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Supplementary Methods

Harvest and Culture of Neonatal Rat Cardiomyocytes

All pups were used, as it is extremely difficult to differentiate between sexes anatomically at this neonatal stage. Spontaneously contracting cells were plated after 1 hour of differential plating to remove non-myocytes. Greater than 99% of the population was determined to be cardiomyocytes by immunostaining with cardiac myosin heavy chain antibody (#ab15, AbCam). The cells were grown in L-15 Leibovitz medium supplemented with 10% fetal bovine serum (FBS) in a standard tissue culture incubator.

Simulated Ischemia-Reperfusion

Cells were treated with reagents of interest 30 minutes prior to start of hypoxia. These reagents include E2, ICI182780 ([ICI], Tocris), SB203580 ([SB], Sigma-Aldrich), rotenone (Sigma-Aldrich), Mito-Q® (a gift from Dr. Ellis Levin), pifithrin-α ([PFT], Sigma-Aldrich), propylpyrazole-triol and diarylpropionitrile ([PPT and DPN, respectively], Tocris). The cells were incubated in an anaerobic chamber (GasPack system, BD Biosciences) for 18 hours, which was purged with 95% N2, 5% CO2 and sealed with an oxygen-consuming palladium catalyst. This created a hypoxic condition of 1.5 % O2. Following hypoxia, the chamber was opened to air, and cells placed in a standard incubator for reoxygenation for 1 hour.

Western Blotting

After H/R, cardiomyocytes were washed and lysed as reported previously.[1] Protein concentration was determined by a bicinchoninic acid-based assay using a commercially available kit (Thermo Fisher Scientific), and 40 μg of protein was loaded onto 10 or 12 % SDS-PAGE gel for electrophoresis. Proteins

were transferred to nitrocellulose membrane, which was blocked and probed with primary antibody against the protein of interest, then with anti-rabbit horseradish peroxidase conjugated secondary antibody (#7074, Cell Signaling Technology) in a standard manner. The membrane was then incubated with chemiluminescence reagents using a commercially available ECL Plus Western Blotting Detection System (Amersham Biosciences) and exposed to film. The band intensity on radiographs was determined by scanning with UN-SCAN-IT™ software (Silk Scientific). Primary antibodies used for western blotting include polyclonal antibody against actin (#4968), serine-15 phosphorylated p53 (#9284), Cyt *c* (#4272), caspase-9 (#9506) , cleaved caspase-9 (#9507) , Bcl-2 (#2876), Bax (#2772) and CoxIV (#4844) from Cell Signaling Technology, and p38β (#sc-6187-R) and p53 (#sc-6243) from Santa Cruz Biotechnology.

For the assessment of the intrinsic apoptotic pathway, the release of Cyt *c* from mitochondria was examined. Mitochondria were isolated by following the protocols of a commercially available Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific). Western blotting was performed on mitochondrial and cytosolic fractions for Cyt *c*, using commercial antibody (#4272, Cell Signaling Technology). Antibody to cytochrome *c* oxidase IV ([CoxIV], #4844, Cell Signaling Technology) was used for a mitochondrial loading control.

Immunofluorescence

Cardiomyocytes grown on glass bottom dishes (MatTek) were stained with MitoTracker® Red CMXR according to the manufacturer's instructions (Invitrogen) at a final concentration of 500 nM. Cells were fixed in 3.7% paraformaldehyde for 15 minutes at 37 °C, permeabilized in 0.2% Triton® X-100 for 10 minutes at room temperature, and incubated with p53 primary antibody (#sc-6243, Santa Cruz Biotechnology) overnight at 4 °C. Then, the cells were incubated with FITC-conjugated secondary antibody (Vector Lab) for 1 hour, mounted with Vectashield (Vector Lab) and examined under fluorescent microscope.

Supplementary Tables

Table 1. Cell counts from apoptosis assay in Fig. 1A

Condition	Apoptotic Cell #	Total Cell #	Mean % Apoptosis	SE
N	7	362	2	1.3
H/R	301	442	68	5.5
H/R+PFT	151	502	30	2.0
H/R+siRNAp53	104	400	26	1.3
N+PFT	40	441	9	5.7
N+siRNAp53	26	521	5	0.3

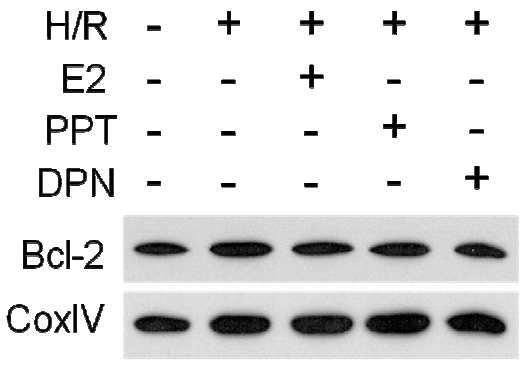
Apoptotic cells were determined by counting the number of green fluorescent, annexin V-positive cells. Total cell # was determined by counting all Hoechst 33342-stained nuclei in the visual field. Cell # represents the numbers averaged from experiments replicated three times. Mean % apoptosis represents the mean percentage of apoptotic cells derived from experiments replicated three times. SE, standard error.

Table 2. Cell counts from apoptosis assay in Fig. 5E

Condition	Apoptotic Cell #	Total Cell #	Mean % Apoptosis	SE
N	19	269	7	4
H/R	169	447	37	5
H/R+E2	78	526	14	3
H/R+PPT	68	257	26	6
H/R+DPN	101	597	17	8

Apoptotic cell # was determined by counting the number of green fluorescent, annexin V-positive cells. Total cell # was determined by counting all nuclei in the visual field. Cell # represents the numbers averaged from experiments replicated three times. Mean % apoptosis represents the mean percentage of apoptotic cells derived from experiments replicated three times. SE, standard error.

Supplementary Figure



Supp. Fig. Bcl-2 expression does not change with E2, PPT, or DPN treatment. Following H/R in the presence or absence of E2, PPT, and DPN (each in 10 nM), cells were lysed and mitochondrial fractions separated for immunoblotting of Bcl-2, a protein that mainly resides in mitochondria, per protocol described in the Methods. Loading control was done by immunoblotting for a mitochondrial marker

protein, CoxIV. Representative immunoblots from triplicate experiments are shown. There was no statistically significant difference among the conditions in band intensity of Bcl-2 or CoxIV (data not shown).

Supplementary Reference

1. Kim JK, Pedram A, Razandi M, Levin ER. Estrogen prevents cardiomyocyte apoptosis through inhibition of reactive oxygen species and differential regulation of p38 kinase isoforms. *J Biol Chem* 2006;**281**:6760-6767.