SAPPLEMENTAL MATERIAL for "**Hydrogen sulfide improves survival after cardiac arrest and cardiopulmonary resuscitation via a nitric oxide synthase 3 dependent mechanism in mice.** (MS ID#: CIRCULATIONAHA/2008/833491)"

Supplemental Methods

Assessment of neurological function—Neurological function was assessed at 24h after CA/CPR or sham surgery using a previously-reported neurological function scoring system with minor modifications [1]. Briefly, five parameters were assessed and scored: level of consciousness (no reaction to pinching of tail = 0, poor response to tail pinch = 1, normal response to tail pinch = 2), corneal reflex (no blinking = 0, sluggish blinking = 1, normal blinking = 2), respirations (irregular breathing pattern = 0, decreased breathing frequency with normal pattern = 1, normal breathing frequency and pattern = 2), coordination (no movement = 0, moderate ataxia = 1, normal coordination = 2), and movement/activity (no spontaneous movement = 0, sluggish movement = 1, normal movement = 2). Total score was reported as the neurological function score (total possible score = 10).

Histological studies—Twenty-four or 72h after CA/CPR or sham surgery, mice were sacrificed, and brains were harvested, fixed in 4% formalin in PBS, and embedded in paraffin. Brains were cut with a microtome in coronal planes including the hippocampus (6 μm thickness). Activation of caspase 3 was assessed by immunohistochemistry in paraffin-embedded brain sections obtained 24h after CA/CPR using a rabbit monoclonal

antibody against cleaved caspase 3 (1:80, Cell Signaling) according to the protocol recommended by the manufacturer. Non-viable neurons were counted in brain sections obtained 72h after CA/CPR stained with hematoxylin and eosin based on cellular morphology as previously reported.[2] Cleaved caspase 3-positive or non-viable neurons in the CA1 sector of the hippocampus were manually counted by an investigator blinded to the treatment group, and the number of these neurons per square millimeters of examined area was reported.

Measurement of protein levels and phosphorylation—LV and cerebral cortex tissue were obtained 15 min after CA/CPR or sham operation, and tissue homogenates were centrifuged for 20 min at 20,000 *g*. Supernatant proteins (15-60 μg) were fractionated on 7 or 12% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked for 1 h in 5% BSA or 2% ECL-Advance reagent (GE Healthcare) and incubated overnight with primary antibodies (Cell Signaling Technology Inc. unless otherwise noted) against total Akt (1:1,000), phospho-Akt (Ser^{473},1:1,000), total NOS3 (1:5,000; BD Biosciences), phospho-NOS3 (Ser^{1179}, 1:5,000), total AMP-dependent kinase α (AMPKα, 1:10,000), phospho-AMPKα (Thr^{172}, 1:10,000), total glycogen synthase kinase 3β (GSK3β, 1:10,000), phospho-GSK3β (Ser^{9}, 1:10,000), cleaved caspase-3 (1:5,000), vinculin (1:2000 Sigma), and β-tubulin (1:2,000). Bound antibody was detected with a horseradish peroxidase-linked antibody directed against rabbit IgG (1:5000; Cell Signaling Technology Inc.) or mouse IgG (1:20,000 Sigma) and was visualized using chemiluminescence with ECL Plus or ECL Advanced kit.

Supplemental Table 1. Group characteristics before cardiac arrest and in the first hour after cardiac arrest and CPR

	Vehicle (n=21)	Na_2S (n=13)	Post-CPR Na_2S (n=11)
Weight, g	25.3±0.3	25.2±0.4	25.4±0.4
HR before CA, bpm	517±16	567±17	510±16
MAP before CA, mmHg	127±3	125±3	129±3
Total dose of Epinephrine, µg	0.7±0.0	0.7±0.0	0.7±0.1
CPR time to ROSC, s	221±10	204±8	228±19
HR at ROSC, bpm	521±4	531±4	506±25
MAP at ROSC, mmHg	54±3	69±5	56±4
HR at 60 min after CPR, bpm	333±13	345±14	341±12
MAP at 60min after CPR, mmHg	48±4	50±3	46±4

Values are mean±SEM. Vehicle, mice subjected to CPR treated with vehicle; Na_2S, mice treated with Na_2S 1 min before CPR; post-CPR Na_2S, mice treated with Na_2S 10 min after CPR; HR, heart rate; MAP, mean arterial pressure; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation. No differences were found statistically significant.

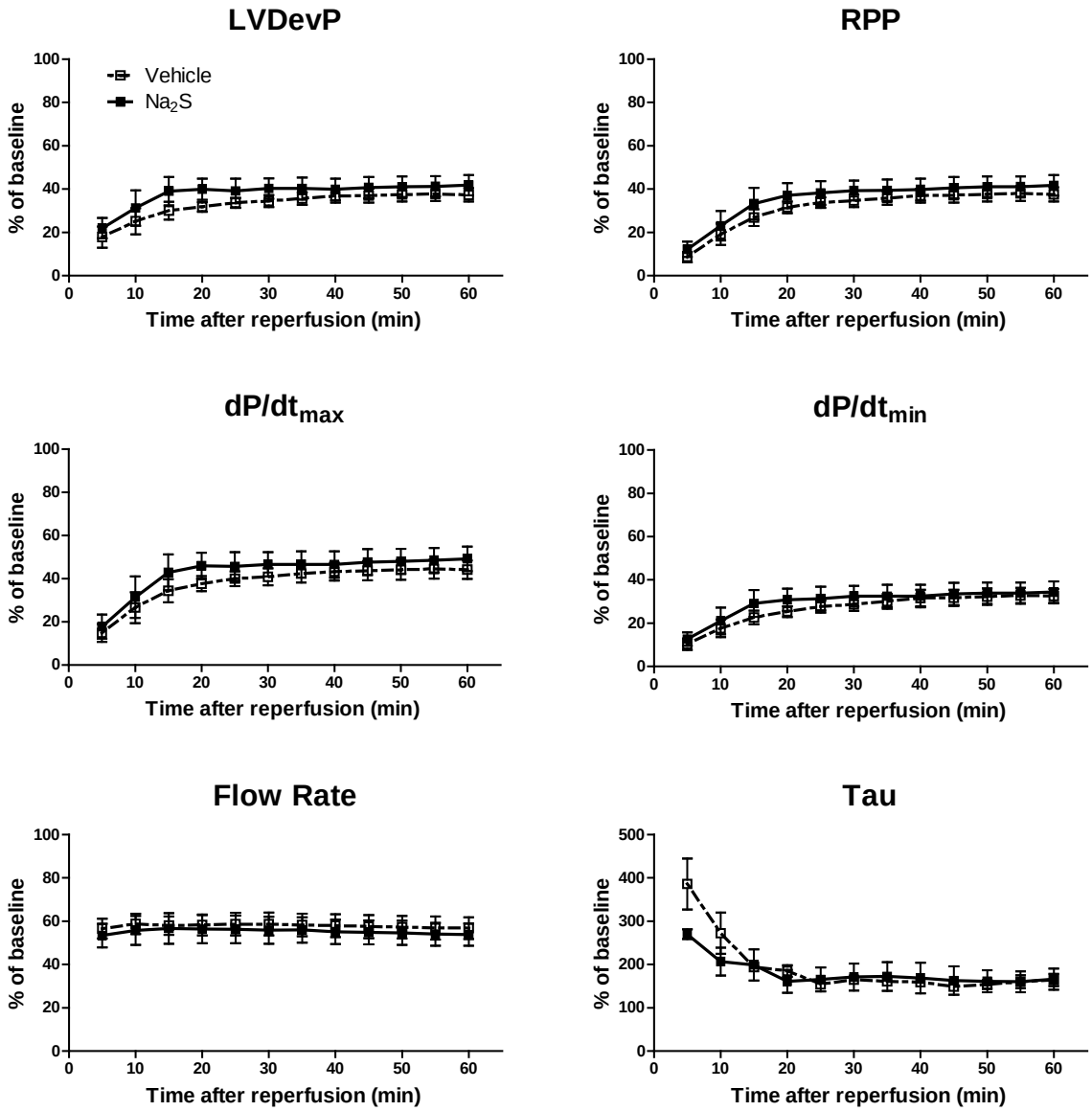
Supplemental Figure Legend

Supplemental Figure 1

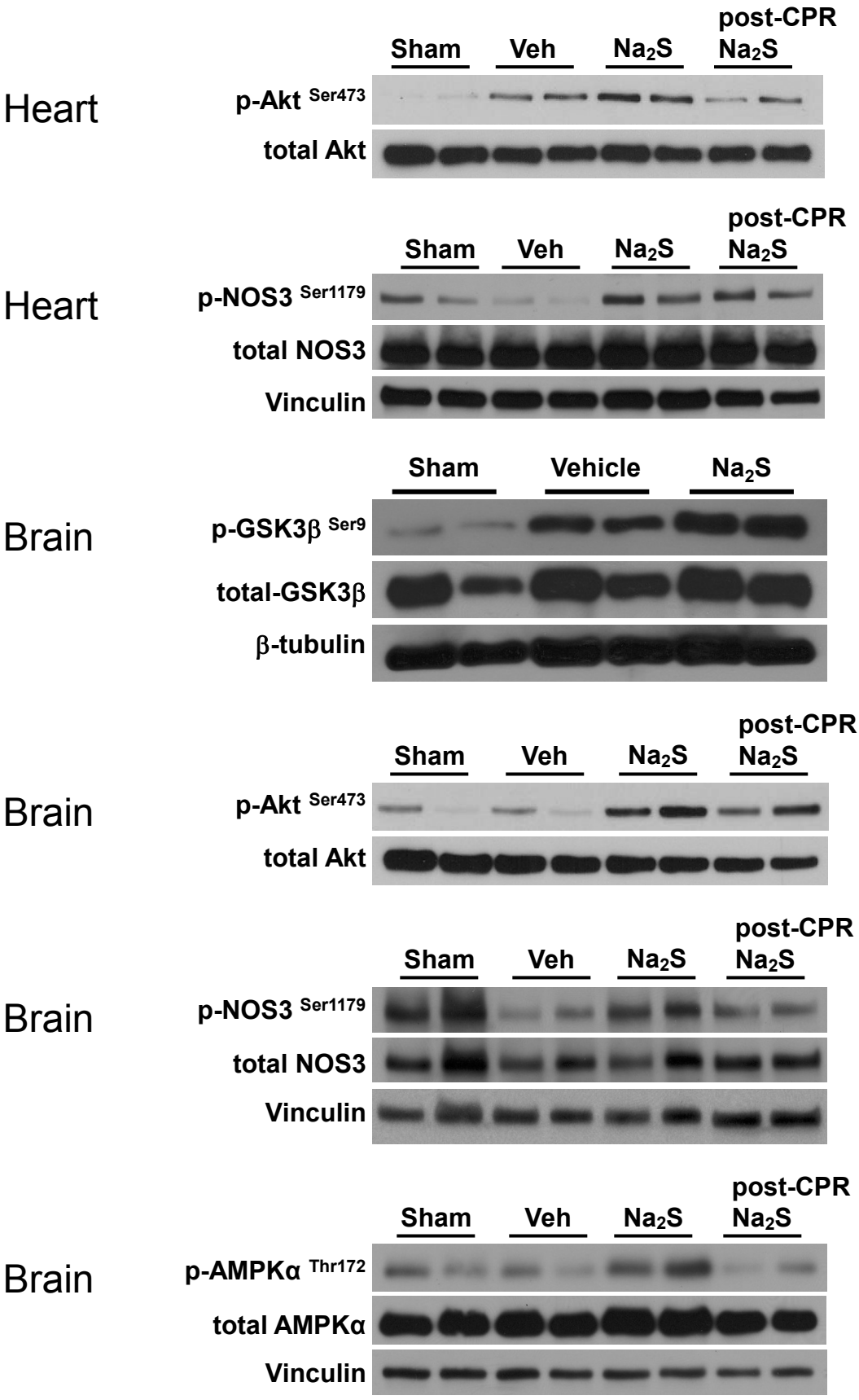
Effects of delayed administration of Na_2S in simulated ischemia-reperfusion in isolated-perfused mouse hearts. Percentage of baseline values are shown for maximal LV developed pressure (LVDevP), rate pressure product (RPP), maximal rate of LV pressure rise (dP/dt_{max}), maximal rate of LV pressure reduction (dP/dt_{min}), the time constant of isovolumic LV relaxation (Tau), and coronary flow rate (Flow Rate). No difference was found between vehicle and delayed administration of Na_2S on all parameters. N=4 each.

Supplemental Figure 2

Additional immunoblots images are shown for phosphorylated (Ser^{473}) and total Akt (heart and brain), phosphorylated (Ser^{1179}) and total NOS3 (heart and brain), phosphorylated (Ser^{9}) and total GSK3β (brain), and phosphorylated (Thr^{172}) and total AMPKα (brain). Expressoin level of vinculin or β-tubulin is shown as loading control for some of the blots. Sham, sham-operated mice not subjected to cardiac arrest; Veh, mice treated with vehicle 1 min before CPR; Na_2S, mice treated with Na_2S 1 min before CPR; post-CPR Na_2S, mice treated with Na_2S 10 min after CPR.



Supplemental Figure 1



Supplemental Figure 2

Supplemental Reference List

(1) Abella BS, Zhao D, Alvarado J, Hamann K, Vanden Hoek TL, Becker LB. Intra-arrest cooling improves outcomes in a murine cardiac arrest model. *Circulation* 2004 June 8;109(22):2786-91.

(2) Kofler J, Hattori K, Sawada M, DeVries AC, Martin LJ, Hurn PD, Traystman RJ. Histopathological and behavioral characterization of a novel model of cardiac arrest and cardiopulmonary resuscitation in mice. *J Neurosci Methods* 2004 June 15;136(1):33-44.