Animals and Experimental Protocol

We studied SHHF (n=25, 19-21 months old, 467±12 g) rats and their controls, Wistar Kyoto (WKY, n=8, 18-21 months old, 453±13 g) rats (Charles River Laboratories, Inc. Wilmington, MA). The SHHF rat is a genetic model of dilated cardiomyopathy with hypertension progressing to HF\(^1\). This model shares common phenotypic features with human HF, including activated renin angiotensin aldosterone system and elevated natriuretic peptides\(^1,2\), elevated tumor necrosis factor α\(^3\), increased left ventricle (LV) end-diastolic volume, decreased LV ejection fraction, activated fetal gene program\(^4\), and elevated XOR activity\(^5\). We treated both SHHF and WKY rats with the XOR inhibitor oxypurinol\(^6\). SHHF and WKY rats were randomly assigned to placebo (n=10 and n=3, respectively) or treatment with oxypurinol (1 mM\(^5,7\), n=15 and n=4, respectively) in drinking water, for four weeks. Echocardiographic measurements were taken at baseline, two weeks, and at the end of the study. In vivo assessment of LV hemodynamics was performed at the end of treatment. Animals were sacrificed and gross anatomical pictures were taken and the hearts were either fixed in paraformaldehyde 4% for histological studies or Animals were sacrificed and hearts were snap-frozen immediately for molecular studies. The Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine approved all protocols and experimental procedures.

Echocardiographic Measurements

LV mass (LVM) was calculated as LVM (mg) = 1.055 x [(AWT + PWT + LVEDd)\(^3\) – (LVEDd)]. LV fractional shortening (FS) was calculated as FS (%) = (LVESe / LVEDd) x 100.

LV hemodynamics
Cardiac preload was indexed as the left ventricular end-diastolic volume (LVEDV) and pressure (LVEDP); cardiac afterload was evaluated as effective arterial elastance (Ea, ratio of LV end systolic pressure to stroke volume), the LV end-systolic volume (LVESV) and pressure (LVESP); and myocardial contractility was indexed by the LV ejection fraction (EF), the slope of the end-systolic pressure-volume relation (Ees), and the peak rate of rise in LV pressure (dP/dt\text{max}) divided by instantaneous developed pressure (dP/dt-IP). Ventricular relaxation was measured as the time constant \( \tau \) for isovolumetric relaxation and peak rate of decline in LV pressure (dP/dt\text{min}). Volume calibration was achieved by bolus injection of 15 % saline at the end of the experiment.

**Measurement of XOR Activity**

XOR activity was measured using the horseradish peroxidase-linked Amplex Red fluorescence assay (Molecular Probes, Invitrogen Detection Technologies), as previously described\(^8\). LV tissue was homogenized with a Polytron (3X15 s) in cell lysis buffer (Cell Signaling Tech., Beverly, MA). The homogenization buffer also contained 1 mM phenylmethyl sulfonfly fluoride (PMSF) and Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN). The homogenate was centrifuged at 4°C at 14,000 g for 30 min. The supernatants were recovered and protein concentrations were determined using BCA reagent (Pierce Biotechnology, Rockford, IL) and bovine serum as a standard. The LV homogenates was added to a working solution containing Amplex Red reagent (50 \( \mu \)M), xanthine (0.1 mM) and horseradish peroxidase type II (0.1 U/ml), and the \( \text{H}_2\text{O}_2 \) production was measured in the absence or presence of oxypurinol (1 mM) in order to subtract the background. The reactions were incubated at 37 °C for 30 minutes. Fluorescence readings were made in duplicate in a 96-well
plate at Ex/Em = 544/590 nm using 100-µl total volume per each well. XOR activity was calculated by using a standard curve and was normalized to the loaded amount of protein concentration.

**Measurement of mRNA Expression by Quantitative Polymerase Chain Reaction (qPCR)**

Fluorescence based real time qPCR was used to determine the mRNA expression of the following genes: XOR, atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), α-myosin heavy chain (α−MHC), β-myosin heavy chain (β−MHC), and α-skeletal actin (αSA). The primer sets for these genes were chosen from the published cDNA sequences. Total LV RNA was isolated, cDNA was synthesized and each sample was run in duplicate on a GeneAmp 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA) and was analyzed using SDS 2.0 software (Applied Biosystems) as we have described9.

**GSH/GSSG Ratio:**

Determination of the GSH/GSSG ratio was performed by using the glutathione assay kit (Cayman chemical, Ann Arbor, MI). Briefly, animals were sacrificed, the hearts harvested and perfused with cold PBS and snap frozen. The tissue was homogenized and the supernatant obtained after centrifugation (10,000 g, 15 min) was deproteinated following the instructions of the manufacturer of the kit. The deproteinated sample was divided in two aliquots: one for measurement of total GSH and the other for measurement of GSSG. The samples were assayed spectrophotometrically at 405 nm.
Measurement of NADPH Oxidase Activity

NADPH-dependent superoxide (O$_2^-$) production was measured in LV homogenates (mentioned in XO activity) using lucigenin-enhanced chemiluminescence (βNADPH 300 µM; at room temperature) on a microplate luminometer (Veritas, Turner Biosystems, Sunnyvale, CA).

A low lucigenin concentration (5 µM) was employed to minimize artifactual O$_2^-$ production due to redox cycling. Briefly, proteins were diluted in modified Krebs-HEPES buffer and βNADPH and lucigenin were added to wells just before reading. Chemiluminescence readings were expressed as integrated light units/mg protein/minute. Experiments were also performed in the presence of the flavoprotein inhibitor diphenyleneiodonium (DPI, 10 µM), or allopurinol (100 µM) or the nitric oxide synthase inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 µM).

Western-Blotting:

Whole heart proteins were prepared and western blots analysis was performed as described$^{10}$. The blots were incubated with primary anti-p47$^{phox}$ antibody (1:500, Upstate, Lake Placid, NY), anti-p22$^{phox}$ antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p67$^{phox}$ antibody, anti-gp91$^{phox}$ antibody (1:500, BD Transduction Laboratories, San Diego, CA), anti-sarcoplasmatic reticulum Ca$^{2+}$ ATPase (SERCA2) antibody (1:500, Affinity BioReagents, Golden, CO), anti-Na$^+/Ca^{2+}$ exchanger (NCX) antibody (1:500, Alpha Diagnostic Inc, San Antonio, TX), anti-phospholambam (PLB) antibody (1:1000, Affinity BioReagents), anti-extracellular signal-regulated kinase (ERK) antibody (1:1000, Cell Signaling Tech.), or anti-pERK antibody (1:1000, Cell Signaling Tech.). A monoclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (1:100000, Research Diagnostic Inc., Flanders, NJ) was used separately as a normalizer. Membranes were incubated for 1 hour with peroxidase-
conjugated chicken anti-rabbit or anti-mouse IgG antibodies (Santa Cruz Biotechnology) in 1:5000 dilutions. Bands were visualized by chemiluminescence (SuperSignal Substrate kit, Pierce, Rockford, IL) and quantified using the NIH Image software.

Reference List


calcium cycling: independent impact of neuronal and endothelial nitric oxide synthases. 