Reductive Stress Impairs Myogenic Differentiation

Namakkal S. Rajasekaran[1,2,4,a*], Sandeep Balu Shelar[1,a], Dean P. Jones[2], John R. Hoidal[3].

[1]Cardiac Aging & Redox Signaling Laboratory, Molecular and Cellular Pathology, Department of Pathology/Center for Free Radical Biology, University of Alabama at Birmingham, Birmingham, AL, USA.

[2]Division of Pulmonary/[4]Division of Cardiovascular Medicine, Department of Medicine, University of Utah School of Medicine, Salt Lake City, UT, USA.

[3]Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Emory University, Atlanta, GA.

[4]Center for Free Radical Biology, University of Alabama at Birmingham, Birmingham, AL, USA.

Running title: Reductive stress impairs differentiation

[a]These authors equally contributed

[*]Corresponding author

Namakkal S. Rajasekaran, PhD

Cardiac Aging & Redox Signaling Laboratory, Center for Free Radical Biology, Division of Molecular & Cellular Pathology, Department of Pathology, UAB, The University of Alabama at Birmingham, BMR2 Room 533, 901 19th Street South, Birmingham, AL 35294-2180. Tel: 205.996.9839|Fax: 205.934.7447|Email: rajnsr@uabmc.edu

Word count: 4680

Material and methods

***Materials**:* L-Sulforaphane, GSH-NEM, N-acetyl cysteine, GSH-GEE, propidium iodide were obtained from Sigma-Aldrich (St, Louis, MO, USA); CM-DCFDA was obtained from Molecular probes (USA).

***Antibodies**:* Anti-catalase was from Calbiochem (San Diego, CA, USA). GSR, HO-1, NQO1, GCLC, GCLM, PAX7, Myh2 and myogenin antibodies were obtained from Abcam (Cambridge, MA, USA). GAPDH, pRB, p27, and PCNA antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-SOD1 and anti-SOD2 were from Enzo Life Sciences (Farmingdale, NY, USA).

GSH/GSSG determination

Cells were harvested, homogenized in 1X MES buffer and centrifuged (5000 rpm, 5 min). The supernatants were mixed with equal amount of 10% meta-phosphoric acid (MPA) and centrifuged to collect the free thiols, and quantified GSH and GSSG as per the manufacturer's instructions (Cayman Chemicals) [1].

Gene expression analysis

RNA isolation and cDNA synthesis: Total RNA was extracted from proliferating or differentiating C2C12 myoblasts using RNeasy extraction kit (Qiagen) following manufacturer's instructions. The genomic DNA was digested using RNase-free DNase (Qiagen). RNA concentration and quality were analyzed by nanodrop spectrometry

(Thermo). cDNA was synthesized using a cDNA RT-kit (Qiagen) and the samples were stored at -20°C until analysis [1, 2].

qRT-PCR: Gene expression analysis was done using gene specific primers and cDNA as a template. SYBR reagent was used to detect amplification of cDNA by qRT-PCR (Roche). Relative gene expression was calculated by normalizing the Ct values with GAPDH [1, 2].

SDS-PAGE and immunoblotting

Cells were harvested, and cell lysates prepared using cytosol extraction buffer containing freshly added protease and phosphatase inhibitors. Protein concentrations were normalized, mixed with 4X loading dye containing 5% β-ME and boiled for 5 min. Proteins were separated on SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk in TBST prior to incubation with primary and secondary antibodies. Protein levels were detected using chemiluminescence detection techniques [1, 2].

ROS determination by flow cytometry

ROS levels were determined using CA-H2DCFDA (Molecular Probes) following the manufacturer's instructions [3]. Briefly, cells were suspended in PBS containing 10 μM of H2DCFDA, and incubated at 37°C for 30 min in dark. Then the cells were washed with PBS and re-suspended in the growth medium and incubated at 37°C for 5-10 min before sorting them (H2DCFDA fluorescence positive) using flow cytometry [3].

shRNA-mediated gene knockdown

C2C12 myoblasts were cultured in proliferation medium to ~70% confluence. Keap1 shRNA constructs #1-4 (OriGene) were transfected into myoblasts using Lipofectamine 3000 (Invitrogen) following manufacturer's instructions. After a 24 h incubation with shRNA constructs, the culture medium was replaced with fresh medium and cells were allowed to grow for 48 h. The transfected myoblasts were either harvested for gene expression analysis or subjected to differentiation [4, 5].

Annexin V/PI apoptosis assay

An Annexin V/PI staining kit (Molecular probes) was used to asses apoptosis following the manufacturer's instructions. Cells were harvested, washed with ice cold PBS and re-suspended in 1x Annexin-binding buffer at a concentration of ~1 X 10^6cells/ml. Then 5.0µl of Annexin V and 1.0µl of propidium iodide (100 µg/ml) were added in 100 µl of cell suspension and incubated in the dark at room temperature for 15 min. Then gently mixed with 400 µl of Annexin-binding buffer, kept on ice and analyzed by flow cytometry within 1.0 hour [1, 6].

Supplementary Figure legends

Figure S1: Sulforaphane mediated RS suppresses reactive oxygen species generation during myoblast differentiation. C2C12 myoblast were cultured in proliferation and differentiation medium with or without SF (1, 3 & 5 µM) and the levels of reactive oxygen species was measured by flow cytometric analysis using redox sensitive dye CM-DCFDA. (A) Histograms of the oxidized CM-DCFDA fluorescence

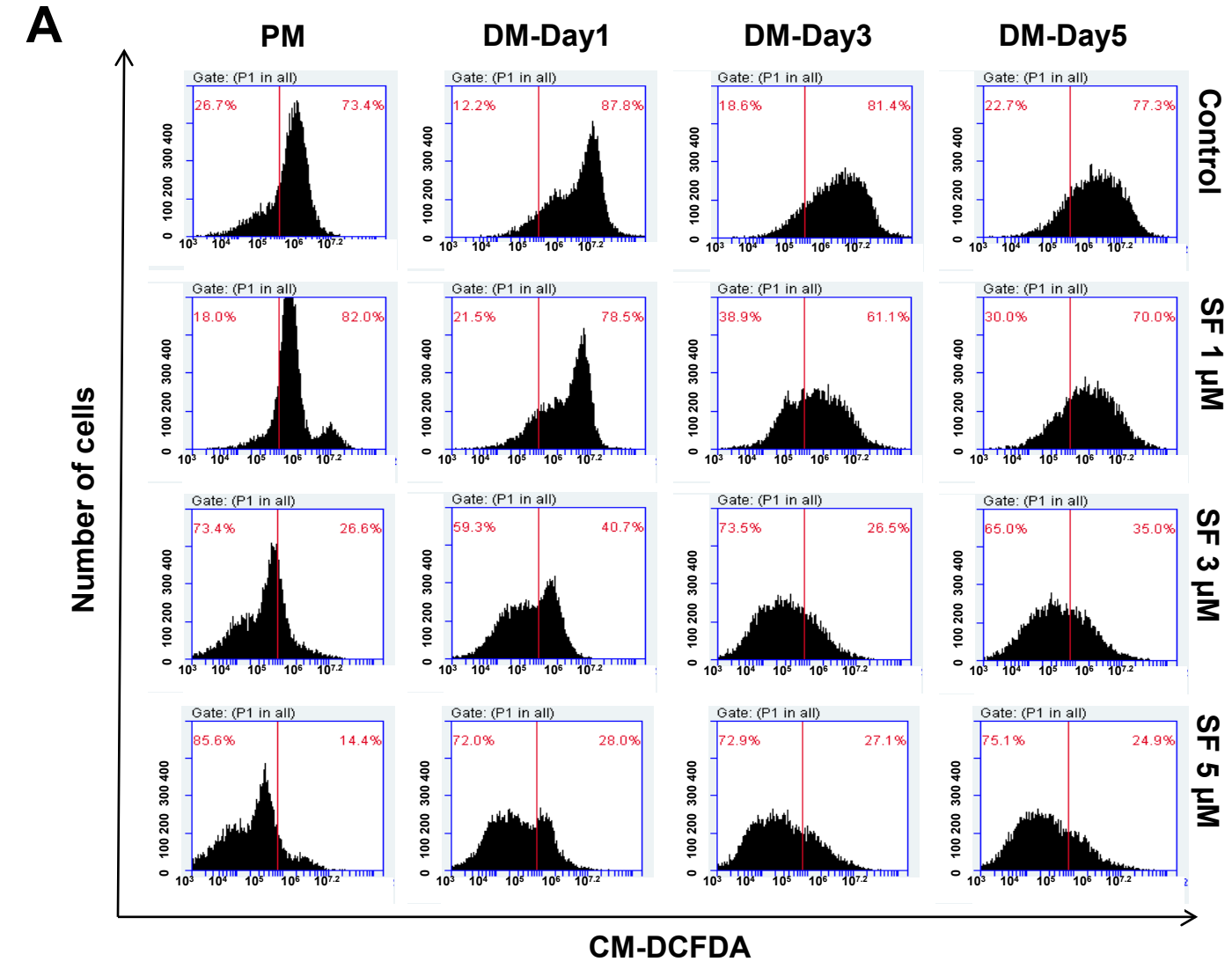
signal (X-axis: fluorescence intensity, Y-axis: number of cells). (B) Bar graphs of % reactive oxygen species measured by change in % fluorescence intensity. (*$p<0.05$, n=3–4).

Figure S2: SF-mediated augmentation of RS does not induce apoptosis in myoblasts. Proliferating (PM) and differentiating (DM-Day1, -Day3 and -Day5) myoblast treated or untreated with SF (1, 3 and 5 μM) were fixed with Annexin V and propidium iodide (PI) and subjected to apoptosis assay by flow cytometry. (A) Scattered plots showing live, early apoptotic, apoptotic and dead cells using Annexin V intensity (X-axis) vs PI intensity (Y-axis). (B) Bar graphs showing percentage (%) number of live, early apoptotic, apoptotic and dead cells. (C) Western blots of apoptosis markers cleaved caspase 3 and cleaved PARP of proliferating (PM) and differentiating (D1 and D5) myoblast treated or untreated with SF (5 μM). PC, positive control (cells treated with 1.0 μM staurosphorin, an apoptosis-inducing agent, for 12 h.

References

1. Shanmugam, G., et al., *A biphasic effect of TNF-α in regulation of the Keap1/Nrf2 pathway in cardiomyocytes.* Redox biology, 2016. **9**: p. 77-89.
2. Bolus, D.J., et al., *Recurrent heat shock impairs the proliferation and differentiation of C2C12 myoblasts.* Cell stress & chaperones, 2018. **23**(3): p. 399-410.
3. Eruslanov, E. and S. Kusmartsev, *Identification of ROS using oxidized DCFDA and flow-cytometry.* Methods Mol Biol, 2010. **594**: p. 57-72.
4. Chien, M.-H., et al., *Keap1–Nrf2 Interaction Suppresses Cell Motility in Lung Adenocarcinomas by Targeting the S100P Protein.* Clinical Cancer Research, 2015. **21**(20): p. 4719-4732.
5. Tóth, A., et al., *The Effect of SERCA1b Silencing on the Differentiation and Calcium Homeostasis of C2C12 Skeletal Muscle Cells.* PloS one, 2015. **10**(4): p. e0123583-e0123583.
6. Li, H., et al., *PFN2a Suppresses C2C12 Myogenic Development by Inhibiting Proliferation and Promoting Apoptosis via the p53 Pathway.* Cells, 2019. **8**(9): p. 959.

Figure S1


A
PM
DM-Day1
DM-Day3
DM-Day5
Control
SF 1 μM
SF 3 μM
SF 5 μM
Number of cells
CM-DCFDA
Gate: (P1 in all)
26.7%
73.4%
12.2%
87.8%
18.6%
81.4%
22.7%
77.3%
18.0%
82.0%
21.5%
78.5%
38.9%
61.1%
30.0%
70.0%
73.4%
26.6%
59.3%
40.7%
73.5%
26.5%
65.0%
35.0%
85.6%
14.4%
72.0%
28.0%
72.9%
27.1%
75.1%
24.9%

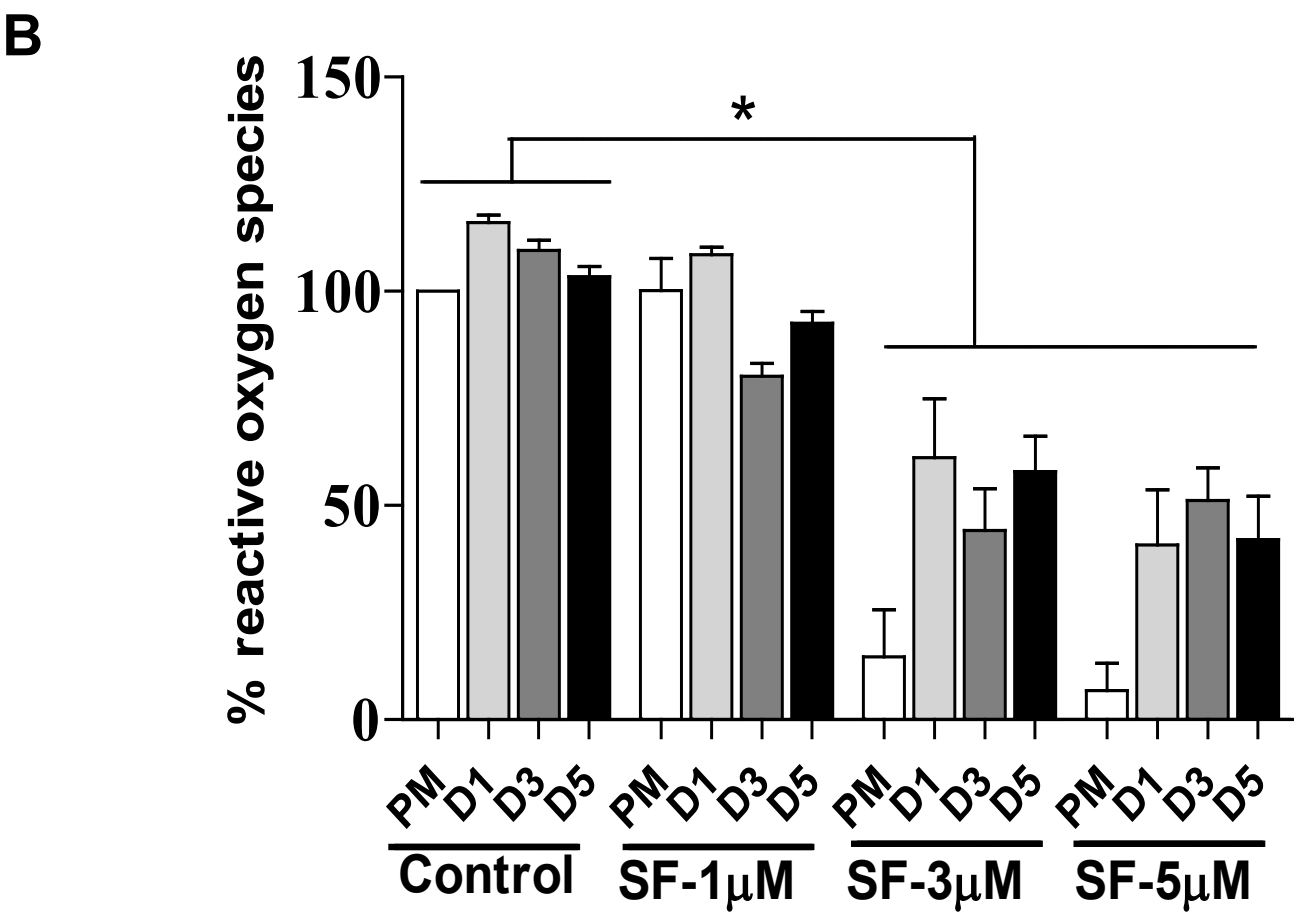
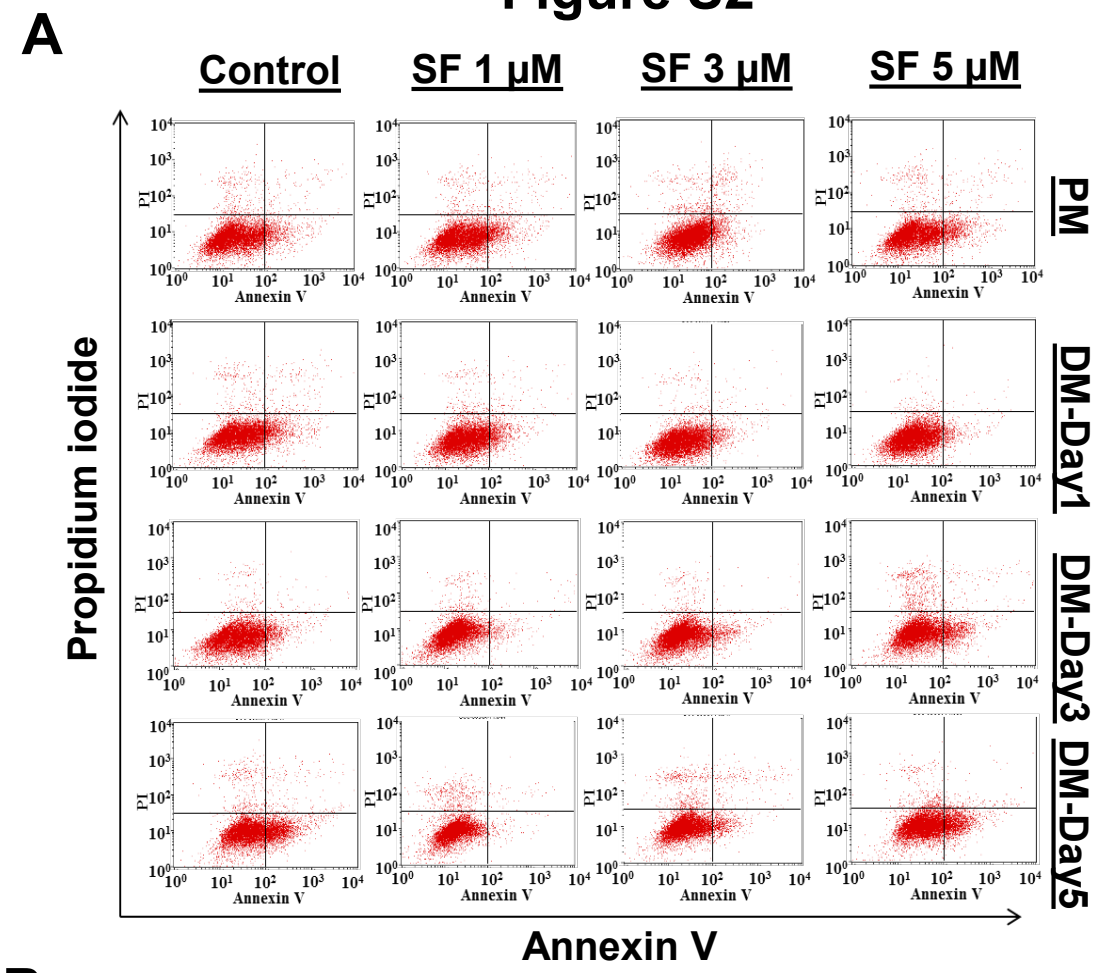
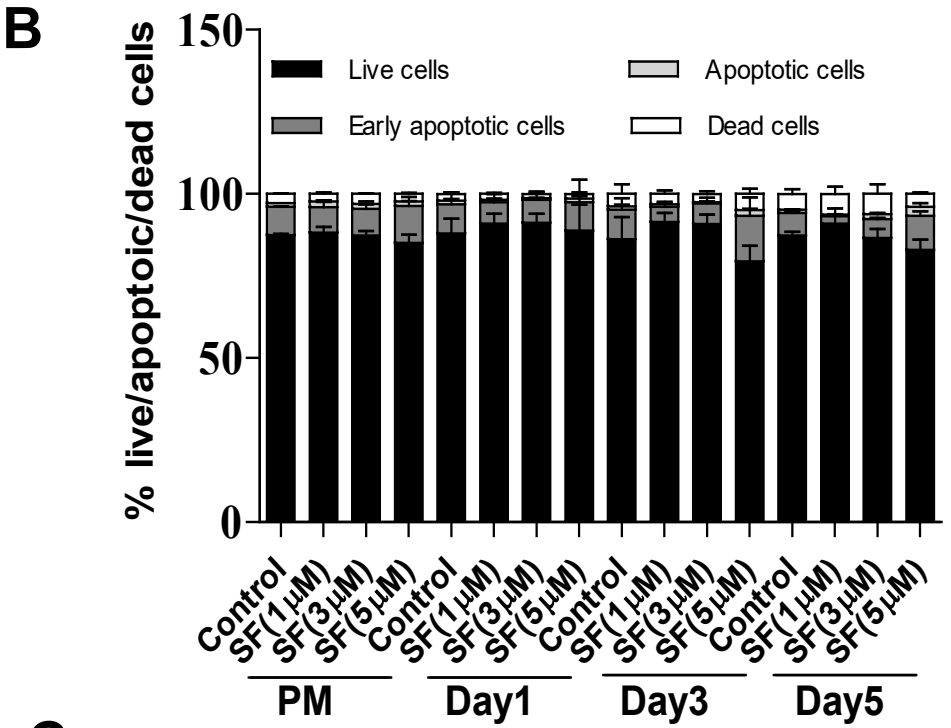
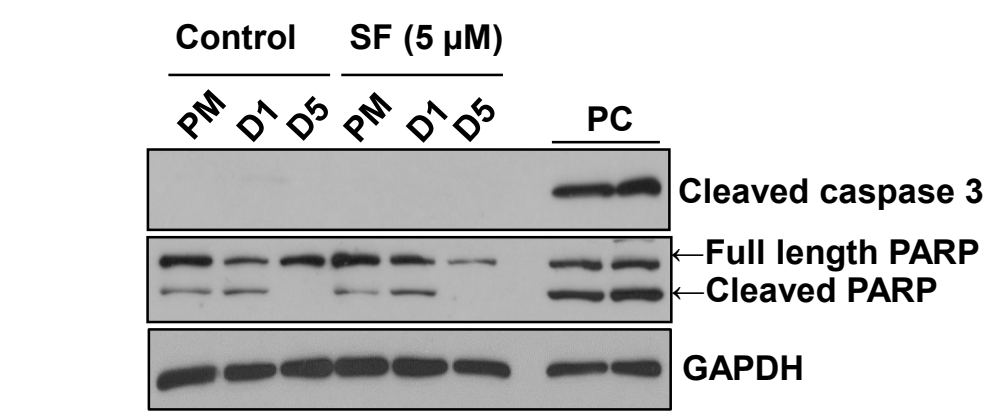

B
% reactive oxygen species
150
100
50
0
*
PM
D1
D3
D5
Control
SF-1μM
SF-3μM
SF-5μM

Figure S2


A
Control
SF 1 μM
SF 3 μM
SF 5 μM
PM
DM-Day1
DM-Day3
DM-Day5
Propidium iodide
Annexin V
PI


B
% live/apoptoic/dead cells
150
100
50
0
Live cells
Apoptotic cells
Early apoptotic cells
Dead cells
Control
SF(1μM)
SF(3μM)
SF(5μM)
PM
Day1
Day3
Day5


C
Control
SF (5 μM)
PM
D1
D5
PC
Cleaved caspase 3
Full length PARP
Cleaved PARP
GAPDH