Supplement Material

Methods and Materials

Cell Cultures

The embryonic rat heart-derived cell line H9c2 was obtained from the American Type Culture Collection. Adult ventricular cardiomyocytes were isolated from 2-month old male Sprague-Dawley rats (Harlan Laboratory), as previously described (1, 2). Briefly, rats were anesthetized with sodium pentobarbital (50mg/kg IP) and heparinized (10,000U/kg IP). The hearts were perfused with preheated modified Krebs-Henseleit buffer (KHB) (in mmol/L: NaCl: 118, KCl: 4.8, HEPES: 25, K2PO4: 1.25, MgSO4: 1.25, glucose: 11, taurine: 5 and BDM: 10, pH7.4) for 5 minutes. Hearts were then perfused with an enzyme solution, which contained 0.7mg/mL collagenase type II (263U/mg), 0.2mg/mL hyaluronidase, 0.1% BSA and 25μM Ca2+, for 10 minutes. Subsequently, the Ca2+ concentration in the perfusion buffer was raised to 100μM, and perfusion continued for 5 additional minutes. Finally, ventricular tissue was excised, minced, pipette-dissociated, and filtered through a 240-μm screen. Cells were harvested and resuspended in 0.2mM, 0.3mM, 0.4mM, 0.5 and 1mM Ca2+-KHB with 1% BSA, each for 5 min (let the cells settle down by gravity). Finally, the cells were centrifuged briefly again, and resuspended in ACCT medium consisting of DMEM containing 2mg/mL BSA, 2mM L-carnitine, 5mM creatine, 5mM taurine, 100IU/mL penicillin, and 100ug/mL streptomycin. Cells were then counted and plated on laminin-coated (10 ug laminin per mL PBS for 1h) glass coverslips or dishes. After 1-2 hours, the dishes were infected with adenoviruses in diluted media, at a multiplicity of infection of 500, for 2 hours before addition of suitable volume of culture media. Transfection efficiency, determined by GFP gene expression in the cultured cardiac myocytes under fluorescence microscopy, was consistently >95% by this method.
Adenoviral Constructs

The dominant-negative mutant of Akt1 cDNA, which encodes Akt1 protein with a hemagglutinin tag at the amino terminus and three amino acid substitutions at lysine 179, threonine 308, and serine 473 to alanine (Akt1AAA) was generously provided by Dr. K. Walsh (Boston University School of Medicine). Adenovirus vector expressing Akt1-AAA (named as Ad.dnAkt) was constructed as described (3-5). The adenovirus vector carrying cardiac Hsp20 cDNA (Ad.Hsp20) was generated by using the AdEasy system (6). The viruses were replicated in 293 cells, purified by Virakit™Adeno (Virapur, ILC) and the viral titers were determined by plaque assay in 293 cells.

Cell Viability and Apoptosis Assay

Cell viability assessment was performed with the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega), as the manufacturer’s instructions. For apoptosis analysis, DNA fragmentation was determined by an ELISA kit (Roche Applied Science, Indianapolis, IN). Cardiomyocytes were lysed by gently dispersing the pellet using a pipette tip that was cut back to prevent shearing of cells and release of nuclear DNA. The extract was then centrifuged at low speed and assayed according to the manufacturer’s instructions. Fold increase was obtained by dividing the measured absorbance of an experimental group by the absorbance of the positive control, provided in the kit.

Drug Treatment in vitro

Doxorubicin (DOX, Sigma Chemical Co.), the mitogen-activated protein kinase kinase (MEK1/2) inhibitor PD98059, p38 inhibitor SB203580, JNK inhibitor II, PI3-Kinase inhibitors
LY294002 and wortmannin, were purchased from Calbiochem (La Jolla, CA). Stock solutions were prepared in de-ionized water (doxorubicin) or DMSO (for inhibitors). Myocytes were seeded and infected with adenoviruses one day before treatment. Then various doses of DOX were added to the cells for various lengths of time. For experiments using combinations of doxorubicin and other inhibitors, cells were pre-incubated with the inhibitors PD98059 (20 μM), SB203580 (20 μM), JNK inhibitor II (40 μM), Ly294002 (10 μM) or wortmannin 100 nM for one hour before addition of doxorubicin. The same volumes of corresponding solvents were added to the controls.

Animal Preparation

Generation of cardiac-specific overexpressed Hsp20 mice has been previously described (7). Male wild-type (WT) and transgenic (TG) mice, inbred on a FVB/N background, were studied at 8 to 10 weeks. Both WT and TG mice were randomly assigned to either the control group or the DOX-treated group. DOX was administered by intraperitoneal (ip) injection at one dose of 20 mg/kg (100μl) or a weekly dose of 3 - 4mg/kg to a cumulative amount of 20 mg/kg. Control mice received injections of saline to a comparable volume 100μl. All procedures were in accordance with institutional guidelines for animal research.

Cardiac Contraction Measurements and Cardiotoxicity Assay

Cardiac function was assessed ex vivo at 4 days (for acute treatment) or 1 week (for chronic treatment) after the last DOX or vehicle injection by Langendorff preparation, as previously described (7). For heart morphometry, hearts were excised at 4 days after Dox injection, and fixed with 10% buffered formalin, embedded in paraffin and cut from the apex to the base. In situ
DNA fragmentation was assessed using the DeadEnd™ Fluorometric TUNEL system (Promega), followed by staining with a mouse anti-α-sarcomeric actin antibody (1:50 dilution, Sigma-Aldrich) and DAPI (Invitrogen) (8). The percentage of TUNEL-positive myocytes was determined by counting 10 random fields per section under a microscope (BX50, Olympus). DNA fragmentation was also determined by an ELISA kit (Roche Applied Science, Indianapolis, IN) with 200 µg heart homogenates, as described above.

**Immunoblotting**

Heart homogenates or cells were harvested in RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 10 µg/ml aprotinin, leupeptin and 5 mM PMSF and incubated for 30 min on ice. Then equal amounts of protein (60-100µg) from each sample were resolved on a 10% or 12% SDS-polyacrylamide gel by electrophoresis. Binding of the primary antibody was detected by peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham), and bands were quantified with densitometry. Rabbit anti-Hsp90 (1:500 dilution), rabbit anti-Hsp70 (1:1000 dilution), mouse anti-Hsp60 (1:4000 dilution), mouse anti-Hsp27 (1:1000 dilution) and rabbit anti-αB-crystallin (1:1000 dilution) (Affinity BioReagents), rabbit anti-p38, anti-JNK, anti-ERK and their phosphorylation antibodies, rabbit anti-Akt and its phosphorylation (pS473 Akt, pT308 Akt) antibodies, rabbit anti-GSK-3β and its phosphorylation (Ser9) (1:1000 dilution), rabbit anti-BAD and its phosphorylation (Ser136) (1:250 dilution) (Cell Signaling Technology, Inc.), mouse anti-PP1(E-9), rabbit anti-PP2A(FL-309) and mouse anti-PP2B-Aα (D-9) (1:250 dilution), mouse anti-Akt1(B-1), anti-Akt2 (F-7) and rabbit p-Akt1/2/3 (Ser 473)-R (1:200 dilution) ( Santa Cruz Bitotech.), as well as mouse Hsp20 antibodies (1:5000 dilution, Research Diagnostics Inc.) were used. Caspase-3 activation was
determined by immunoblotting with measurement of cleaved caspase-3 (Asp175) (rabbit anti-cleaved caspase-3, 1:500 dilution, Cell Signaling). α-actin (1:1000 dilution, Sigma) was probed in each membrane as a loading control.

**Akt Kinase Activity Assay**

*In vitro* Akt kinase activity assay were performed by Western blotting, using phosphor-GSK-3α/β (Ser21/9) antibody (1:250 dilution) (Akt Kinase Assay Kit, Cell Signaling Technology, Inc.). Briefly, 20 μl of immobilized phosphorylated-Akt (Ser473) antibody bead was added to 200 μl (1mg) of heart homogenate, and incubated with gentle rocking overnight at 4 ºC. The mixture of beads-sample was microcentrifuged at 13,000rpm for 30 seconds, and the pellet was washed with 500 μl of 1 × Cell Lysis buffer twice, followed by washing pellet with 500 μl of 1 × Kinase buffer twice. Suspend the pellet in 50 μl of 1 × Kinase buffer supplemented with 1 μl of 10mM ATP and 1 μg of GSK-3 fusion protein, incubating for 30 min at 30 ºC. Terminate reaction with 50 μl of 2 × SDS sample buffer. Heat the sample to 95-100 ºC for 2-5 min. Vortex, then centrifuge for 5 min at 13,000rpm. Load 30 μl of sample per well on 12%SDS-PAGE gel. Except that phosphor-GSK-3α/β (Ser21/9) antibody (1:250 dilution) was probed, Hsp20 and Ser-473 Akt antibodies were also probed in the each membrane.

**Co-immunoprecipitation**

For the co-immunoprecipitation experiments, protein lysates, extracted from mouse cardiac homogenates or cultured cardiomyocytes with 1 × cell lysis buffer (Cell Signaling, #9803) supplemented with 1mM PMSF and protease inhibitor cocktail (Sigma), were centrifuged for 30 min at 13000rpm at 4ºC. The anti-Hsp20 or anti-Akt1 or anti-Akt2 antibody (4μg) was added to
1ml diluted cell lysates (\(\mu\text{g}/\mu\text{l}\)), incubated overnight on a rotary wheel at 4 °C. Protein G PLUS-agarose beads (Santa Cruz Bitotech.) were added (1\(\mu\text{g}\) antibody/10 \(\mu\text{l}\) agarose beads) and incubated for an additional 1-2 h at 4 °C. The agarose beads were washed 6 times with the cell lysis buffer, solubilized in 2×SDS-sample buffer, boiled at 95 °C for 5 min, analyzed by SDS-PAGE and processed for immunoblotting.

**Oxidative Stress and ROS Assay**

Antioxidant enzymes were measured by Glutathione Peroxidase (GPx) Assay Kit and Superoxide Dismutase (SOD) Assay Kit II (Calbiochem). Reactive oxygen species (ROS) levels were determined by the fluorescence indicator DCFH, as described elsewhere (9, 10). When DCFH is added to the heart homogenate, ROS in the homogenate will lead to the oxidation of DCFH, producing the fluorescent product DCF. To rule out the potential artifact of ROS generation, the specificity of DCF fluorescence signal was confirmed by adding different doses of SOD, an antioxidant enzyme provided in the SOD Assay Kit II, into the DOX-treated WT heart homogenates (Online Figure IV). In the present study, 1\(\mu\text{M}\) DCFH was incubated with 1 ml of 200 \(\mu\text{g}\) heart homogenate for 1 h, and fluorescence was recorded using a fluorometer (1420 multilabel counter, PerkinElmer Life Science) equipped with a 96-well plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. For measurement of ROS formation in cardiomyocytes, H9c2 cells were grown in 96-well plates (10,000 cells/well) and transfected with various doses of pcDNA3-Hsp20 containing mouse cardiac Hsp20 cDNA or control pcDNA3.1 for 48 h, followed by addition of DOX (0.5\(\mu\text{M}\)) for 24 h (since our Ad.Hsp20 vector contains GFP expression cassette, which will interfere with the fluorescent product DCF, we cloned Hsp20 cDNA into the pcDNA3.1 (+) at the sites of Hind III and Xba I). Then these
treated cells were incubated with DCFH (10 \mu M) for 1h. Data were collected by fluorometer, as described above.

**Statistical Analysis**

All values are expressed as mean ± SEM. Statistical significance was determined with one-way ANOVA, followed by Duncan multiple range comparison test using Super ANOVA (Abacus Concepts, Inc). Survival curves after DOX injection were created using the Kaplan-Meier method and compared by a log-lank test. Differences were considered statistically significant at a value of P<0.05.

**References**


Online Figure I. Effects of Hsp20 on the expression of major Hsps in the DOX-treated hearts. Hearts were excised on day 4 after acute administration of DOX (20 mg/kg), homogenized with lysis buffer, and subjected to Western-blotting analysis. There were no differences in the expression of major Hsps between WT and Hsp20-TG hearts after treatment without or with DOX. (n=4).
Online Figure II. Effects of Hsp20 on the expression of MAPK pathway in the DOX-treated hearts. Hearts were excised on day 4 after acute administration of DOX (20 mg/kg), homogenized with lysis buffer, and subjected to Western-blotting analysis. There were no differences in the activation of MAPK pathway between WT and Hsp20-TG hearts after treatment without or with DOX (n=4).
Online Figure III. Reciprocal immunoprecipitation experiments using Akt1, Akt2 or Hsp20 antibodies showed Hsp20 not only interacted with Akt1, but also interacted with Akt2 in the murine heart. Pre-immunoprecipitated heart homogenate was used as positive control (+), immunoprecipitation without any antibodies (only added protein G agarose beads) was used as negative control (-). Antibodies Akt1 (B-1), Akt2 (F-7), and p-Akt1/2/3 (Ser473)-R were from Santa Cruz Biotech Inc (1:200 dilutions for immunoblotting). Hsp20 antibody was from RDI (1:4000 dilutions). For immunoprecipitation, antibody Akt1 or Akt2 or Hsp20 (4 μg) was added to 1mg (1ml) pre-cleared heart homogenates (detailed in Methods).
Online Figure IV

Online Figure IV. Various doses of SOD were added to the 1 ml mixture of 1μM DCFH/200 μg DOX-treated WT heart homogenates, and incubation for 1 h at room temperature. Fluorescent intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.