBST overnight at room temperature. The blots were incubated with an anti-secondary antibody (1:5000) in TBST, immunoblots were detected by SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and visualized after exposure to X-ray film.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated TRIzol reagent (Invitrogen, Life technologies, Carlsbad, CA), Reverse transcription was performed using SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was conducted using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Triplicate samples per condition were analyzed on an Applied Biosystems StepOnePlusTM Real-Time PCR System using absolute quantification settings. The primers sequences were as follows: Mouse β-actin (forward: 5'- GAT CTG GCA CCA CAC CTT CT-3' and reverse: 5'-GGG GTG TTG AAG GTC TCA AA-3'), Mouse SOD2 (forward: 5′-ATG GTG GGG GAC ATA TT-3′ and reverse: 5′-GAA CCT TGG ACT CCC ACA GA-3′), Mouse PGC-1α (forward:5′- CCG AGA ATT CAT GGA GCA-3′ and reverse: 5′-TTT CTG TGG GTT TGG TGT-3′). Amplification of cDNA started with 10 minutes at 95 ˚C, followed by 40 cycles of 15 seconds at 95 ˚C and 1 minute at 60 ˚C

ATP Measurements.

Total ATP levels were monitored using an ATP Colorimetric/Fluorometric Assay Kit (K354) as per the manufacturer's instructions (BioVision, Milpitas, CA, USA). The cells (1×10^6) were lysed by quickly pipetting up and down a few times in 100 μL of ATP assay buffer and then centrifuged under ice-cold conditions at 15,000 g for 2 minutes to pellet the insoluble materials. The supernatant was collected and 2 - 50 μL of this supernatant was added to a 96-well plate, with the final volume topped up to 50 μL / well with ATP assay buffer. ATP reaction mix was made (ATPassay buffer 44 μL, ATP probe 2 μL, ATP converter 2 μL and developer mix

2 μL), and 50 μL of this reaction mix was added to each well containing a test sample. Then the plate was incubated at room temperature for 30 minutes in the dark, and the OD was measured at 570 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry for total reactive oxygen species (ROS) and superoxide measurement

Cultured RAW264.7 were seeded 1.5×10^5 cells/well in duplicate on 12-well plates. Next day, cells were treated with vehicle, CCCP (70 μM) or CCCP plus NL-1 (20 μM). Then, cells were washed with PBS wash collected by centrifugation with 5% FBS containing PBS. Remove supernatant from cells and carefully wash cells with 1X Wash Buffer. Samples were then centrifuged at 1000 rpm for 3 min and the pellets were resuspended in 500 μL of ROS/Superoxide Detection Solution 2X (ROS-ID® Total ROS/Superoxide Detection Kit, Enzo Life Sciences, Farmingdale, NY, USA, ENZ-51010). Cells were incubated for 30 min at 37 ˚C in the dark. Measurements were performed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer.

Flow cytometry for mitoSOX red mitochondrial superoxide

Cultured RAW264.7 were seeded 1.5×10^5 cells/well in duplicate on 12-well plates. Next day, cells were treated with CCCP (70 μM) or CCCP plus NL-1 (20 μM) for the indicated times. After for 9 hours, cells were incubated with 5 μM Mitosox red (MitoSOX™ Red Mitochondrial Superoxide Indicator, for live-cell imaging, (Invitrogen, Life Technologies, Grand Island, NY, M36008) for 30 min at 37 ˚C in the dark. After 30 minutes of loading, samples were the centrifuged at 1000 rpm for 3 minutes and the pellets were resuspended in 500 μL of 5% FBS containing PBS. Measurements were performed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer.

Confocal microscopy

RAW264.7 cells were seeded at 5 × 10^5 cells / well on coverslips in 6-well plates and treated with reagents for 6 hours. After reagent treatment, media was removed by washing with PBS and cells were incubated with serum free media containing 100 nM Mitotracker red (Mitochondrial marker deep red, Invitrogen, Life Technologies, Carlsbad, CA, M22426), 75 nM LysoTracker Green (LysoTracker™ Green DND-26, Invitrogen, Life Technologies, Carlsbad, CA, L7526) or 5 μM Cell ROX red (CellROX® Deep Red Reagent for oxidative stress detection, Invitrogen, Life Technologies, Carlsbad, CA, C10422) for 30 minutes at 37 ˚C in the dark. And then, cells fixed for 20 minutes in 4 % formaldehyde, rinsed 3 times in PBS. A nuclear counterstaining was made with a solution of 1 μg/mL Hoechst 33258 stain for 5 minutes and mounting on a slide Fluorescence Mounting Medium (DAKO North America Inc, Carpinteria, CA, United States, S3023). Olympus FV1000 MPE microscope was used to acquire images.

Annexin V (AV)/Propidium Iodide (PI) staining assay

Cell death was analyzed by PI staining with flow cytometry. Cultured RAW264.7 were seeded 1 × 10^5 cells / well in duplicate on 12-well plates. Next day, cells were treated with CCCP (25 μM) or CCCP plus NL-1 (20 μM) for the indicated times. After for 12 hours, the cells were harvested, washed with phosphate-buffered saline (PBS), and stained with the Annexin V (AV)/Propidium Iodide (PI) Apoptosis Detection Kit (Becton Dickinson, San Jose, CA, USA, 556547). Measurements were performed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer.

lactate dehydrogenase (LDH)-release assay

Cultured RAW264.7 were seeded 1 × 10^5 cells / well in duplicate on 12-well plates. Next day, cells were treated with CCCP (25 μM) or CCCP plus NL-1 (20 μM) for the indicated times. After reagent treatment, In the high control wells, 10 μL cell lysis solution (LDH-Cytotoxicity Colorimetric Assay kit II, BioVision, 155 S Milpitas Boulevard, Milpitas, CA 95035, USA, #K313-500) was added, and the plate was

shaken for 1 minutes. Quantitative analysis was performed on the cell culture supernatant (20 μL/well). For each well, 100 μL of LDH Reaction Mix (LDH Assay Buffer : WST substrate Mix = 50 : 1) was added. Absorbance was then measured at 450 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA) to calculate LDH release percentage.

Cell viability assay

Cell viability was determined by the MTS assay using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, G3581). Cells were seeded at 1 × 10^4 cells / well in 96-well plates. After reagent treatment, 20 μL of MTS solution was added to each well. Plates were incubated for an additional 2 - 4 hours at 37 ˚C. Absorbance at 490 nm was then measured using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) to calculate the cell survival percentages.

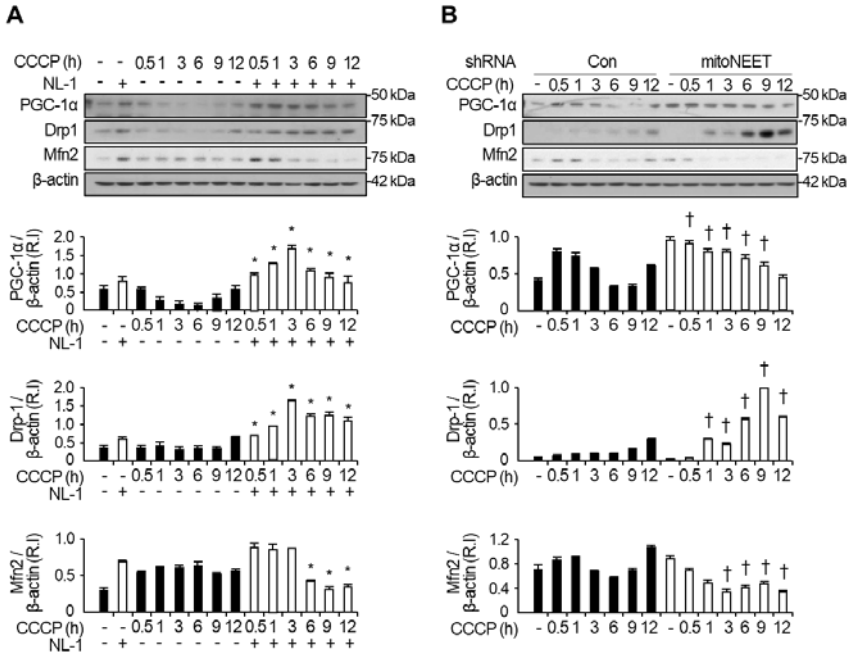
Statistical analysis

All results were confirmed in at least three independent experiments; data from one representative experiment are shown. Quantitative data are shown as means ± standard deviation and significance of statistical analysis was determined with two-tailed, unpaired Student's *t*-test. *P*-values ⟨0.05 were considered significant.

Reference

1. Kwon MY, Hwang N, Lee SJ, Chung SW (2019) NOD2 attenuates ER stress-induced cell death in VSMCs. BMB Rep 52, 665-670

Supplementary Figure 1.



Supplementary Figure 1. Inhibition of mitoNEET maintains mitochondrial quality via the balance of mitochondrial dynamics.

RAW264.7 cells, control shRNA-expressing or mitoNEET shRNA-expressing RAW264.7 cells were treated with vehicle, CCCP (25 μM), or CCCP plus NL-1 (20 μM) for variable time points. Total protein was harvested and the expression levels of mitochondrial biogenesis proteins, PGC-1α and dynamic proteins, Drp-1 (a fission protein), mitofusin 2 (Mfn2; a fusion protein) were analyzed by western blotting (A-C). All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for CCCP vs. CCCP plus NL-1. †$P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of CCCP.