ONLINE SUPPLEMENT

Gene Transfer of nNOS to the Paraventricular Nucleus Reduces the Enhanced Glutamatergic Tone in Rats with Chronic Heart Failure

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Methods

General Surgery for Hemodynamic, RSNA Measurement and Microinjection

On the day of the experiment (5 days after viral injection),), the rat was anesthetized with urethane (0.75g/kg, i.p.) and α-chloralose (70mg/kg, i.p.). The left femoral vein was cannulated with polyethylene tubing for injection of supplemental anesthesia. The left femoral artery was cannulated and connected via a pressure transducer (Gould P23 1D) to a computer-based data recording and analyzing program (PowerLab) to record mean arterial blood pressure (MAP) and heart rate (HR).

The left kidney was exposed through a left retroperitoneal flank incision, and a branch of the renal nerve was isolated from the adipose and connective tissues. The distal end of the nerve was ligated, and the nerve was placed on a bipolar platinum electrode. The nerve-electrode junction was insulated electrically from the surrounding tissues with Wacker gel (St Louis, MO). The electrical signal from the electrode was linked via a high impedance probe (H1P5) to a Grass P511 band-pass amplifier (gain, 10000) with high- and low-frequency cutoffs of 1,000 Hz and 100 Hz. The output from the Grass amplifier was directed to a Grass integrator, which rectifies the signal and integrates the raw nerve discharge. The output of the Grass integrator was displayed as an integrated voltage that is proportional to the renal nerve discharge. The average rectified signal [resistor-capacitor circuit (RC) filtered with a time constant of 0.5 s] was then recorded and stored for later analysis in a computer-based data-acquisition system (Mac-Lab). Efferent RSNA at the beginning of the experiment was defined as basal nerve discharge. The RSNA recorded at the end of the experiment (after the rat was injected with hexamethonium, 30mg/kg, iv.) was defined as background noise. The value of RSNA was calculated by subtracting the background noise from the actual recorded value and changes found in integration of the nerve discharge during the experiment were expressed as a percentage from basal value. Responses of MAP and HR were expressed as the difference between the basal value and the value after each dose of a drug.

For placement of microinjection cannulas into the PVN, the anesthetized rat was placed in a stereotaxic apparatus (David Kopf Instruments, Tujanga, CA). A longitudinal incision was made on the head and bregma was exposed. A small burr hole was made in the skull to allow access to the PVN. The coordinates for the PVN, determined using the Paxinos and Watson atlas, were 1.5mm posterior to bregma, 0.4mm lateral to midline, and 7.8mm ventral to the dura. A thin needle (0.2mm OD) connected to a 0.5µl microsyringe (Hamilton) was lowered into the PVN. An inhibitor of NOS, L-NMMA, or NMDA was injected into the PVN in three doses (50, 100, and 200pmol) in random order. Subsequent injections were made at least 20min after prior injections to allow MAP, HR, and RSNA to return to basal levels. In a separate group rats (n=5), the vehicle control, 100nl of artificial cerebrospinal fluid (aCSF) was microinjected into the PVN. The effects on RSNA, MAP and HR were observed.

In a separate group of rats, the vehicle control, 100nl of artificial cerebrospinal fluid (aCSF) was microinjected into the PVN and RSNA, MAP and HR were monitored. The vehicle control, 100nl of aCSF microinjected into the PVN, had no effects on RSNA, MAP and HR. At the end of the experiment the brains were subjected to histological evaluation to determine the injection sites. Injections within the boundaries of the PVN were used in the data presented. Typically injections outside the boundaries

of the PVN did not produce any changes in RSNA, MAP and HR. The spread of the dye was typically within the boundaries of the PVN.

NADPH-diaphorase Activity as a Marker of NOS Activity

The rat was perfused through the left ventricle of the heart with heparinized saline followed by 4% paraformaldehyde. The brain sections of 30µm were cut with a cryostat. Every third section was kept from the anterior commissure (0.4mm posterior to bregma) posterior to where the optic tracts were observed to be in their most lateral position on the ventral surface of the brain (2.6mm posterior to bregma). The sections were collected in 0.1M phosphate, containing 0.3% Triton X-100, 0.1mg/ml nitroblue tetrazolium and 1.0mg/ml ß-NADPH. The sections were placed in an oven at 37°C for 1hr. Following the reaction, the sections were rinsed in phosphate buffer and mounted onto slides. The presence of NADPH-diaphorase in the PVN was examined under a microscope. The density of the staining was evaluated by counting the number of cells that were positively stained for NADPH-diaphorase.

Real-time RT-PCR for the Measurement of nNOS and NR_1 Receptor mRNA

Total RNA extracted from the punched tissue was subjected to reverse transcription. The cDNA was amplified by real-time quantitative RT-PCR with the BioRad iCycler IQ system (Biorad Laboratories). All primer pairs were designed using BeconDesign 4.0 (Biorad Laboratories). For nNOS (GenBank: NM 052799), the sense primer was 5'-GCGGAGCAGAGCGGCCTTAT-3', the antisense primer was 5'-TTTGGTGGGAGGACCGAGGG-3'; for NR_1 receptor (GenBank: NM 017010), the sense primer 5'-ATAGTGACAATCCACCAAGAGCC-3', the antisense primer was 5'-GTAGCTCGCCCATCATTCCGTT-3'; for rpl19 (GenBank: NM 031103), the sense primer 5'-CCCCAATGAAACCAACGAAA-3', the antisense primer was 5'-ATGGACAGTCACAGGCTTC-3'. Relative mRNA expression of nNOS or NR_1 receptor was calculated using the Pfaffl equation relates expression of the target gene to expression of a reference gene (rpl19).

Western Blot Assay of nNOS and NR_1 Receptor Protein

The punched tissues were incubated with lysis buffer (10mmol/L Tris, 1mmol/L EDTA, 1% sodium dodecyl sulfate, 0.1% Triton X-100 and 1mmol/L phenylmethanesulfonylfluoride). Samples were loaded on the 7.5% SDS-PAGE gel to be subjected to electrophoresis. Then the fractionized proteins on the gel were electrophoretically transferred onto the PVDF membrane. The membrane was probed with primary antibodies (rabbit anti-nNOS, NR_1 receptor and GAPDH antibody, Santa Cruz, CA) overnight at 4°C. The membrane was incubated with a peroxidase-conjugated secondary antibody (Pierce). The signals were visualized using an enhanced chemiluminescence substrate (Pierce) and detected by digital image system (UVP BioImaging). The signals were quantified by Kodak 1D software (Eastman Kodak Company). The expression of nNOS or NR_1 receptor protein was calculated as the ratio of intensity of the nNOS or NR_1 receptor band relative to the intensity of GAPDH band.

Cell Culture

The NG108-15 (neuroblastoma X glioma) hybrid cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G and streptomycin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 Cells were seeded in 6 well plates and grown until 60-70% confluent before treatment with indicated concentrations of with AdnNOS, in a dose-dependent manner (10^4~10^8pfu/ml, 24hrs) for 24hrs. Cells were homogenized in lysis buffer (10mmol/L Tris, 1mmol/L EDTA, 1% sodium dodecyl sulfate, 0.1% Triton X-100 and 1mmol/L phenylmethanesulfonylfluoride). Lysates (30-40μg) were processed to measure nNOS and NR_1 receptor protein by Western blot.

Immunofluorescent Staining of NG108 cells

Adherent NG108 cells were grown on laminin coated 6mm Transwel-Clear™ inserts (Corning, Costar) overnight. Cells were fixed and then permeabilized with 0.2% Triton X-100 for 20min. 10% normal donkey serum was used for blocking for 1hr followed by incubation with primary antibody at 4°C overnight. All antibodies used were diluted in 1% blocking solution. For nNOS and NR_1 receptor double immunostaining, dilutions of 1:200 for mouse anti-nNOS and 1:200 for goat anti-NR_1 receptor were used. Secondary antibody consisted of a 1:200 dilution of Cy2-conjugated donkey anti-mouse IgG and Cy3-conjugated anti-goat was used for 2hrs. Coverslips were then mounted onto slides using Fluoromount G (Southern Biotechnology). Labeled cells were visualized by Olympus fluorescence microscope equipped with digital camera

Results

Table S1: Baseline values of morphology and hemodynamics in rats with heart failure and sham-operated rats

Parameters	Sham-AdβGal (n=10)	CHF-AdβGal (n=10)	Sham-AdnNOS (n=10)	CHF-AdnNOS (n=10)
Body weight (g)	372±16	396±21	367±19	391±12
Heart weight (g)	1.1±0.1	1.8±0.3*	1.2±0.1	1.9±0.3*
Infarct size (% of epicardial LV)	0	41±6*	0	42±7*
LVEDP (mmHg)	2±2	26±2*	3±2	23±4*
Basal mean blood pressure (mmHg)	92±5	87±3	89±3	86±4
Basal heart rate (bpm)	329±18	359±39	336±19	351±21
Basal integrate RSNA (μV.s)	4.3±0.7	5.4±0.2	4.1±0.1	4.8±0.4

Values are presented as mean±SE; * indicates $P<0.05$ versus sham rats. LV: left ventricle; wt: weight.