Methods

Two different models of the failing heart were used: the explanted failing human heart removed at transplant and a high coronary ligation model of HF in the rat. Details of the methods for the high LAD ligation have previously been reported.[1] This model produces mild to moderate heart failure at 9 weeks characterized by decreased fractional shortening and increased heart/body weight ratio. Rat left ventricular size and fractional shortening were measured by echo as previously described.[14] The HF rats had a fractional shortening of 14.2 ∀ 1.6% compared to 45.4 ∀ 2.6% in the controls (n=5-6, $p < 0.001$). LVDd was 1.2 ∀ 0.5 cm in HF vs. 0.80 ∀ 3.2 (n= 5-6, $p < 0.005$). The animal protocol was approved by the University of California, Davis Animal Research committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Human heart samples, collected at the time of transplantation at the Methodist Hospital, Houston, Tx, were the gift of Dr. Guillermo Torre-Amione. This tissue bank of failing hearts has IRB approval at Methodist Hospital. The protocols involving human normal and failing heart samples were reviewed by the University of California, Davis IRB and approved as exempt, category 4. All explanted hearts were preserved by immediately freezing them in liquid nitrogen. Failing left ventricular heart samples came from adults identified as having either dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (ICM). Significant coronary disease was excluded in those hearts identified as DCM Normal, adult human heart samples were collected from brain dead donors by a private company (T-Cubed). Heart samples were provided to the authors without identifying information, but with a brief medical history, gender and age. The left ventricle was used for all studies.

Western Blots - were performed as previously described.[2] Mouse monoclonal anti-OPA1 (BD Biosciences), anti-DRP1 (BD Biosciences), anti-MFN1 (Abnova Corporation), anti-MFN2 (Abnova Corporation), and rabbit polyclonal anti-Fis1 (Imgenex) antibodies were all used at 1:1000 dilution. Mouse monoclonal anti-GAPDH antibody (Assay Designs) was used as a loading control. Chemiluminescence detection was performed using an ECL detection kit (Pierce).

Electron Microscopy - Standard transmission electron microscopy and immuno-electron microscopy were performed as previously described.[3] Briefly, rat left ventricles were fixed in 4% paraformaldehyde. For standard EM, tissues then were fixed in osmium tetroxide and embedded in an epoxy resin using standard protocols. For immuno-EM, sections were blocked with 1% fish gelatin and then incubated in anti-OPA1 (BD Biosciences) overnight at 4EC at 1:50. Secondary antibodies were used at 1:100. The sections were stained in UA and lead citrate. Ultra thin sections were viewed on a Philips CM 120 Biotwin. Digital images of sequential fields were collected for analysis.

Mitochondrial Measurements - Digital images of multiple samples were collected from sequential fields during EM analysis. To determine the population and size of the mitochondria, the EM images were analyzed with Photoshop CS3, using the counting and area analysis function, in an approach similar to that reported by other investigators.[4,5] For area measurement, the mitochondria were circled by the lasso tool and then the area of the circles were calculated and converted to their actual values using the scale bar.

Apoptosis - was detected using the Cell Death Detection assay (CDD, Roche), which measures DNA fragmentation by assessing oligosome formation. The TUNEL assay (Roche) was used to assess the percent of cells undergoing apoptosis. Cytochrome c release was assessed by fractionating the cells and determining the amount of cytochrome c in the cytosol by western blot, as previously described. [1] All apoptosis measurements were made 10 h after simulated ischemia.

shRNA and Constructs - A short hairpin RNA (shRNA) against the rat OPA1 sequence (1992-2020, 5 GATGAAGTCATCAGTCTGAGCCAGGTTAC-3) was used to silence OPA1 expression in H9c2 cells. The oligonucleotide pair was annealed, ligated into the pENTR/U6 vector and used to transform competent *Escherichia coli cells* following an established protocol (Invitrogen). For the control group, shRNA against LacZ (5 AGCGAAACCGATTTTAACTGGGGAGTCTT-3) was made as well. The rat OPA1 full length cDNA was introduced into pIRES-EGFP vector (Clontech) for OPA1 over expression, while the empty vector (EGFP alone) was used as control. The pIRES-EGFP vector expresses both the subcloned gene, OPA1, and EGFP. The resulting plasmids were transfected into H9c2 cells using Lipofectamine following Invitrogen protocols. [6]

RNA and Real Time PCR - RNA was isolated from heart samples using Trizol (Invitrogen). Real time PCR was performed as previously described.[7] Primers were from Applied Biosystems (TaqMan Gene Expression Assays). GAPDH was used as a control, as described previously.[7]

H9c2 Cells - H9c2 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% O2 / 5% CO2 at 37EC. Simulated ischemia was carried out as previously described.[8] Briefly, cells were placed in ischemic buffer and placed in an anaerobic chamber as previously detailed for 10 h.[8] At the end of simulated ischemia, the cells were removed from the chamber and returned to normal media. Cyclosporine A (CsA, Sigma) treatment (500 nM) was used in some experiments.

Confocal Microscopy - Mitochondria were labeled with organelle Lights Mito-OFP reagent (Invitrogen), which is a fluorescent protein fused to a mitochondrial targeting sequence and cloned into baculovirus for delivery. To determine mitochondrial fusion activity, cells were visually scored under a fluorescent microscope into three morphological classifications. Highly tubular refers to cells in which greater than 95% of the mitochondrial mass existed as tubules as opposed to spherical fragments. Tubular refers to cells in which more than 40% but less than 95% of the mitochondrial mass existed as tubules. Fragmented refers to cells that contain spherical mitochondrial fragments with no more than 40% short tubules found.

Statistics - Results were compared by ANOVA followed by a Student-Neuman-Keuls test for multiple comparisons. For normalized data an ANOVA on Ranks was used. The student's t test was used for comparisons involving only two groups. Results are expressed as mean ∀ SEM. A $p < 0.05$ was considered significant.

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