Neuronal Nitric Oxide Synthase Protects Against Myocardial Infarction-Induced Ventricular Arrhythmia and Mortality in Mice

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SUPPLEMENTAL MATERIAL

Methods

Induction of MI and Ischemia/Reperfusion (I/R)

Adult nNOS−/− mice and their WT littermates (males, 3-4 months old) were randomly selected to undergo coronary artery ligation to induce MI or sham surgery (same procedure without coronary artery ligation) as described previously. Mice were anesthetized, intubated, and mechanically ventilated. A left intercostal thoracotomy was performed and the left coronary artery was ligated by placing a suture (8-0) around it. The lungs were then hyper-inflated using positive end-expiratory pressures (3 cm H2O), and the thorax was closed. Infarct size was determined at the end of the study. In the case of myocardial I/R, the left coronary artery was occluded for 45 minutes to induce ischemia and the suture was loosened to allow for reperfusion for 45 minutes.

Hemodynamic Measurements

Heart function was measured 2 and 30 days post-MI in anesthetized mice as described previously. Briefly, a Millar pressure transducer catheter (1.4-Fr) was placed in the right carotid artery for measurement of arterial blood pressure and heart rate. The catheter was then advanced into the left ventricle for measurement of left ventricular (LV) systolic and end-diastolic
pressures, as well as the LV maximal rate of pressure development (+dP/dt) and maximal rate of pressure relaxation (-dP/dt). All measurements were recorded using PowerLab Chart Program (ADInstruments, Colorado Springs, CO).

Measurement of Apoptosis and Superoxide Production

Caspase-3 activity was measured using a caspase-3 assay kit (BIOMOL, Plymouth Meeting, PA) as previously described. Terminal deoxynucleotidyl transferase d-UTP nick end labeling (TUNEL) staining was performed on paraffin-embedded heart sections using an In Situ Cell Death Detection Kit (Roche, Indianapolis, IN) as previously reported. NADH-dependent O$_2^-$ generation was measured in cell lysates by lucigenin-enhanced chemiluminescence (20 µg of protein, 100 µmol/L β-NADH, 5 µmol/L lucigenin) with a multilabel counter (Victor$^3$ Wallac). The light signal was monitored for 5 seconds, and counts per second (CPS) were presented as NADH oxidase activity that was inhibitable by diphenyliodinium (10 µM).

Telemetry

Animals were anesthetized and a small telemetric biopotential transmitter (EA-F20, Data Sciences International, St. Paul, MN) was implanted into the abdomen. Leads (cathodal and anodal) were placed subcutaneously in a standard lead I position and sutured on each side of the chest wall. A receiver (RMC-1) was placed under the cage of each animal and connected to the data acquisition system (Dataquest A.R.T, Data Sciences International) for off-line analysis. After a 2-day period during which mice were allowed to adapt to the presence of the transmitter, mice were subjected to MI and ECG was measured over a 10-day period.
ECG Analysis

Ventricular arrhythmias were analyzed offline according to the Lambeth Convention guidelines for the analysis of experimental arrhythmias. Ventricular premature beats (VPBs) were defined as singlet or doublet premature QRS complexes in relation to the P wave. Ventricular tachycardia (VT) was defined as a run of three or more premature QRS complexes and ventricular fibrillation (VF) was defined as conversion from a clear sinus rhythm without major artifact to a signal for which individual QRS complexes could no longer be distinguished from one another, usually following a clear brief period of tachycardia. For analysis of telemetric data, ECG was evaluated from 05:00-06:00, 09:00-10:00, 17:00-18:00, and 21:00-22:00 recordings from each day. All ventricular arrhythmias were expressed as the number of events (singlets, doublets and VT) per hour. The incidence and duration of VF were noted in the hour preceding death. For analysis of I/R-induced arrhythmias, the number of singlet and doublet VPBs, and the incidence and duration of VT were quantified.

Adult Cardiomyocyte Isolation

Cardiomyocytes were isolated from the hearts of adult WT and nNOS−/− mice. Hearts were mounted on a Langendorff apparatus and perfused with digestion buffer containing 45 \( \mu g/mL \) of liberase blendzyme IV (Roche). Following digestion, cells were re-suspended and exposed to a series of sedimentation and resuspension steps in buffer containing increasing concentrations of Ca\(^{2+}\) (12.5 \( \mu M \)-1.0 mM). Healthy, rod-shaped myocytes were used for subsequent experiments.
Intracellular Ca\(^{2+}\) Transients

Free intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)) were measured in isolated ventricular cardiomyocytes using fura-2-AM as previously described\(^5\) with modifications. Cells were loaded with 1 μM fura-2-AM and placed into a perfusion chamber at room temperature. The chamber was placed on an inverted microscope (Nikon, Melville, NY) and perfused continuously at approximately 2 mL/min in a buffer containing (in mM) 120 NaCl, 5.4 KCl, 1.2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), 20 NaHCO\(_3\), and 5.6 glucose at pH 7.4. Cells were then paced at twice diastolic threshold voltage at a frequency of 0.5-4.0 Hz using a 2.5 ms duration pulse. Fluorescence intensity at 510 nm was measured using a Deltascan monochrometer system (Photon Technology International, London, ON). [Ca\(^{2+}\)]\(_i\) was determined from the ratio of recorded fluorescence intensity with 340/380 nm excitation by the methods of Grynkiewicz\(^6\)

L-Type Ca\(^{2+}\) Current

Whole-cell L-type Ca\(^{2+}\) currents were measured as described previously\(^7\) with modifications. The extracellular saline solution contained (in mM) 130 NaCl, 5 KCl, 20 Hepes, 10 Glucose, 2 CaCl\(_2\), 1 MgCl\(_2\), with pH adjusted to 7.4 with NaOH. The pipette solution contained (in mM) 135 CsCl, 20 Hepes, 1 MgCl\(_2\), 10 TEACl, 0.1 EGTA (pH 7.2). Ca\(^{2+}\) channel current was recorded using nystatin (300 μg/ml) perforated whole cell patch-clamp configuration. Cell capacitance was determined for each cell by applying a 10 mV hyperpolarizing pulse from the holding potential of -40 mV and integrating the resulting current trace. Currents were normalized to cell capacitance (pA/pF) to account for differences in cell size.
References


