Supplemental Material

Supplemental Methods

Cardiac differentiation from iPSCs

Human Induced pluripotent stem cells (iPSCs) were dissociated into single cells by treatment with 1 mL Accutase solution (Millipore-Sigma SCR005) for 3-5 min at room temperature. iPSCs were then plated on matrigel-coated six-well plates and cultured with fresh E8 medium (Thermo Fisher, Waltham, MA, USA) supplemented with 10 µM Rho-associated protein kinase (ROCK) inhibitor Y-27632 (S1049, Selleck Chemicals, Houston, TX, USA) for 24 hours. On day 0, iPSCs were differentiated into iPSC-cardiomyocytes with a chemically defined cardiomyocyte differentiation protocol[49]. Briefly, iPSCs were first treated with a small molecule inhibitor of GSK3β signaling, CHIR99021 (6 µM; S2924, Selleck Chemicals), to activate the canonical Wnt signaling pathway in RPMI1640 (11875093, Thermo Fisher) medium containing B27 without insulin (A1895601, Thermo Fisher). Three days later, cells were treated with a Wnt signaling inhibitor, Wnt-C59 (2 µM; S7037, Selleck Chemicals), until day 5. Afterwards, RPMI1640 supplemented with B27 plus insulin (A3582801, Thermo Fisher) media without any small molecules was changed every 2 days. To purify cardiomyocytes, cells were glucose-starved for 5 days to metabolically select iPSC-CMs[50]. At day 30 iPSC-CMs were dissociated with TrypLE (12605036, Thermo Fisher) and plated on cover slips for immunostaining or reseeded on a matrigel-coated seahorse plate for respiration analysis using the Seahorse analyzer.

Immunofluorescence Staining

iPSC-CMs at day 30 were cultured on the matrigel-coated coverslips. Cells were fixed with 4% paraformaldehyde (sc-281692, ChemCruz, Santa Cruz, CA, USA) for 15 min at

room temperature, permeabilized with 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 15 min, and blocked with 10% goat serum (G9023, Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature. The cells were then stained with the following primary antibodies at 4°C overnight: rabbit anti-cardiac troponin T (1:100 dilution; ab45932, Abcam, Cambridge, MA, USA) (cardiomyocyte specific marker) and mouse anti-TOM20 (1:100 dilution; sc-17764, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (mitochondrial marker). After three washes with PBS-T (PBS containing 0.05% Tween-20) for 15 min, the cells were incubated in the dark for one hour at room temperature with the following secondary antibodies: Alexa Fluor 594 goat anti-rabbit immunoglobulin G (IgG) (1:250, Thermo Fisher Scientific) and Alexa Fluor 488 goat anti-mouse IgG (1:250, Thermo Fisher Scientific) for 1 hour at room temperature. After three washes with PBS-T for 15 minutes, cells were mounted with ProLong Gold Antifade Mountant with DAPI (P36935, Thermo Fisher Scientific) and imaged using a Zeiss confocal microscope (LSM880, Carl Zeiss, Germany).

Mitochondrial respiration analysis for iPSC-CMs

iPSC-CMs at day 30 were seeded onto matrigel-coated Seahorse 24-well assay plates at a density of 100,000 cells/well and allowed to grow for 4 days in RPMI supplemented with B27 culture medium (control media). The cells were then treated with 50 µM 4-hydroxynonenal (4HNE) or control media for 24 hours. Mitochondrial oxidation was evaluated at day 35 by analysis of oxygen consumption rate (OCR, pmol/min) using the Seahorse XF24 Extracellular Flux Analyzer (100840-000, Agilent, Santa Clara, CA, USA). One hour prior to the assay, the culture medium was replaced with XF media (Agilent,

Santa Clara, CA, USA). OCR was obtained by plotting changes in oxygen concentration over time and obtaining the gradient. After establishing baseline OCR, additional measurements were made following sequential automatic injections of the following: 1 µM oligomycin (an ATP synthase inhibitor), 1 µM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, a mitochondrial uncoupler), and 0.5 µM rotenone/antimycin A (mitochondrial electron transport chain complex I and III inhibitor). Oxidative phosphorylation was calculated by subtracting the oligomycin rate (proton leak) from the basal rate. Reserve respiratory capacity was determined as maximal OCR (uncoupled) minus baseline OCR. Non-mitochondrial respiration was measured as OCR after addition of Rotenone and Antimycin.

Cell counting

HL1 cells were cultured in 6-well plates. Immediately prior to 4HNE treatment, a set of wells were set aside to establish the initial cell count. The cells were washed and detached with trypsin-EDTA, then counted with a hemocytometer. The remaining wells were treated with 4HNE and/or carvedilol, and after 24 hours, their cells were counted the same way.

Mitochondrial membrane potential assay

HL-1 cells were treated with 4HNE and/or carvedilol for 24 hours. 30 minutes before harvest, 20 nM of tetramethylrhodamine, methyl ester (TMRM) (T668; ThermoFisher Scientific) was added to each culture well. At harvest, the cells were

washed, detached with trypsin-EDTA, then analyzed with a flow cytometer (DXP10; Cytek Biosciences).

Mitochondrial mass determination

HL-1 cells were treated with 4HNE and/or carvedilol for 24 hours, washed with DPBS, and were treated with 50 nM MitoTracker Red CMXRos (M7512; ThermoFisher Scientific) in Hank's Balanced Saline Solution (HBSS) for 15 minutes. The cells were harvested with trypsin-EDTA and assayed with a flow cytometer (DXP10; Cytek Biosciences).

Fluorescence imaging of mitochondria

HL-1 cells were treated with 4HNE and/or carvedilol for 24 hours, followed by treatment with 50 nM MitoTracker Red CMXRos and 1:2000 diluted Hoechst 33342 nuclear stain (I36007, ThermoFisher Scientific). Staining was carried out for 15 minutes in culture medium. The cells were washed and fixed with 4% formaldehyde in PBS for 15 minutes at room temperature and imaged with a fluorescence microscope. Qualitative analysis of mitochondrial fragmentation was performed by an investigator blinded to the sample identity.

Carvedilol dose determination

Appropriate dose of carvedilol was determined by preliminary testing. HL1 cells were co-treated with 200 μM 4HNE and 1, 10, or 100 μM carvedilol. MFF and OPA1 were assayed by western blot. MFF and OPA1 were increased by 10 μM, but

decreased by 100 μM carvedilol. Thus, we determined 10 μM carvedilol is the highest dose at which potential deleterious effects do not manifest.

Supplemental Results

Mitochondrial fission and fusion proteins are increased in RV failure

Inherent to the heterogeneity in patients related to disease etiology, duration, severity, medication use and mitochondrial heterogeneity within a cell, we noted significant variability between patients within each group and therefore only noted a trend toward an increase in expression of fission proteins DRP1 and MFF in RV failure (Supplemental Fig IIa-d). Similarly, the expression of fusion protein OPA1 long form trended toward an increase while MFN2 increased significantly in RV failure (Supplemental Fig IIe-g).

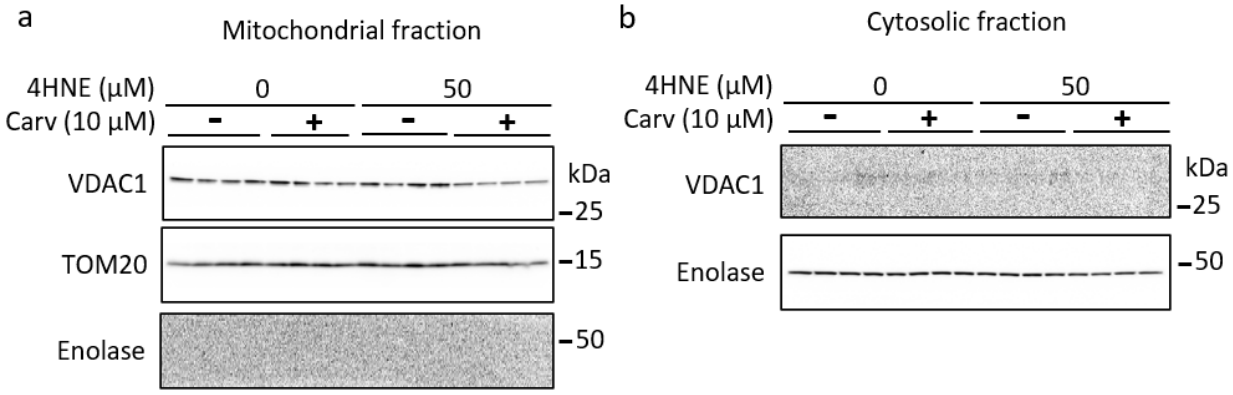
4HNE inhibits mitochondrial dynamics

4HNE treatment increased the whole-cell expression of fission proteins (DRP1: by 164%, $p<0.0001$; MFF: by 140%, $p=0.0002$) (Supplement Fig VIa, c, d), and a trend toward an increase in the expression of the fusion proteins OPA1 long isoforms and MFN2 (Supplement Fig VIb, e, g). 4HNE increased the expression of OPA1 short isoforms which control energetic efficiency via optimal cristae organization (control 1±0.12 vs. 4HNE 1.86±0.06, $p=0.0001$) (Supplement Fig VIf). In contrast, despite whole-cell level increase in fission proteins, mitochondrial localization of the fission and fusion proteins were inhibited by 4HNE (Supplement Fig VIh-n): DRP1 (control 1±0.06 vs. 4HNE 0.56±0.03, $p<0.0001$), MFF (control 1±0.07 vs. 4HNE 0.68±0.04, $p=0.0014$), and MFN2 (control 1±0.04 vs. 50 μM 4HNE 0.66±0.03, $p=0.0390$), while both long and short isoforms of

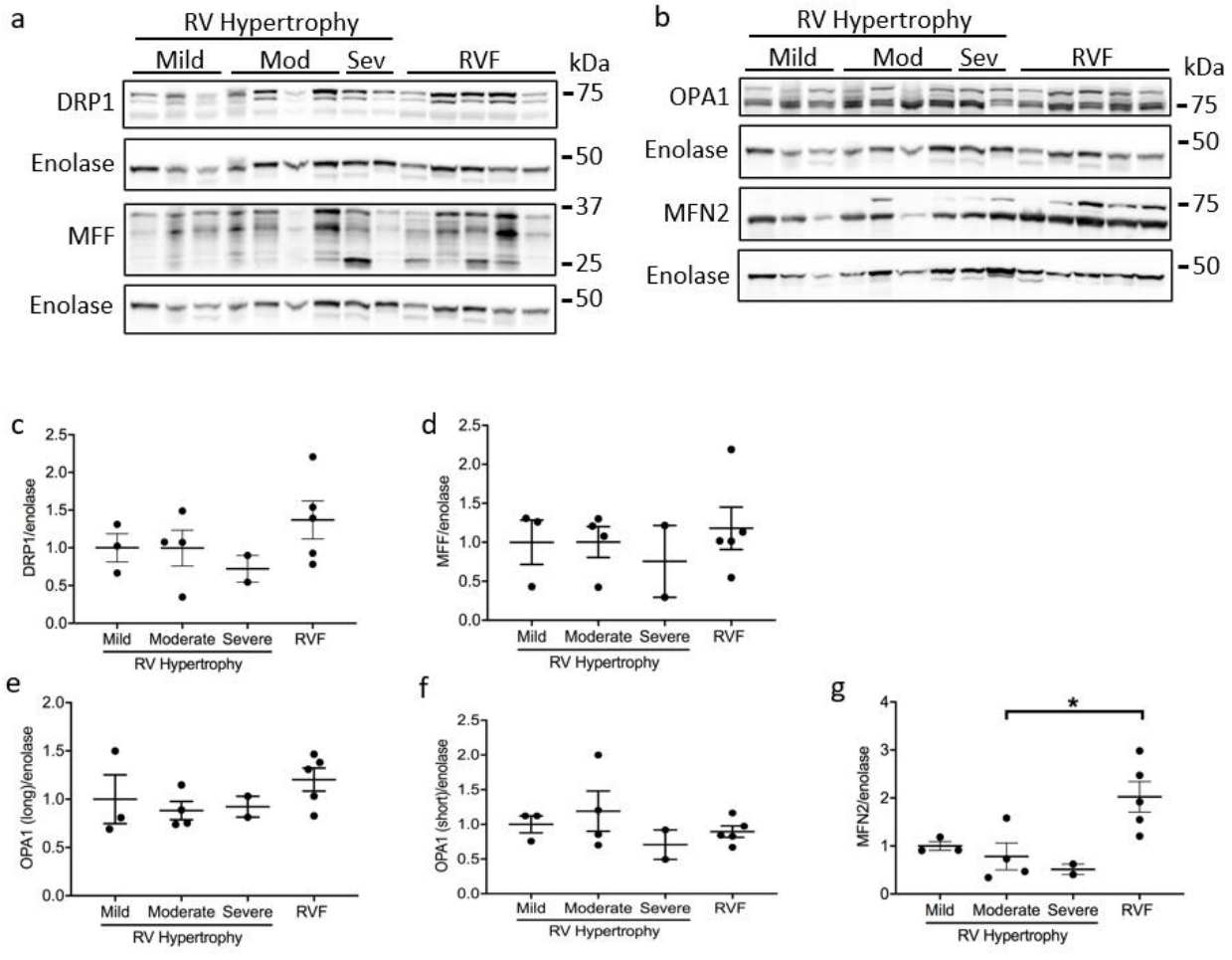
OPA1 were not affected. Carvedilol further decreased fission protein DRP1 and MFF mitochondrial localization, but did not affect fusions proteins OPA1 and MFN2. These data demonstrate that 4HNE alters the mitochondrial morphology and blocks mitochondrial localization of mitochondrial dynamics proteins.

Supplemental Figures

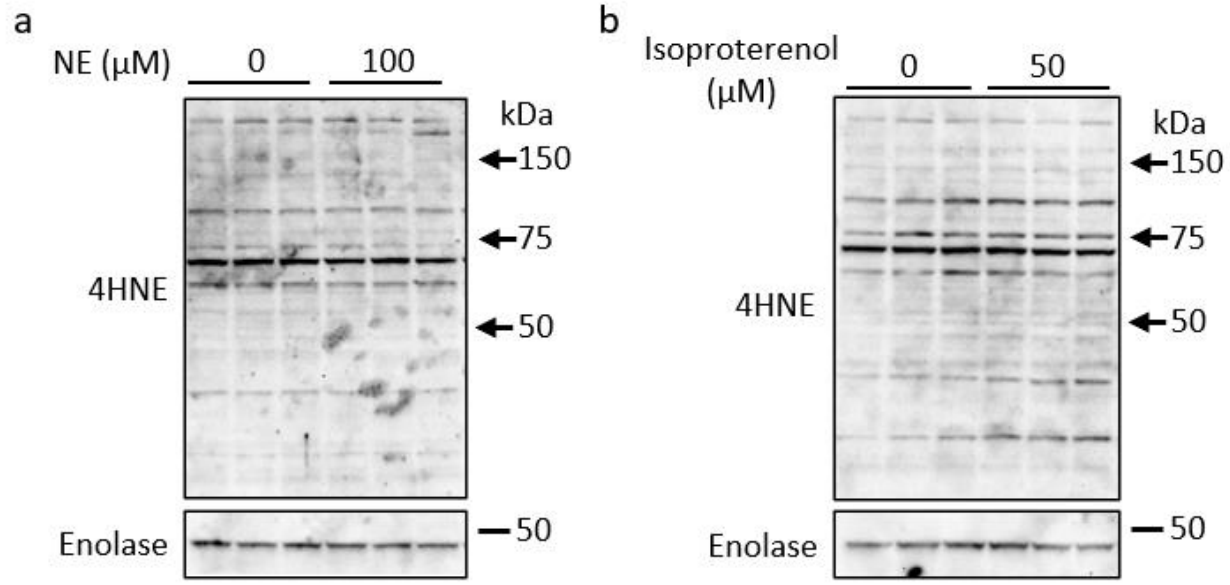
Supplemental Figure I. Mitochondrial fraction was free of cytosolic content. Purity of mitochondrial and cytosolic fractions were tested by probing with mitochondrial- and cytosolic-specific proteins. (a) In the mitochondrial fraction, mitochondrial proteins VDAC1 and TOM20 had strong signals, but the cytosolic protein Enolase was absent. (b) Conversely, in the cytosolic fraction, VDAC1 was absent but Enolase showed a strong signal. 4HNE – 4-hydroxynonenal; Carv – carvedilol.



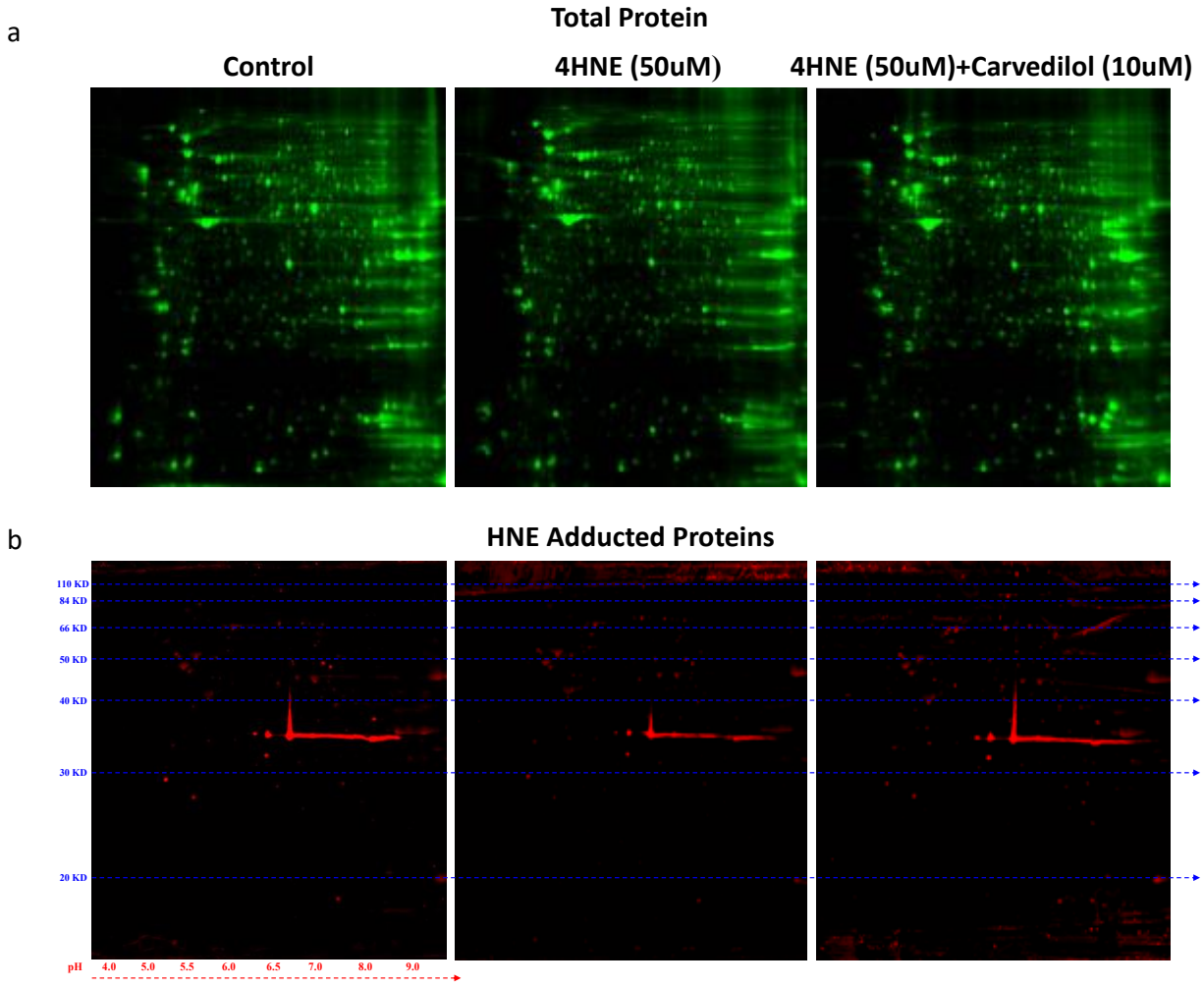
Supplemental Figure II. Patients with severe RV hypertrophy and RV failure demonstrate altered expression of mitochondrial dynamics proteins. Representative western blots of mitochondrial (a) fission and (b) fusion proteins. In RV failure, mitochondrial fission protein (c) DRP1 expression trended toward an increase, (d) Fission protein, MFF, did not change. (e, f) Mitochondrial fusion protein OPA1 did not change across the groups, but (g) MFN2 increased in RV failure (N=2-5/group). RV – right ventricle; RVF – right ventricular failure; Mod – moderate; Sev – severe. Data are presented as mean±SEM. *p<0.05.



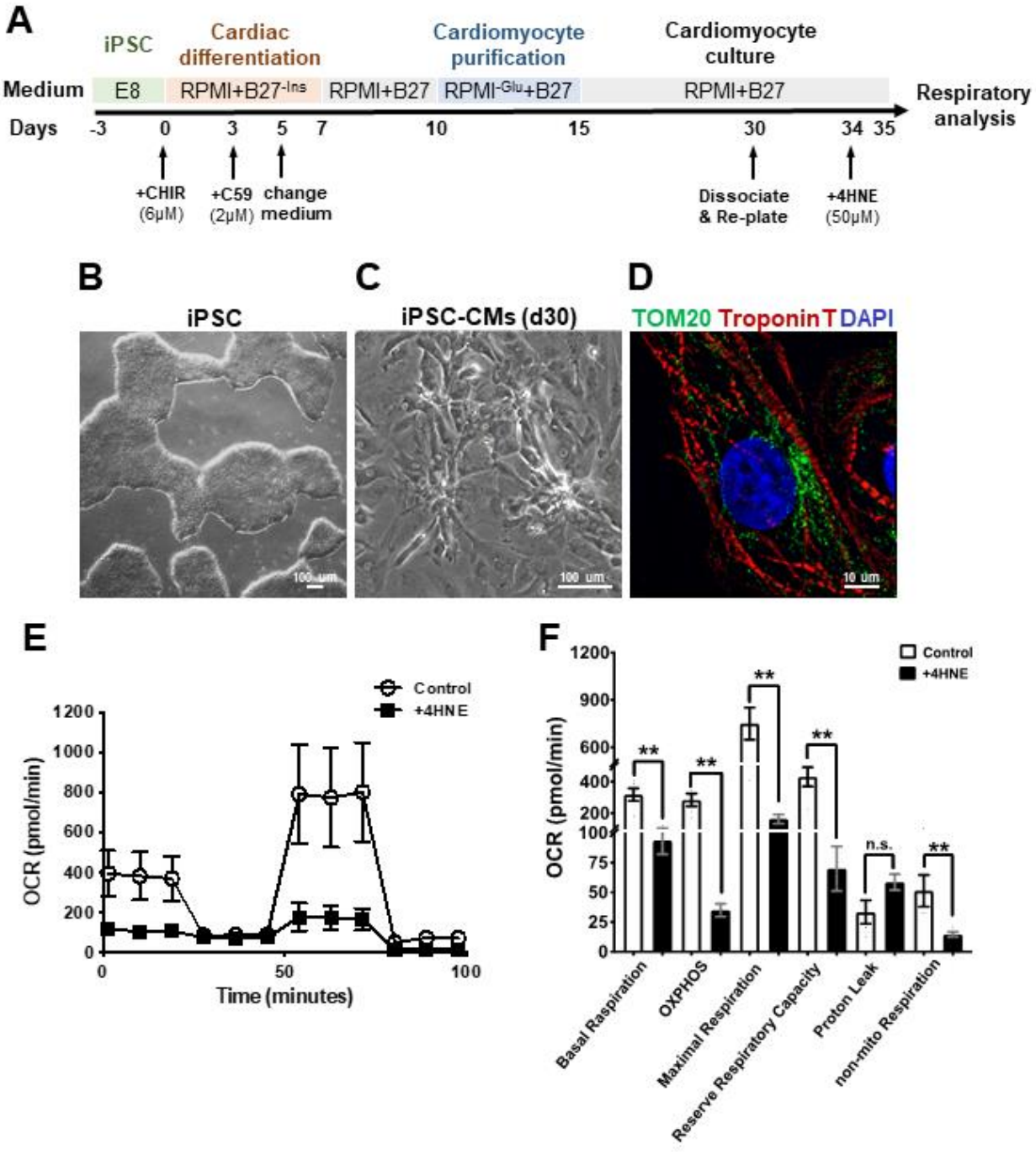
Supplemental Figure III. 4HNE is not increased by Norepinephrine or isoproterenol. (a, b) HL1 cells were treated for 24 hours with 100 μM norepinephrine or 50 μM isoproterenol. 4HNE expression did not increase with the treatment. NE – norepinephrine; 4HNE – 4-hydroxynonenal.



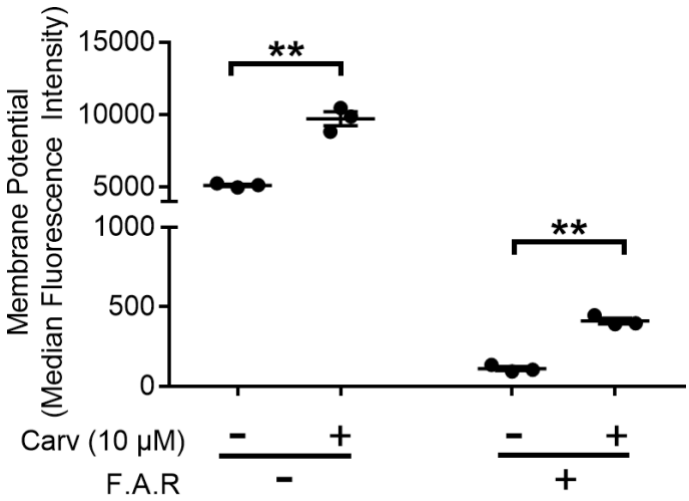
Supplemental Figure IV. Identification of 4HNE adducted proteins. (a) 2D-gel electrophoresis and western blot were used to detect total protein in HL1 cardiomyocytes treated with 4HNE (50 µM) and 4HNE+carvedilol (10 µM) and compared with control. (b) 2D-gel electrophoresis and western blot were used to detect the proteins with increased 4HNE-adducts. 4HNE - 4-hydroxynonenal.



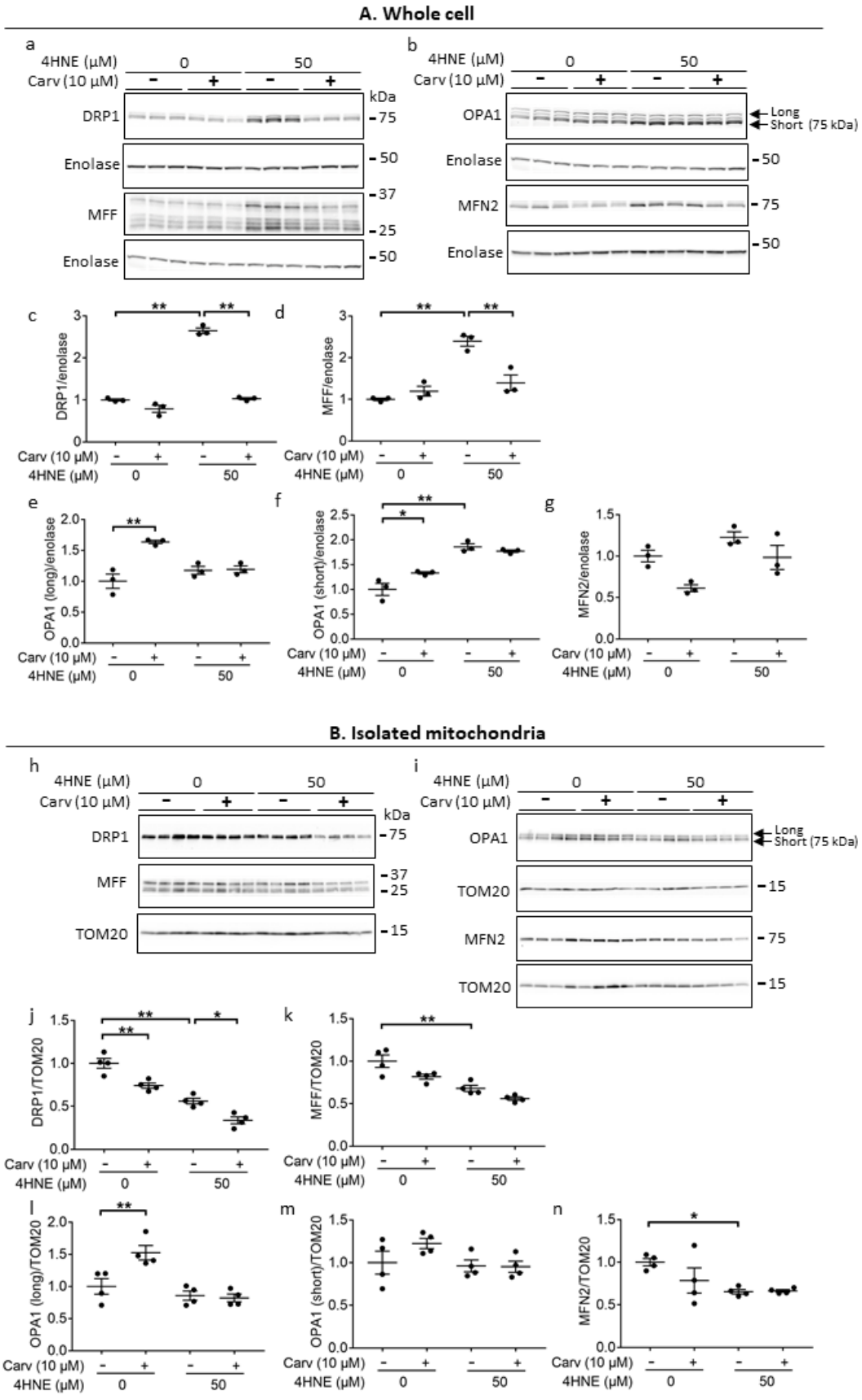
Supplementary Figure V: The effect of 4HNE on mitochondrial respiratory capacity of iPSC-derived cardiomyocytes. (a) Schematic depicting the process of generating cardiomyocytes from iPSCs by temporal modulation of Wnt signaling. Phase contrast image of iPSC colonies at (b) day 0 and (c) at day 30 of differentiation. (d) Confocal fluorescent image of an iPSC-derived cardiomyocytes expressing cardiomyocyte-specific markers troponin T (red) and mitochondrial import receptor subunit TOM20 (green). Nuclei are stained with DAPI (blue). (e) Representative oxygen consumption rate graphs of iPSC-cardiomyocytes at day 35 treated with either control medium or 4HNE for 24 hours, respectively, in response to oligomycin, FCCP, and rotenone/antimycin A. (f) Quantification of oxygen consumption rate parameters representing mitochondrial function in control (n=5) and 4HNE-treated iPSC-cardiomyocytes (n=5). Data are represented as mean ± SEM. Between-group comparisons performed using Mann-Whitney U test. ** $p < 0.005$ or n.s. not significant $p > 0.05$. 4HNE – 4-hydroxynonenal; iPSC – induced pluripotent stem cell; FCCP - Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; OXPHOS – oxidative phosphorylation.


A
iPSC
Cardiac differentiation
Cardiomyocyte purification
Cardiomyocyte culture
Medium
E8
RPMI+B27-Ins
RPMI+B27
RPMI-Glu+B27
RPMI+B27
Respiratory analysis
Days
-3
0
3
5
7
10
15
30
34
35
+CHIR (6μM)
+C59 (2μM)
change medium
Dissociate & Re-plate
+4HNE (50μM)
B
iPSC
C
iPSC-CMs (d30)
D
TOM20 TroponinT DAPI
100 um
100 um
10 um
E
Control
+4HNE
OCR (pmol/min)
Time (minutes)
F
Control
+4HNE
OCR (pmol/min)
**
n.s.
Basal Raspiration
OXPHOS
Maximal Respiration
Reserve Respiratory Capacity
Proton Leak
non-mito Respiration

Supplemental Figure VI. Increased mitochondrial membrane potential with carvedilol treatment is due to the accumulation of carvedilol in the mitochondrial matrix. HL1 cells were treated with carvedilol and with a combination of FCCP, antimycin, and rotenone (F.A.R) to abrogate the mitochondrial proton gradient. The control group had near abolishment of the membrane potential following treatment with F.A.R. However, carvedilol-treated cells still showed significantly elevated membrane potential compared to control, indicating the presence of negatively charged molecules (carvedilol) in the matrix of the mitochondria. N=3/group. F.A.R - FCCP, antimycin, and rotenone; Carv – carvedilol. Data are presented as mean±SEM. **p<0.01.



Supplemental Figure VII. 4HNE inhibits mitochondrial dynamics proteins. HL1 Cardiomyocytes were treated with 50 μM 4HNE for 24 hours and with 10 μM of carvedilol. A. Whole cells: (a, b) Representative western blot images for fission and fusion proteins are shown, (c, d) 4HNE increased expression of fission proteins DRP1 and MFF, and carvedilol returned the expressions to baseline. (e, f) 4HNE did not affect the expression of the fusion protein, long OPA1 isoforms, but increased short OPA1 isoforms. Carvedilol increased both long and short OPA1 isoforms, but only under control conditions. (g) 4HNE did not affect the expression of the fusion protein MFN2 (N=3/group). B. Mitochondrial fraction: (h-i) Representative western blot images for fission and fusion proteins are shown. In contrast to whole cell, (j, k) 4HNE decreased mitochondrial recruitment of both DRP1 and MFF; (l-n) 4HNE did not affect the recruitment of long and short OPA1 isoforms, but it decreased that of MFN2. Carvedilol did not rescue 4HNE-mediated inhibition in mitochondrial recruitment of dynamics proteins (N=4/group). Carv – carvedilol; 4HNE - 4-hydroxynonenal. Data are presented as mean±SEM. *$p<0.05$, **$p<0.01$.


A. Whole cell
a
4HNE (μM)
0
50
Carv (10 μM)
DRP1
Enolase
MFF
Enolase
kDa
75
50
37
25
50
b
4HNE (μM)
0
50
Carv (10 μM)
OPA1
Long
Short (75 kDa)
Enolase
MFN2
Enolase
50
75
50
c
DRP1/enolase
d
MFF/enolase
e
OPA1 (long)/enolase
f
OPA1 (short)/enolase
g
MFN2/enolase
Carv (10 μM)
4HNE (μM)
B. Isolated mitochondria
h
DRP1
MFF
TOM20
75
37
25
15
i
OPA1
Long
Short (75 kDa)
TOM20
MFN2
TOM20
15
75
15
j
DRP1/TOM20
k
MFF/TOM20
l
OPA1 (long)/TOM20
m
OPA1 (short)/TOM20
n
MFN2/TOM20

Supplemental Tables

Supplemental Table I. Antibodies used to assess protein expression.

Antibody	Catalog Number/Company	Dilution
4-hydroxy-2-nonenal (4HNE)	HNE11-S; Alpha Diagnostic International	1:1000
Voltage-dependent anion-selective channel protein 1 (VDAC1)	Ab14734; Abcam	1:2000
PPARG coactivator 1α (PGC1α)	NBP1-04676; Novus Biologicals	1:1000
Dynamin 1-like (DRP1/DLP1)	611113; BD	1:2000
Mitochondrial fission factor (MFF)	17090-I-AP; Proteintech	1:1000
Mitochondrial dynamin like GTPase (OPA1)	612607; BD	1:2000
Mitofusin 2 (MFN2)	H00009927-M01; Abnova	1:1000
Enolase	sc-15343; Santa Cruz Biotechnology	1:2000
Translocase of outer mitochondrial membrane 20 (TOM20)	42406; Cell Signaling sc-17764; Santa Cruz Biotechnology	1:2000
Troponin T	ab45932, Abcam	1:100

Supplemental Table II. Buffers and reagents for measuring oxygen consumption.

	Reagents	**Final concentration**
Oxygraph buffer	20 mM HEPES, 5 mM K_3PO_4, 0.2 mM EDTA, 2.5 mM $MgCl_2$, 10 mM KCl, 0.25 M sucrose, 1 mg/mL fatty acid free-bovine serum albumin; pH 7.4	-
Malate	1 M malate	5 mM
Glutamate	2 M glutamate	5 mM
Succinate	1 M succinate	20 mM
ADP	0.5 M ADP	200 µM
Oligomycin	4 mg/ml oligomycin	0.5 µM
FCCP	1 M FCCP	1 µM
Antimycin A	5 mM antimycin A	1 µM

Supplemental Table III. 4HNE modification of metabolic and mitochondrial proteins.

Category	4HNE-Modified Protein Name	4HNE/ Control (FC)	4HNE+ Carv/ Control (FC)
Metabolism	Cytochrome b-c1 complex subunit 1, mitochondrial	1.61	4.47
	Alpha-enolase	1.26	2.26
	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	2.11	2.23
	Acyl-coenzyme A thioesterase 2, mitochondrial	2.21	1.79
	Glycerol-3-phosphate phosphatase	2.3	1.09
	Nucleoside diphosphate kinase A	2.17	3.36
Structure	Desmin	2.2	1.26
	Tubulin-folding cofactor B	3.26	7.22
	Vinculin	2.17	4.74
Survival	Stress-70 protein, mitochondrial	2.66	9.48
	Heat shock protein 90-ß	1.99	10.79
	Heart shock protein 70	8.45	8.24
Others	Serum albumin	1.97	1.61
	Vacuolar protein sorting-associated protein 29	3.36	2.16
	Ubiquitin-like modifier-activating enzyme 1	2.24	1.57

Proteins with increased levels of 4HNE modification detected in 4HNE-treated HL1 cardiomyocytes vs. control by 2D-gel electrophoresis and western blot were identified using mass spectrometry. The same proteins were also assessed in 4HNE+Carvedilol treated HL1 cells. There was 100% protein score confidence interval for all proteins (>95% is considered significant). 4HNE - 4-Hydroxynonenal; FC - Fold change; Carv - carvedilol.