Supplementary Material

Methods

Echocardiography

All rats were anesthetized in a gas chamber using isoflurane (Webster veterinary, Sterling, MA) delivered at an initial rate of 3 L/min mixed with 2 L/min of oxygen. After proper anesthesia was reached, rats were transferred and fixed in a supine position to a heating pad where heart rate, respiratory rate and body temperature were monitored and controlled. In the pad, isoflurane rate was decreased to 1 L/min administered via a facemask. A Visualsonic Vevo 770 3.0.0 system was used for the ultarsound. Long axis imaging was obtained using B mode echocardiography and putting the probe on the anterior chest wall following the angle of the normal heart axis. Short axis was obtained putting the probe in the perpendicular position used for long axis. Short axis was changed from B mode to M mode when papillary muscles were clearly seen. Images were analyzed using Vevo 770 3.0.0 software. Measurements were performed at least three times in each rat and an average of measurement was used. Systolic function was evaluated using B mode in the long axis to estimate the ejection fraction and using M mode in the short axis to estimate the fractional shortening.

Isolation of Myocytes

The rats were anesthetized with sodium pentobarbital (100 mg/kg i.p. with 4000 U/kg heparin). After suppression of spinal cord reflexes, the heart was exposed via a midline thoracotomy, and removed. For isolation of myocytes, the hearts were cannulated and perfused through the aorta with Ca^{2+}-free bicarbonate buffer containing (in mmol/L) 120 NaCl, 5.4 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 5.6 glucose, 20 $NaHCO_3$, 10 2,3-butanedione monoxime, and 5 taurine, gassed with 95% O_2 - 5% CO_2, followed by enzymatic digestion with collagenase type 2 (1 mg/ml;

Worthington, NJ) and protease type XIV. Ventricular myocytes were obtained by mechanical disruption of digested hearts, filtration, centrifugation, and resuspension in a Tyrode solution containing (in mM) 0.125 $CaCl_2$, 144 NaCl, 1 $MgCl_2$, 10 HEPES, 5.6 Glucose, 1.2 $NaHPO_4$, 5 KCl, pH 7.4. Myocytes were incubated with 5 µmol/L fura-2/AM (Molecular Probes, Eugene, OR) then transferred to an inverted microscope (Nikon TE 200), continuously superfused with Tyrode solution. Ca^{2+} transients ($[Ca^{2+}]_i$) were measured in myocytes stimulated at 0.5, 1, 2 3 and 4Hz. All experiments were conducted at 37°C.

Calcium measurements

$[Ca^{2+}]_i$ was measured using fura-2 AM excited at wavelengths of 365 and 380 nm. The emission fluorescence was reflected through a barrier filter (510±15 nm) to a photomultiplier tube. The fura-2 fluorescence ratio, the ratio of the photon live count detected by the excitation at 365 nm compared with 380 nm, represents $[Ca^{2+}]_i$.

Calcium leak assessment

Ventricular myocytes were loaded with fura-2 and paced by field stimulation at the different frequencies in normal Tyrode's solution until cellular Ca^{2+} transients reached a steady state. After the last pulse, the bath solution is rapidly switched to 0 Na^+-0 Ca^{2+} (Na^+ replaced by Li^+) Tyrode. In the control condition, $[Ca^{2+}]_i$ is monitored while 0 Na^+-0 Ca^{2+} Tyrode buffer is applied at least 40 seconds to eliminate transsarcolemmal Ca^{2+} fluxes, creating a closed system with a steady-state $[Ca^{2+}]_i$. Then a rapid pulse of caffeine (10 mmol/L) is added to cause SR Ca^{2+} release. After the cell recovers, it is stimulated again in the same conditions, but the 0 Na^+-0 Ca^{2+} Tyrode solution contains 1 mmol/L tetracaine. Under this condition, RyR is blocked and a shift (decrease) in the fura-2 signal (cytosolic $[Ca^{2+}]$) is observed. In this condition, the leak is blocked and the difference in $[Ca^{2+}]_i$ between tetracaine and control condition corresponds to diastolic Ca^{2+} efflux. The amplitude of the caffeine-induced Ca^{2+} transient is used to estimate the

total $[Ca^{2+}]_i$. The load–leak relationship is estimated by grouping cells with similar total SR $[Ca^{2+}]$ load.

Western blotting

Hearts were cannulated and perfused through the aorta with Krebs solution to wash the blood, and then rapidly frozen in liquid nitrogen. Equal amounts of protein (60 µg) were resolved by NuPAGE 3-8% Tris-acetate gels (Invitrogen Life Technologies, Carlsbad, CA). Proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA) and then blocked overnight by incubation with 5% nonfat dry milk in Tris-buffered saline (TBS). After washing with TBS containing 0.1% Tween 20 (TBS-T buffer), the blots were incubated with primary antibodies, including mouse anti-RyR2 (Affinity Bioreagents) anti phospho-RyR2 Ser2809 (Badrilla, UK), anti NOS1, NOS2 and NOS3 (BD Biosciences) and xanthine oxidase (Neomarkers).

S-Nitrosylation

Briefly, hearts were homogenized in HEN buffer (250 mmol/L HEPES, 1 mmol/L EDTA and 100 µmol/L neocuproine, pH 7.7). Free thiols residues were blocked with S-methyl methanethiosulfonate. Proteins were precipitated with cold acetone and then re-suspended in HEN buffer containing 0.5% SDS and labeled with biotin-HPDP with or without sodium ascorbate. For direct detection of biotinylated RyR2, 60 µg of each sample was loaded in a 3-8% Tris-acetate gel, electrophoretically resolved and followed by immunoblotting with anti-biotin antibody (Cell Signaling). In order to separate *S*-nitrosylated RyR2 after labeling, biotinylated proteins were incubated with streptavidin-agarose beads. The bound proteins were eluted and resolved electrophoretically in a 3-8 % Tris-acetate gel, followed by immunobloting with anti-RyR2 antibody.

Legends to figures.

Supplementary figure1.

Echocardiography data from Wistar-Kyoto (WKY) and spontaneously hypertensive-heart failure rats (SHHF) at 18 months of age. Ejection fraction and fractional shortening are significantly decreased in SHHF rats compared to WKY controls. ** *P*=0.0008. *** *P*<0.0001. Numbers in the white box represent the n of the sample.

Supplementary figure 2.

Detection of S-nitrosylation using anti-nitrosocysteine antibody.

Controls performed to validate the specificity of anti-SNO antibody. The upper left panel shows a control myocytes stained with anti SNO antibody. The upper right panel shows a myocyte treated with SNAP (1 mmol/L, 30 min), a NO donor. The lower left panel shows a control cell pre-treated with $HgCl_2$, (0.2 % in PBS, 30 min) a compound that degrades SNO bonds. Finally, the lower right panel displays a cell treated with normal rabbit serum instead of the rabbit polyclonal anti-SNO antibody.