On-line Supplementary Information

Role of AIF in cardiac apoptosis in hypertrophic cardiomyocytes from Dahl salt sensitive rats

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Supplemental Methods

Materials

Antibodies were obtained from the following sources: AIF, caspase-9, and cytochrome c (BD Pharmingen, Franklin Lakes, NJ), cytochrome c oxidase subunit IV (COX IV), Bcl-2, and α-tubulin (Cell Signaling, Danvers, MA), GAPDH (Sigma-Aldrich, St. Louis, MO), Hsp70 (Calbiochem, Gibbstown, NJ), and anti-mouse HRP and anti-rabbit HRP (Jackson Immuno Research Laboratory, West Grove, PA). Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD.fmk) was obtained from Promega (Madison, WI) and 4-amino-1,8-napthalimide (4-AN) was obtained from Biomol International (Plymouth Meeting, PA).

Generation of hypertrophic and non-hypertrophic adult cardiomyocyte cultures

To generate pressure-overload cardiac hypertrophy, female Dahl salt-sensitive (DSS) rats (Harlan Sprague Dawley, Indianapolis, IN) were fed a 6% NaCl diet from 6 to 12 weeks of age. A high salt diet induces significant pathological cardiac hypertrophy in DSS rats. The clinical characterization of this animal model has been published by our laboratory [19], and we have used

this model to establish hypertrophic cultures [19, 20]. Control rats without cardiac hypertrophy were age-matched DSS females fed normal rat chow.

To prepare cultures of hypertrophied and non-hypertrophied cardiomyocytes, rats were anesthetized with ketamine. The hearts were removed rapidly using sterile technique, attached to the Langendorff apparatus and perfused retrogradely for 5 minutes with calcium-free perfusion buffer containing minimum essential medium (Joklik's modification) supplemented with 5 mM taurine, 2 mM creatine, 5 mM HEPES, and 20 u/L insulin. The hearts were then enzymatically dissociated with perfusion buffer containing 0.3% collagenase for 45 minutes, minced further and dissociated in incubation buffer (perfusion buffer with 0.2% BSA and 0.3 mM $CaCl_2$) containing 0.3% collagenase. The supernatants containing dissociated cardiomyocytes were washed twice with incubation buffer and the myocyte fractions separated and plated on laminin-coated plates (10 μg/ml) at $2x10^5$ cells/cm^2 with serum-free DMEM supplemented with 5 mM taurine, 5 mM creatine, 2 mM L-carnitine, 25 mM HEPES and 20u/L insulin. After one hour of plating, unattached (damaged or dead) cells were removed by changing the media. The cells were used after 24 hours of plating. A different heart was used for each culture.

Induction of apoptosis

Apoptosis was induced by hypoxia/reoxygenation, which is similar to the pathological condition in heart failure. In addition, to investigate whether AIF-induced apoptosis is stimuli specific, we also performed experiments with staurosporine (1μM), which has been shown to induce apoptosis in virtually all cells that posses an intact death pathway [46], including the recent demonstration in neonatal cardiomyocytes [47]. We have used this apoptotic stimulus extensively in both normal and hypertrophic cardiomyocytes in previous investigations [19, 22].

A hypoxic condition was created by incubating the cells in an air-tight chamber infused with 5% CO_2/95%N_2 gas at 37°C for the periods specified in each experiment. Intrachamber oxygen (<2%) was verified with a VTI Oxygen Analyzer (Vascular Technology) at the end of the experiment. At the end of the hypoxic phase of the procedure, cells were removed from the incubation chamber and reoxygenated in room air at 37°C. There were two main experimental groups: those in which cells were exposed to a specified period of hypoxia followed by reoxygenation (eg., 6H/6R) and normoxic controls (C).

Quantitative Analysis of Cellular Viability and Apoptosis

Apoptosis was quantified by annexin V staining. Cell viability was determined using propidium iodide exclusion and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Ten µl/ml annexin V-FITC (Pharmingen) was added directly to the media containing 2.0 mM $CaCl_2$, and incubated for 15 minutes at 37°C. Non-viable cells were identified by co-staining with 0.5 µg/ml propidium iodide. The number of viable cells was calculated by subtracting all non-viable cells from the total number of cells. Approximately 1000 cells were counted per dish (10 random microscopic fields at x250 magnification).

MTT assay was performed according to the manufacturer's instructions (Sigma, St. Louis, MO), and as previously published. Briefly, cells were treated with MTT for 3 hours. Formed formazan crystals were dissolved with MTT solvent while plates were agitated on a shaker for 30 minutes in the dark. Absorbance was measured at 570nm with a reference wave length at 690 nm. MTT assay can no longer be considered a strictly mitochondrial assay, because MTT reduction is associated not only with mitochondria but also with the cytoplasm and with non mitochondrial membrane.

Subcellular fractionation and immunoblots

The cells were collected in mitochondria buffer (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM EGTA, 20 mM HEPES) supplemented with 1 mM DTT and protease inhibitors (Complete Cocktail, Boeringer Mannheim). The cells were mechanically disrupted with the glass tissue homogenizer on ice, and centrifuged at 350 g for 10 minutes to pellet cell debris and nuclei. The supernatant was again centrifuged at 10,000g for 30 minutes to obtain the mitochondrial fraction. Final ultracentrifugation at 100,000g for 60 minutes was done to obtain the supernatant (cytosolic fraction). Protein concentration was determined using the Bradford method (Bio-Rad). Samples with equal amounts of protein (100 μg) were separated by SDS-PAGE and transferred to an Immobilin-P transfer membrane (Millipore), which was probed with specified antibodies. Visualization of the antigens was done with a chemiluminescence detection system (NEN Life Science Products). COX IV, α –tubulin or GAPDH was used as internal control. In adult cardiomyocytes, there is a very high level of cytochrome c because, in part, of a high density of mitochondria. Thus, in some immunoblots for cytochrome c, we load 10 times less total protein from the mitochondrial fraction compared to the total protein in the cytosol (please see Figure S3, where we loaded 10 μg of total protein from mitochondria vs. 100 μg from the cytosol). We also conducted a comparative analysis of AIF release from N-CM and H-CM, which was run in the same gel and blotted on the same membrane. The western blots were quantified by the NIH Image J program.

Caspase and PARP activity assay

Cells were collected and incubated in caspase lysis buffer (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 10μg/ml leupeptin, 2 μg/ml aprotinin) for 15 minutes on ice. They were then mechanically disrupted and centrifuged at 15,000g for 20 minutes to obtain cell lysates. Equal amounts of protein were incubated with 50 μM of caspase substrate in caspase reaction buffer mM 50 HEPES (pH 7.4), 75 mM NaCl, 0.1% CHAPS, 2 mM DTT in a 96-well microplate at 37°C for 60 minutes. Caspase-3 activities were measured using synthetic caspase substrate AcDEVD-pNa. Release of pNa was measured at 405 nm wavelength by spectrometer, and adjusted to the background. PARP activity was measured from cell extracts with an ELISA based, PARP Universal Colorimetric Assay Kit (R & D System) according to the manufacturer's instruction.

Supplemental Figure Legends

Supplemental Figure 1: Cell viability of cultured non-hypertrophic and hypertrophic cardiomyocytes over eight days. A. Quantitative analysis of the viability of non-hypertrophic (dark shaded) and hypertrophic cardiomyocytes (light shaded) over 8 days in a normoxic environment. Cell viability was assessed by MTT assay at specific time points. N-CM=non-hypertrophic cardiomyocytes, H-CM=hypertrophic cardiomyocytes. N=6, * $p<0.05$. **B** and **C.** Western blots of subcellular fractions in non-hypertrophic **(B)** and hypertrophic (**C**) cardiomyocytes after 0, 6, 12 and 24 hours incubation with vehicle.

Supplemental Figure 2: Time courses of AIF and cytochrome c release from mitochondria in non-hypertrophic and hypertrophic cardiomyocytes after apoptotic stimulation. Western blot **(A)** and quantitative **(B)** analyses of subcellular fractions in non-hypertrophic and hypertrophic cardiomyocytes after H/R. The blots were run in the same gel to compare non-hypertrophic and hypertrophic cardiomyocytes directly. Cytosolic and mitochondrial AIF levels were normalized to α-tubulin and COX IV levels, respectively. N=4, * $p<0.05$. Western blot **(C)** and quantitative **(D)** analyses of subcellular fractions in non-hypertrophic and hypertrophic cardiomyocytes after staurosporine treatment. N=4, * $p<0.05$.

Supplemental Figure 3: Time course of release of cytochrome c after staurosporine treatment in non-hypertrophic cardiomyocytes. A. Western blot analysis of subcellular fractions in non-hypertrophic cardiomyocytes after staurosporine treatment. To detect changes in the level of mitochondrial cytochrome c, mitochondrial cytochrome c protein was diluted 1:10 compared to cytosolic cytochrome c protein. **B.** Quantitative analysis of mitochondrial and

cytosolic cytochrome c release after staurosporine treatment in arbitrary units referenced to a-tubulin and COX IV. N=4, * $p<0.05$.

Supplemental Figure 4: Effect of caspase inhibition and 4-AN on cardiomyocytes. A. Quantitative analysis of cytosolic cytochrome c levels with various caspase inhibitions, AdCrmA (100 moi) and zVAD.fmk (25 μM), after H/R in non hypertrophic cardiomyocytes. Cytosolic cytochrome c levels were normalized to α-tubulin. N=4, NS=not significant. **B.** Western blot analysis of subcellular fractions in non-hypertrophic (left panel) and hypertrophic (right panel) cardiomyocytes comparing the effect of PARP inhibition by 4-AN to vehicle and control.

Supplemental Figure 5: Localization of Bax and BCl2 family members in non hypertrophic and hypertrophic cardiomyocytes after fractionation.

A. Endogenous levels of Bcl-2, Bax and Bak in subcellular fractions of non- hypertrophic and hypertrophic cardiomyocytes. Cytosolic and mitochondrial contamination was checked with α-tubulin and COX respectively. N=4. **B**. Endogenous level of EndoG in cardiomyocytes and non cardiomyocytes.

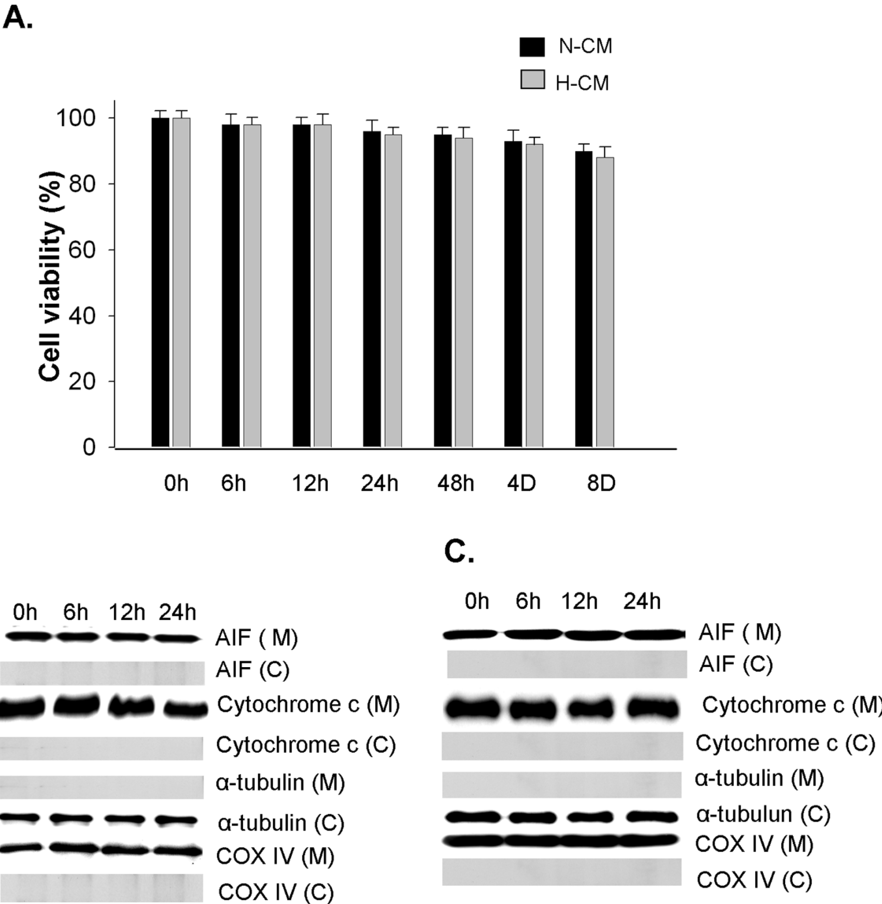
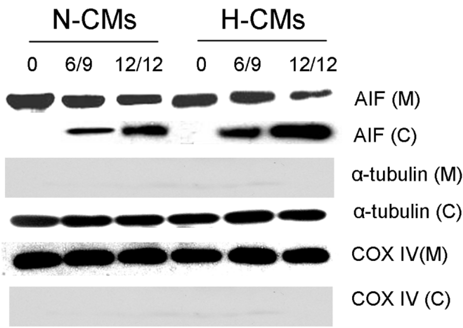
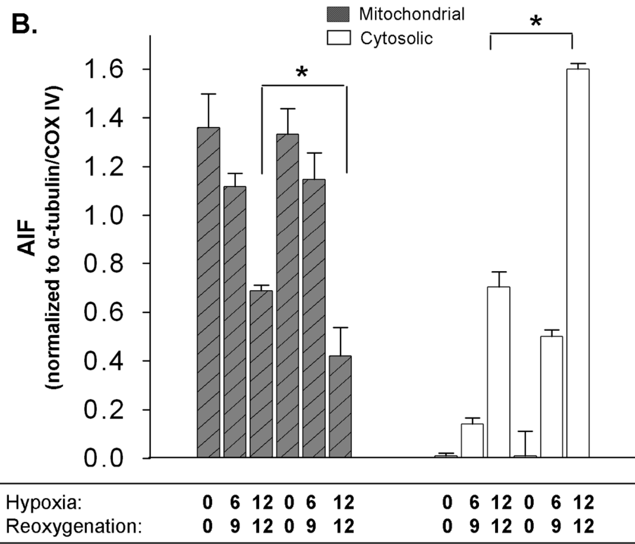
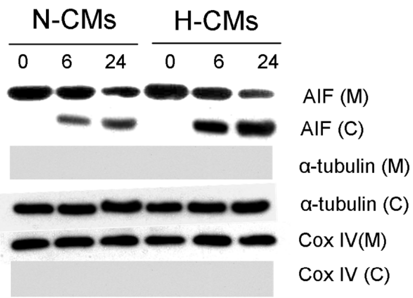


Figure S1







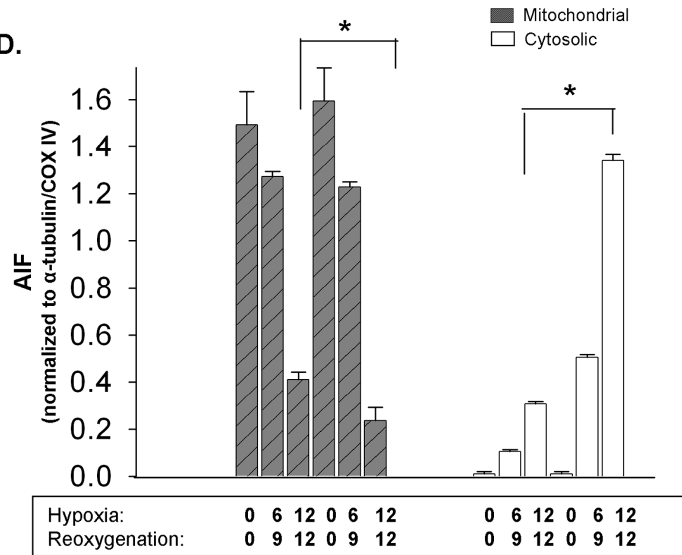
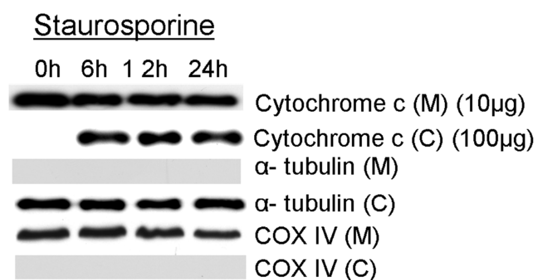


Figure S2



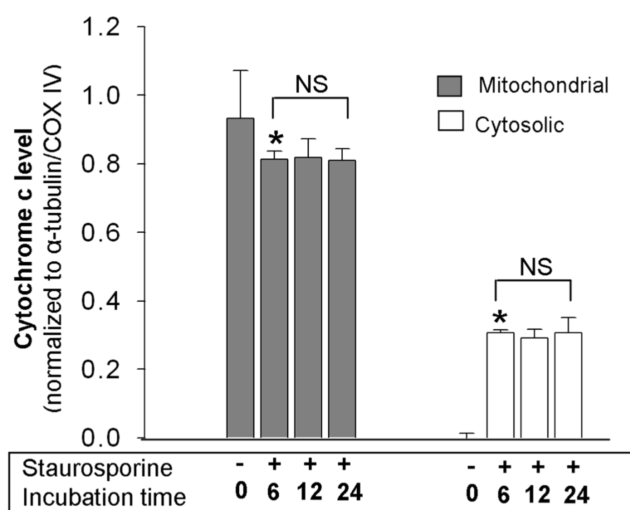
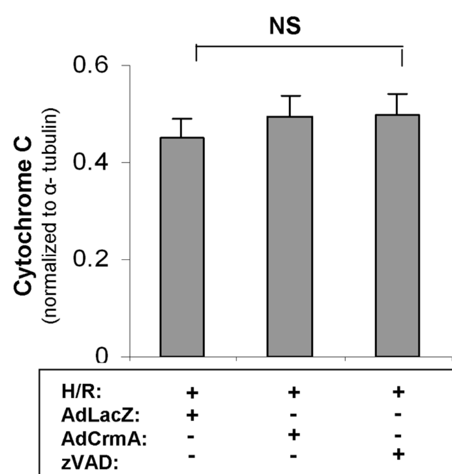


Figure S3



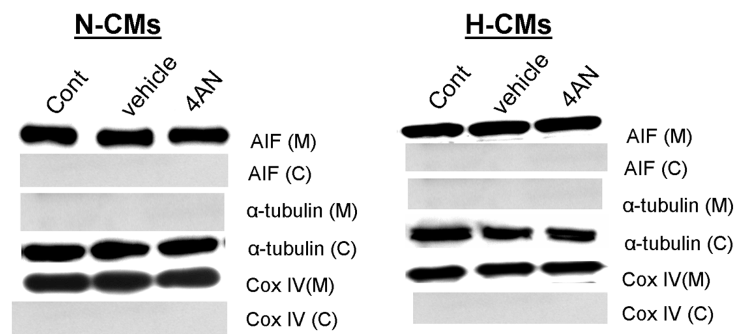


Figure S4

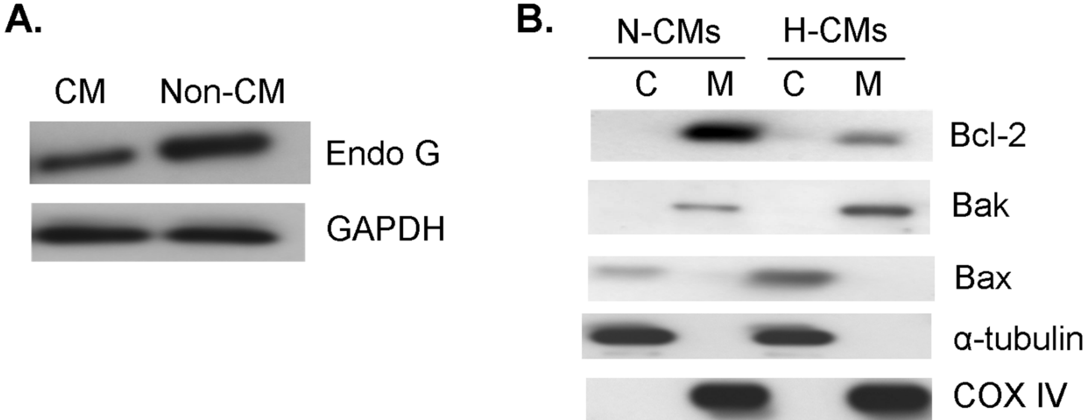


Figure S5