Data supplement

Expanded materials and methods

Surgical procedures

ICV cannulae: Mice were anesthetized with ketamine/xylazine (80 mg/kg ketamine+10 mg/kg xylazine, I/P). Following placement into a stereotaxic apparatus (Kopf Instruments, CA), an incision was made through the skin, fascia was removed and the skull was exposed. After the skull was leveled in the rostral/caudal direction, a small, stainless steel screw was inserted into the skull and the cannula was inserted through a drilled hole, using the following stereotaxic coordinates: 0.3 mm posterior to bregma, 1.0 mm lateral from midline and 2.8 mm from the surface of the skull. Dental acrylic was used to fix the cannula in place and the skin was sutured over the hardened dental acrylic cap. Mice were treated with analgesics and allowed to recover for 1-week before induction of myocardial infarction.

Experimental myocardial infarction (MI)

Mice were pre-medicated with atropine sulfate (0.04 mg/kg SC) and anesthetized 5 min later with 2% v/v isoflurane/oxygen. Mice were placed on a heating pad in supine position and the trachea was intubated using a 24-guage intravenous catheter with a blunt end. Anesthesia was maintained by supplementing oxygen (1.8 L/min) and 0.7%-2.0% isoflurane at a rate of 105/min and with a tidal volume of 2.1-2.5 ml using a rodent ventilator (Harvard Apparatus, Inc., MA). A left lateral thoracotomy was performed by blunt incision in the fourth intercostal space, the left anterior descending (LAD) coronary artery was ligated using a prolene monofilament with a tapered needle (Ethicon USP 7-0, Johnson & Johnson Co., NJ) 2-3 mm from the tip of the left auricle with the aid of a dissecting microscope (Zeiss) to induce MI. Following coronary artery ligation (CAL), the chest was closed, and the mouse was allowed to recover. In sham-operated

animals, the suture was placed under the LAD artery and removed without ligation of the vessel. Mice were monitored and studied for a period of 4-weeks following surgery.

Osmotic mini-pump implantation: Immediately following MI, while still under anesthesia, a subcutaneous incision was made upon the back of the mouse, a pocket was made between the skin and muscle and washed with sterile saline. Single 28-day osmotic mini-pumps with a polyvinyl tubing (0.027"I.D. x 0.045"O.D.) attached to the pump injector were implanted in each mouse. The tubing was inserted under the skin and connected to the cannula in the lateral ventricle and fixed into place with dental acrylic cement. The skin was sutured closed and the mice were treated with analgesics.

At the end of the study, mice from both protocols were anesthetized under isoflurane and sacrificed by decapitation. Blood samples were collected for plasma norepinephrine (NE) measurement. Hypothalamus and brainstem samples were collected for gene expression studies. Mice that were used for electron spin resonance spectroscopy (ESR) studies were injected with heparin (100U/25 g body wt.) prior to exposing them to carbon dioxide and were then perfused transcardially with ice-cold buffer, and these brain regions were collected and processed for ROS measurement. For immunofluorescence studies, anesthetized mice were perfused transcardially with ice-cold heparinized saline, followed by formalin. The tissues were stored in formalin until further processed.

Metabolic studies

To study relative fluid and sodium balances between different treatment groups, we performed metabolic studies by individually placing animals in custom-designed metabolic cages with free access to food, water and 1.8% NaCl solution, in a temperature-controlled environment and a

12/12 h light–dark cycle. Three days after acclimation, water and salt solution intake were measured and 24 h-urine collections were obtained for the duration of the study. Sodium intake (mEq) was calculated as salt solution intake multiplied with salt content/equivalent weight of sodium. All sodium chloride is expressed as sodium. Urine samples were collected under saturated oil in graduated conical tubes to avoid evaporative losses and their volumes recorded. All aliquots of urine were immediately frozen at -20°C until urinary sodium (U_{Na}) was determined by a Nova electrolyte 16+ analyzer (Nova Biomedical, Waltham, MA). Total 24-hour U_{Na} excretion was calculated by multiplying the measured concentration with the daily urine volume.

Echocardiography

Cardiac function and development of CHF were analyzed by echocardiography 24 h and 4-weeks after induction of MI. Echocardiography was performed in mice anesthetized with 1.5% isoflurane/oxygen with Toshiba Aplio SSH770 system (Toshiba Medical Systems, CA) fitted with a PLT 1202 linear transducer (12 MHz), which generates two-dimensional images at frame rates ranging from 300 to 500 frames per second. Left ventricular (LV) wall thickness, LV end-diastolic dimension (LVD), LV end-systolic dimension (LVS), LV end-systolic posterior wall thickness (PWS), and LV end-diastolic posterior wall thickness (PWD) were measured using two-dimensional short-axis imaging. LV percent fractional shortening (%FS) was calculated as: %FS = (LVD – LVS)/LVD x100%. The portion of the LV that displays akinesis was electronically planimetered and expressed as a percent of the total LV silhouette to estimate the size of the infarct. Only the mice that had an infarct size of 40-50% were used for the study.

Electron Spin Resonance (ESR) Studies

One of the most sensitive and definitive methods of ROS production is ESR. In this study, we utilized an established technique for superoxide anion ($O_2^{\bullet-}$) and $OONO^{\bullet}$ measurement in the hypothalamus/brainstem using ESR and the spin trap. Two different spin probes were used for ESR studies. 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH) was used to measure $O_2^{\bullet-}$ levels, and 1-hydroxy-3-carboxypyrrolidine (CPH) was used to measure peroxynitrite ($OONO^{\bullet}$) levels. All ESR measurements were performed using an EMX ESR eScan BenchTop spectrometer and super-high quality factor (Q) microwave cavity (Bruker Company, Germany).

Sample preparation for ESR studies: The dissected hypothalamus/brainstem from each animal was placed into a 24-well plate containing Kreb's HEPES buffer (KHB) (20 mM, pH 7.4) Tissue pieces were then washed twice with the same buffer to remove any trace contamination. Samples were then incubated at 37^{o}C with specific spin probes for 30 minutes.

Total tissue $O_2^{\bullet-}$ production: Tissue pieces were incubated at 37^{o}C with CMH (200 μM) for 30 minutes. Aliquots of the incubated probe media were then taken in 50 μl glass capillary tubes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) for determination of total ROS production, under the following ESR settings: field sweep 50 G; microwave frequency 9.78 GHz; microwave power 20 mW; modulation amplitude 2 G; conversion time 327 ms; time constant 655 ms; receiver gain 1 x 10^5. For superoxide production, samples were pre-incubated at 37^{o}C with PEG-SOD (50 U/ml) for 30 minutes, then CMH (200 μM) for an additional 30 minutes. Aliquots of the incubated probe media were taken in 50 μl glass capillary tubes for determination of total superoxide production. Addition of PEG-SOD to CMH allowed competitive inhibition of the $O_2^{\bullet-}$-CMH oxidation reaction by the quenching of $O_2^{\bullet-}$ radicals. Since it is cell permeable, PEG-SOD can competitively inhibit the CMH-$O_2^{\bullet-}$ interaction both

intracellularly and extracellularly, thus allowing accurate measurement of total tissue $O_2^{\bullet-}$ production. To determine actual total tissue superoxide production, the values obtained from incubation with PEG-SOD and CMH were subtracted from the values obtained from incubation with CMH only.

Tissue OONO$^\bullet$ production: Tissue pieces were incubated at 37^{o}C CPH (500 μM) and the reaction was initiated by adding cysteine (100 μM) for 30 minutes. The reaction of OONO$^\bullet$ with the thiols of free cysteine is the first direct reaction of OONO$^\bullet$, and cysteine is the amino acid that reacts the fastest with peroxynitrite. Aliquots of the incubated probe media were then taken in 50 μl glass capillary tubes for determination of OONO$^\bullet$ production following the parameters previously outlined.

Double-labeling immunofluorescence

Fluorescent immunohistochemistry was performed as described previously [24] with minor modifications. Anesthetized mice were perfused transcardially with heparinized saline followed by 10% neutral buffered formalin. Brains were then collected and stored in 4% paraformaldehyde until analyzed. The brains were embedded in paraffin and 10 μm thick sections were cut on Superfrost plus slides (Fischer Scientific) and incubated overnight at 56˚C. The slides were then deparaffinized in xylene, and rehydrated in descending grades of ethanol. Antigen retrieval was performed by incubating slides in Reveal Decloaker, pH 6.0, for 30 min at 120°C in a decloaking chamber (Biocare Medical, Concord, CA) at 17 to 19 lb/in^2 and cooled to 90°C. Following equilibration to RT, slides were incubated in 0.2% fish skin gelatin (FSG) (Sigma-Aldrich, St. Louis, MO) in PBS for 10 min. This solution was also used to wash slides between all incubations. Tissues were blocked in a humidity chamber for a minimum of 30 min with blocking solution consisting of 2% donkey serum (Sigma), 1% bovine serum albumin

(BSA) (Sigma), 0.05% FSG, 0.1% Triton X-100 (Sigma), and 0.05% Tween 20 (Bio-Rad) in PBS. To identify neuronal cells, the slides were incubated overnight at 4°C with a 1/100 dilution of mouse polyclonal anti-neuronal nuclear protein (NeuN) (Molecular Probes). Slides were then incubated with 1/300 dilution of biotinylated mouse anti-goat immunoglobulin G (IgG) or goat anti-rabbit IgG (Molecular Probes) for 30 min at RT and then incubated with 1/500 dilution of streptavidin conjugated to Alexa Fluor 594 (Invitrogen).

For dual staining, slides were incubated in blocking solution for 30 min, incubated with rabbit polyclonal anti-nNOS or anti-3-nitrotyrosine (3-NT) or an antibody that recognizes c-Fos, FosB, Fra-1, or Fra-2 (c-Fos K25, Santa Cruz) in 0.2% FSG in PBS at 4°C overnight. Slides were then incubated with 1/300 dilution of biotinylated goat anti-rabbit IgG (Molecular Probes) for 30 min at RT and then incubated with 1/500 dilution of streptavidin conjugated to Alexa Fluor 488 for 30 min at RT. Slides were mounted with ProLong Gold antifade reagent (Molecular Probes) and allowed to set for overnight at 4°C. The lack of nonspecific staining was confirmed by using no primary antibody controls (data not shown).

Supplementary table 1: Primers used for real-time RT-PCR in this study

Category	Transcript	Sense	Antisense
	18S	GACTCAACACGGGAAACCTC	CTCCACCAACTAAGAACGGC
Cytokines	TNF	AGTCCGGGCAGGTCTACTTT	GGTCACTGTCCCAGCATCTT
	IL-1β	TCACAGCAGCACATCAACAA	TGTCCTCATCCTGGAAGGTC
	IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
	IL-10	CTTGCACTACCAAAGCCACA	TGGGAAGTGGGTGCAGTTAT
Nitric oxide synthases	nNOS	CTCACCCCGTCCTTTGAGTA	GGTCGCTTTGACTCTCTTGG
	eNOS	ACTTCGTTCGGTTGACCAAG	GCAGGATGCCCTAACTACCA
	iNOS	GGCAGAATGAGAAGCTGAGG	GAAGGCGTAGCTGAACAAGG
Nox homologues	NOX2	TGTGGCTGTGATAAGCAGGA	CTTGAGAATGGAGGCAAAGG
	NOX4	TCTGCATGGTGGTGGTATTG	GCCAGGAGGGTGAGTGTCTA
AngII receptors	AT1R	CTGCGTCTTGTTCTGAGGTG	ACTGGTCCTTTGGTCGTGAG
	AT2R	GCCTGCATTTTAAGGAGTGC	ACGGCTGCTGGTAATGTTTC