Supplemental Materials and Methods

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Animal Models and Procedures.

A tetracycline-responsive binary α-MHC transgene system was used to allow temporally regulated expression of ASK1 in cardiomyocytes of the heart.[1] Dox was administered in the food with a special diet formulated by Purina (625 mg/kg in pellets). In all experiments that required ASK1 protein induction, Dox was removed from the food at weaning, resulting in induced expression of ASK1 a few weeks later in young adulthood. All experimental procedures with animals were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Transverse aortic constriction (TAC) of the transverse aorta was performed as previously described.[2] Transthoracic echocardiography to measure cardiac dimensions and pressure gradients across the aortic constriction was performed as described previously.[3] Pressure gradients were calculated as $4 \times V_{max}^2$ (m/s) where V_{max} is the velocity of the blood flow across the aortic constriction measured by Doppler. Fractional shortening (FS) from echocardiographic measurements was calculated using left ventricle dimensions in end of systole and diastole (LVES and LVED, respectively) according to the formula: FS = [(LVED - LVES)/LVED] x 100 (%). Azlet 1002 osmotic minipumps (Cupertino, CA) either filled with isoproterenol (60 mg/kg/day in phosphate-buffered saline) or phosphate-buffered saline were implanted under the skin for 2 weeks following a routine surgical procedure.

The surgical procedure for ischemia-reperfusion (I/R) injury and myocardial infarction (MI) injury in the mouse were described previously.[4] Briefly, a suture was tied with a slip-knot around the left coronary artery, and mice were revived by removal from anesthetic during 60 min of ischemia, after which the knot was released and the heart was reperfused for 24 h (I/R). The MI procedure was identical except that the ligature was permanent with no reperfusion. Mice were sacrificed by CO_2 asphyxiation, and hearts were analyzed as previously described using 2% triphenyltetrazolium chloride in saline and 2% Evan's blue dye infusion to identify area at risk, infarct area, and area of perfusion for the I/R procedure.[4]

Histological Analysis, Cell Size Measurement, and TUNEL.

For histological analysis, adult hearts were fixed in 10% formalin/phosphate-buffered saline and dehydrated for paraffin embedding. Fibrosis was detected with Masson's Trichrome staining on 5-μm paraffin sections. Blue collagen staining was quantified using Metamorph software. For cell surface area measurements, membranes were stained with TRITC- or FITC-labeled lectin from *Triticum vulgaris* (Sigma), and nuclei were labeled with TO-PRO 3 iodine (Molecular Probes, Carlsbad, CA). Cellular areas were quantified with ImageJ 1.33 software (Scion Corp., Frederick, MD). Assessment of TUNEL from paraffin sections was performed with TMR Red In Situ Death Detection Kit (Roche Diagnostics) according to the manufacturer's instructions (Roche Diagnostics).[3]

Luciferase reporter assays in mouse hearts

The NFAT-luciferase reporter mouse was described previously.[2] In brief, ASK1 transgenic mice were crossbred with NFAT-luciferase reporter mice and ventricles were excised and stored at –70°C. The frozen hearts were homogenized in 1 ml luciferase assay buffer (100 mM KH_2PO_4, pH 7.8, 0.5% Nonidet P-40, and 1 mM DTT). Homogenates were centrifuged at 3,000 *g* for 10 min at 4°C and the supernatants assayed for luciferase activity as described previously.[2]

Western Blotting

Protein extraction from mouse heart and subsequent Western blotting followed by enhanced chemiluminescence detection was performed as previously described.[2,4] Antibodies including anti-JNK1/2, anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-ERK1/2, anti-phospho-ERK1/2, anti-MKK4, anti-phospho-MKK4, and anti ASK1 (Thr 845) were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-ASK1 antibody and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical Analysis

Results are shown as means ± SEM. Paired data were evaluated by Student's *t* test. A one-way or two-way ANOVA with the Bonferroni's post hoc test or repeated-measures ANOVA was used for multiple comparisons. $p < 0.05$ was considered significant.

References

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