Alternative Oxidase Attenuates Cigarette Smoke-Induced Lung Dysfunction and Tissue Damage

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ONLINE DATA SUPPLEMENT

Supplemental Materials and Methods

Cigarette smoke (CS) exposure

The chronic smoke protocol exposed animals for 5 h per weekday over a period of nine months with three smoking phases of 87 min daily, interrupted by room-air exposure for 40 min. CS contained 100-120 mg/m^3 total suspended particles (TSP) and 500-600 parts per million (ppm) carbon monoxide. The acute smoke protocol exposed mice to the smoke of 8 cigarettes for 30 min. The smoke (675 ml/min) was mixed with room air (1800 ml/min) by animal ventilators (7025 rodent ventilator, Hugo Basile, Biological Research Instruments, Comerio, Italy) and directed to the exposure chamber. The procedure was repeated hourly, five times per day for three consecutive days. Between each exposure, the animals were removed from the exposure chamber and placed in their regular cages for the remainder of each hour. The concentration of cigarette smoke inside the chamber was 800-1000 mg/m^3 of total suspended particles (TSP) and 300-800 ppm carbon monoxide.

Lung function measurements

Mice were anesthetized with ketamine and xylazine (ketamine 105 mg/kg (Ursotamin, 100 mg/ml, Serumwerk Bernburg AG, Bernburg, Germany), xylazine 7 mg/kg (Rompun, Bayer Vital GmbH, Leverkusen, Germany). The trachea was cannulated for lung function measurements. Data acquisition was done using flexiWare software v7.1 (SCIREQ, Montreal, Canada).

Isolating and culturing primary mouse embryonic fibroblasts (MEFs)

Retrovirus packaging cell lines PA317+hTERT and PA317+E7 were cultured and media from both lines combined and filtered (Minisart NML Syringe filters, pore size 0.45 µm) (Sigma-Aldrich, Darmstadt, Germany, 16555-K). Virus-containing media were mixed with 1000x polybrene (Millipore, Merck KGaA, Darmstadt, Germany, TR-1003-G) and added to primary MEF cultures. Infected cells were selected by G418 (Millipore, Merck KGaA, Darmstadt, Germany, 345812) containing media. Cell were maintained in DMEM high glucose (4.5 g/l) (Lonza, Basel, Switzerland, 12614,) supplemented with 10% FBS (Thermo-Fisher Scientific, Vantaa, Finland, Gibco, 12070), 100 U/ml penicillin, 100 µg/ml streptomycin (Lonza, Basel, Switzerland, DE17602E), 2 mM GlutaMAX (Gibco, Thermo-Fisher Scientific, Vantaa, Finland, 35050038).

For experiments, iMEFs were grown in DMEM (Thermo-Fisher Scientific, Vantaa, Finland, A14430) supplemented with 10 mM glucose (glc) (Darmstadt, Germany, Sigma, G7021) or 10 mM galactose (gal) (Darmstadt, Germany, Sigma, G0750), 10% FBS (Gibco, Thermo-Fisher Scientific, Vantaa, Finland, 12070), 100 U/ml penicillin, 100 µg/ml streptomycin (Lonza, Basel, Switzerland, DE17602), 1 mM sodium pyruvate (Sigma-Aldrich, Darmstadt, Germany, S8636), and 2 mM L-glutamine (Lonza, Basel, Switzerland, BE17-605E) according to a previous publication (1).

Generation of cigarette smoke condensate (CSC)

Vectis s.r.l. Cava dei Tirreni (Salerno, Italy) used a CERULEAN SM 450 smoking machine and CORESTA monitor test pieces (CM7, Imperial Tobacco PLC, Hamburg, Germany). The particulate matter was collected on glass-fiber filters and its mass was determined by weighing. CSC was dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Darmstadt, Germany, D2650) and stored at -20 °C as described elsewhere (2).

Protein extraction and Western blotting

Proteins were extracted from frozen iMEFs, resuspended in solubilization buffer containing 1% dodecylmaltoside (DDM) (Sigma-Aldrich, Darmstadt, Germany, D4641), 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, Darmstadt, Germany, 93482) and protease inhibitor mini tablet mix (Thermo-Fisher Scientific, Vantaa, Finland, 88665). Protein content was estimated using the Bradford assay (Bio-Rad, Basel, Switzerland, B6916) with absorbance measured in a SpectraMAX 190 Microplate Reader (Molecular Devices, San Jose, California).

The normalized lysate was fractionated on gradient (4-20% mini-protean TGX stain-free) (Bio-Rad, Basel, Switzerland, 4568095) or 12% polyacrylamide gels and transferred onto a 0.2 µm polyvinylidene difluoride membrane (PVDF) (Bio-Rad, Basel, Switzerland, 17404156) using Trans-Blot Turbo Transfer System (Bio-Rad, Basel, Switzerland) or onto a nitrocellulose membrane (PerkinElmer, Waltham, Massachusetts, USA, NBA085C001EA) using a Thermo-Fisher Scientific Owl HEP Series Semidry Electroblotting System (HEP-3) (Thermo-Fisher Scientific, Vantaa, Finland). After incubation with 1.5% non-fat dry milk in tris-buffered saline 0.1%-Tween20 (TBST) for 1 h, the membrane was washed once with TBST and incubated overnight with primary antibodies diluted in 5% BSA-TBST.

Antibodies used: **α**-tubulin (Cell Signaling Technology, 3873, 1:2,000), total OXPHOS cocktail (Abcam, Cambridge, UK, ab110413, 1:250), AOX (custom-raised in rabbit, 21st Century Biochemicals, Marlborough, Massachusetts, USA, 1:40,000), caspase-3 (Cell Signaling Technology, Danvers, Massachusetts, 9662, 1:1,000) and cleaved caspase-3 (Cell Signaling Technology, Danvers, Massachusetts, 9661S, 1:1,000).

Secondary antibodies were anti-mouse (Jackson ImmunoResearch, West Grove, Pennsylvania, USA, 115-035-146, 1:10,000) and anti-rabbit (Jackson ImmunoResearch, 111-035-144, 1:20,000) peroxidase-conjugated IgG. Blots were developed using the Clarity ECL Western Blot Substrate kit (Bio-Rad, Basel, Switzerland, 1705060). Quantitative analyses were performed using the Image Lab software (Bio-Rad, Basel, Switzerland). The densitometric value of OD units of each band was related to OD units of corresponding **α**-tubulin signal.

Respirometry

Substrates and inhibitors were added in the following order: (i) CSC (18.75-75 µg/ml); (ii) digitonin (30 µg, DC141), (iii) sodium pyruvate (2256), sodium glutamate (27647) and sodium malate (M1000) (5 mM each), (iv) adenosine diphosphate (ADP, 2 mM) (A5285), (v) rotenone (150 nM) (R8875), (vi) succinic acid (17 mM) (3674), (vii) antimycin A (AA, 30 ng/ml) (8674), (viii) n-propyl gallate (nPG, 200 µM) (P3130), (ix) N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD, 1 mM) (T3134) plus (x) sodium L-ascorbate (1 mM) (A7631), (xi) sodium azide (40 mM) (S8032). If not stated otherwise, chemicals were purchased from Sigma-Aldrich, Darmstadt, Germany. Respirometry in intact cells was carried-out in DMEM, 10 mM glucose, followed by CSC (37.5-75 µg/ml), oligomycin (1.5 µg/ml) (75351), and uncoupling oxidative phosphorylation by carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (C2920) stepwise titration (2.5-6 µM). Respiration was inhibited by 300 µM rotenone or 2.5 µM antimycin A (AA).

Measuring reactive oxygen species (ROS)

For MitoSOX measurements $6x10^4$ iMEFs/well were seeded in a 24-well microplate in DMEM high glucose. After 24 h, cells were washed with PBS followed by 1 h of preincubation in DMEM 10 mM glucose. Then cells were incubated for 10 min with 5

μM MitoSOX, washed with PBS and incubated for 30 min or 3 h in DMEM 10 mM glucose containing the indicated concentrations of CSC. Fluorescence was measured using a TECAN 200 microplate reader set to 510 nm excitation (bandwidth: 10 nm) and 580 nm emission (bandwidth: 35 nm) wavelengths.

The amount of ROS was also estimated using the EMXmicro ESR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) with the spin probe CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine; Noxygen, Elzach, Germany) (0.5 mM). CMH reacts specifically with superoxide and peroxinitrite. The portion of superoxide was determined by incubation of samples with polyethylene glycol-conjugated superoxide dismutase (50 U/ml pSOD in ESR-Krebs HEPES buffer) as the difference between the spectral peak-to-peak amplitudes of the samples incubated with and without pSOD.

Supplemental References

1. Gohil VM, Sheth SA, Nilsson R, Wojtovich AP, Lee JH, Perocchi F, Chen W, Clish CB, Ayata C, Brookes PS, Mootha VK. Nutrient-sensitized screening for drugs that shift energy metabolism from mitochondrial respiration to glycolysis. *Nat Biotechnol* 2010;28:249–255.
2. Giordano L, Deceglie S, D'Adamo P, Valentino ML, La Morgia C, Fracasso F, Roberti M, Cappellari M, Petrosillo G, Ciaravolo S, Parente D, Giordano C, Maresca A, Iommarini L, Del Dotto V, Ghelli AM, Salomao SR, Berezovsky A, Belfort R, Sadun AA, Carelli V, Loguercio Polosa P, Cantatore P. Cigarette toxicity triggers Leber's hereditary optic neuropathy by affecting mtDNA copy number, oxidative phosphorylation and ROS detoxification pathways. *Cell Death Dis* 2015;6:e2021.

Supplemental Figures

Supplemental Figure E1. Testing the effect of chronic cigarette smoke (CS) exposure and AOX on lung function.

(**A**)-(**E**) Respiratory system mechanics measured using SCIREQ FlexiVent system.
All data shown as mean±SEM; **p≤0.005 by two-way ANOVA. # refers to paired *t* test on smoke exposed groups.

Supplemental Figure E2. Quantitation of total caspase-3 protein in cultured iMEFs exposed to CSC.

Quantitation of blots shown in figure 3D (total caspase-3 normalized against **α**-tubulin). See also figure 3D, 3E.
All data shown as mean±SEM.

Supplemental Figure E3. CSC toxicity effect on mitochondrial respiration and ROS production in cultured iMEFs.

(**A**) Endogenous oxygen consumption measured in intact WT and AOX iMEFs grown on galactose medium supplemented with CSC for 48 h and normalized to the protein content.
(**B**) Mitochondrial superoxide production measured using MitoSOX Red after 30 min of CSC exposure. Fluorescence was measured using a TECAN 200 microplate reader set to 510 nm excitation (bandwidth:10 nm) and 580 nm emission (bandwidth: 35 nm) wavelengths.
(**C**) Representative spectra as obtained by ESR for quantification of mitochondrial-derived superoxide radicals.

All data shown as mean±SEM; *p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.0001 by two-way ANOVA analysis.

Supplemental Figure E4. Acute effect of CSC on mitochondrial respiration *in vitro*.

(**A**) Oxygen consumption normalized to the protein content of intact iMEFs in respiration buffer B.

(**B**) Oxygen consumption of iMEFs treated with carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) in DMEM containing glucose (10 mM).

(**C**) Rotenone-sensitive oxygen consumption of permeabilized iMEFs driven by PGM plus ADP in respiration buffer B.

(**D**) Antimycin A- and n-propyl gallate (nPG)-sensitive oxygen consumption of permeabilized iMEFs driven by succinate plus rotenone in respiration buffer B.

(**E**) Sodium azide-sensitive oxygen consumption of permeabilized iMEFs, driven by ascorbate/TMPD in respiration buffer B.

All data shown as mean±SEM; *p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.0001 by two-way ANOVA analysis vs. WT untreated; ###p≤0.0005, ####p≤0.0001 by two-way ANOVA analysis vs. AOX untreated.

Supplemental Figure E5. Entire blots including molecular weight markers for cropped Western blot bands shown in Figure 3D.

(**A**) Membrane and molecular weight marker as probed for caspase-3.

(**B**) Membrane and molecular weight marker as probed for cleaved caspase-3. Please note, a second protein has been probed on this membrane accounting for extra bands in the lower molecular weight range.

(**C**) Membrane and molecular weight marker as probed for AOX.

(**D**) Membrane and molecular weight marker as probed for **α**-tubulin.

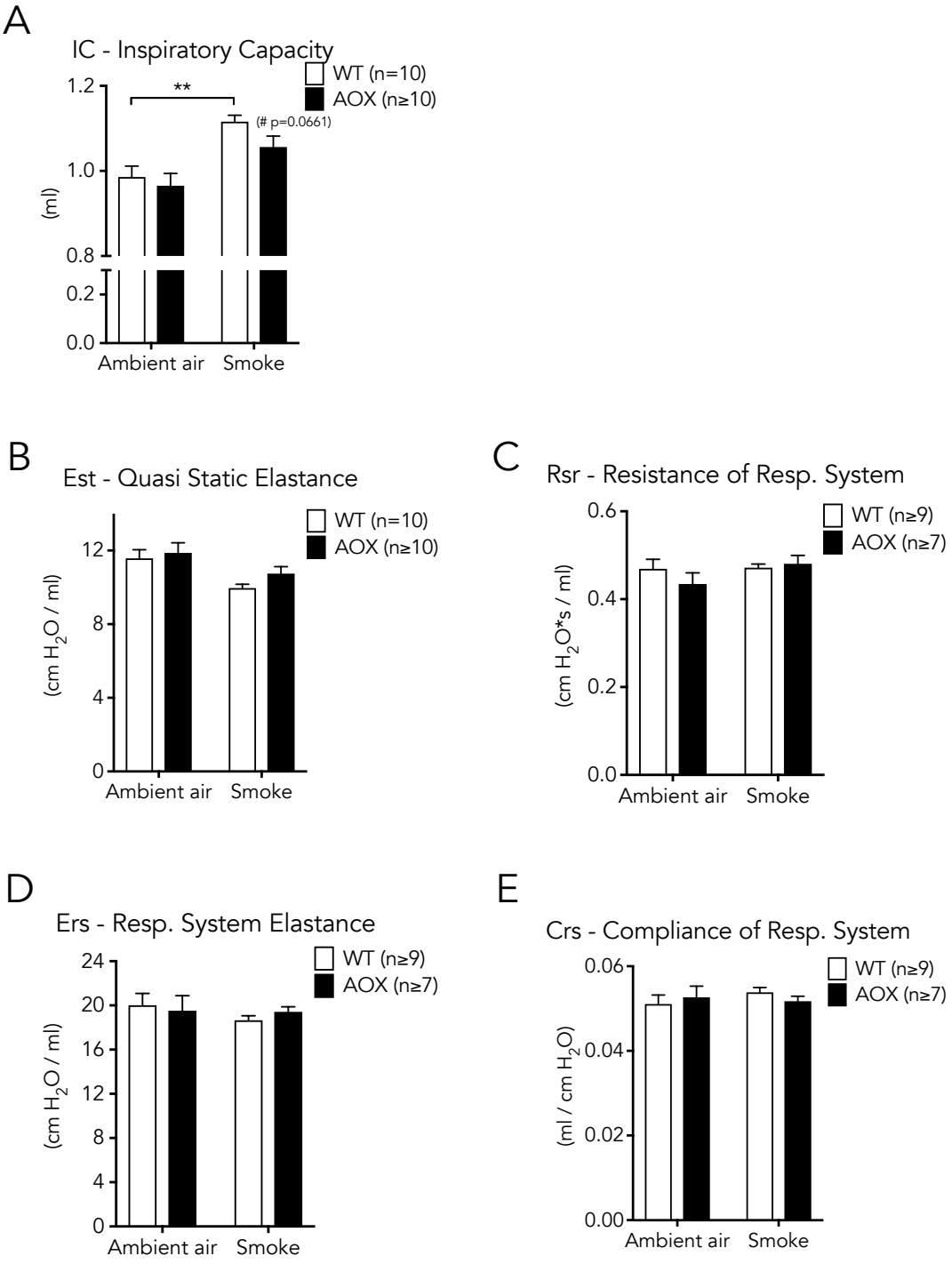
Supplemental Figure E6. Entire blots including molecular weight markers for cropped Western blot bands shown in Figure 4D.

(**A**) Full membrane and molecular weight marker as probed for subunits of the complexes of the mitochondrial OXPHOS system.

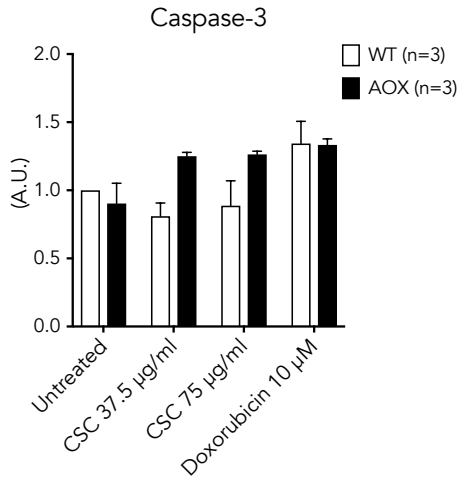
(**B**) Membrane and molecular weight marker as probed for AOX.

(**C**) Membrane and molecular weight marker as probed for **α**-tubulin.

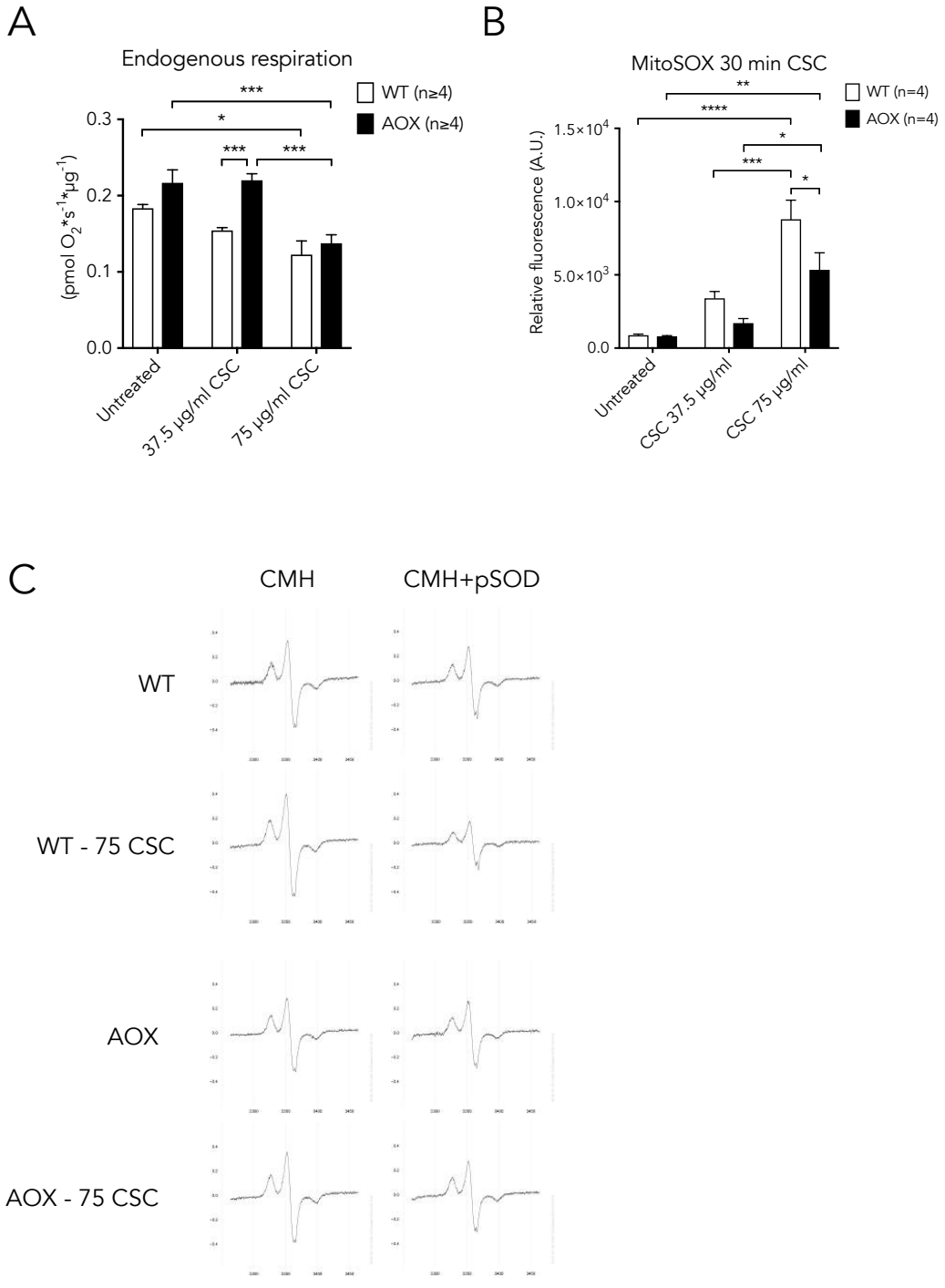
Supplemental Figure E1



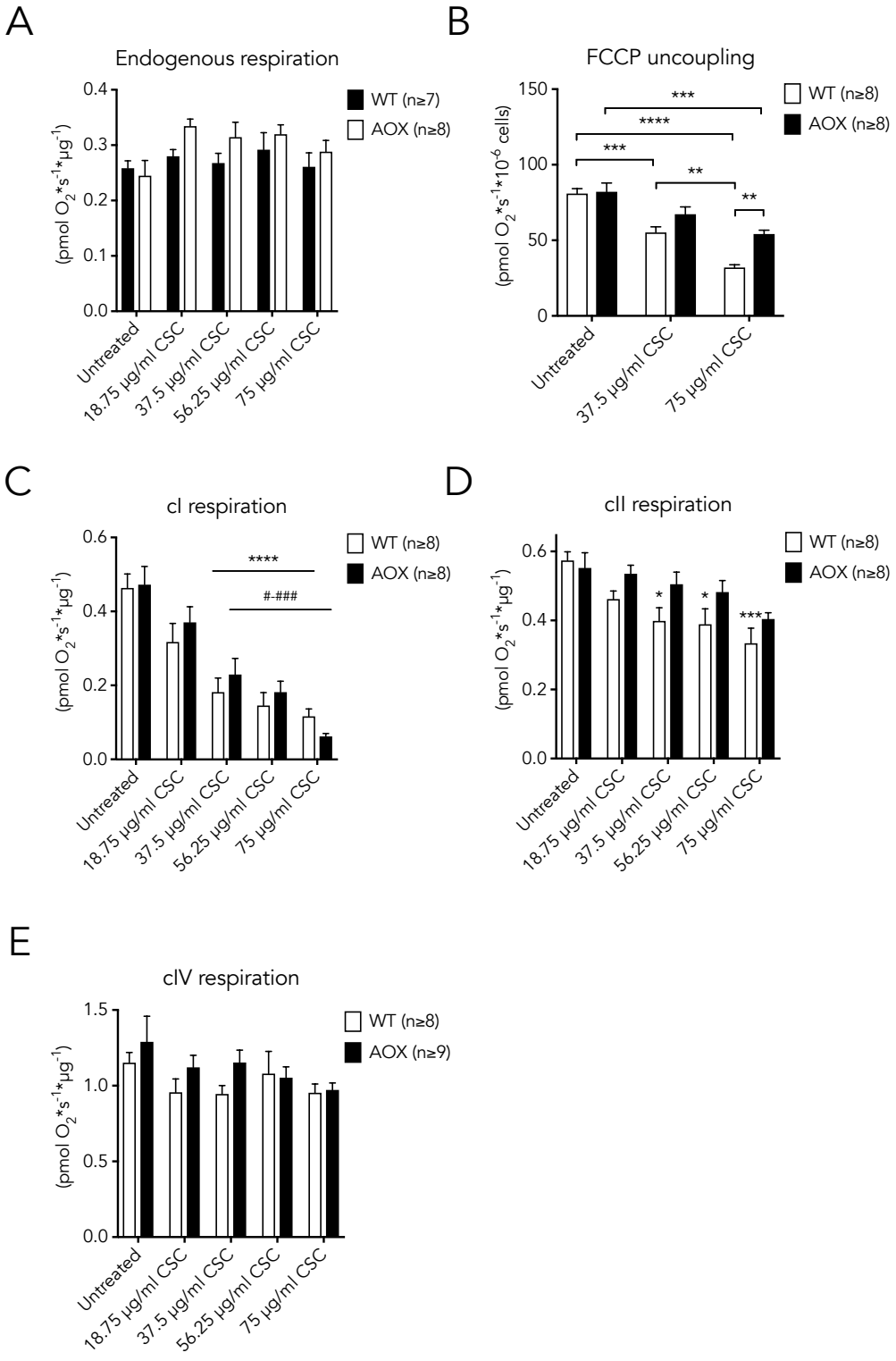
Supplemental Figure E2



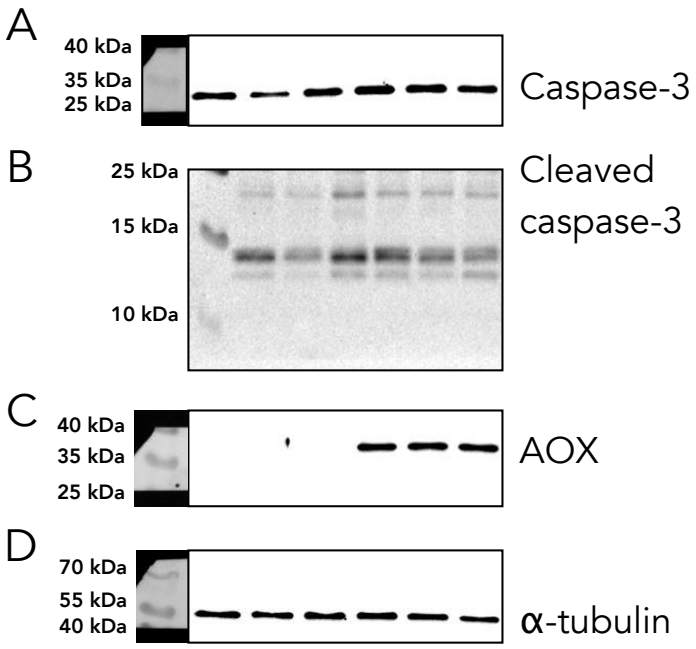
Supplemental Figure E3



Supplemental Figure E4



Supplemental Figure E5



Supplemental Figure E6