Supplementary Material

to

The role of decreased cardiolipin and impaired electron transport chain in brain damage due to cardiac arrest

Surgical Procedures

All procedures were performed according to the animal protocol approved by the IACUC. Adult male Sprague–Dawley rats (465–530 g, Charles River Production), housed in a rodent facility under 12 h light–dark cycle with unrestricted access to food and water. Bendavia was a gift from Stealth Peptides Inc.

Rats were anesthetized with 4% isoflurane for 5min in a plexiglass chamber and orotracheally intubated with a 14-gauge intravenous catheter (B. Braun Medical Inc., Bethlehem, PA, USA). Rats are placed on a thermostatically regulated heating pad and rat temperature was kept at 37 °C using the heating pad and a heating lamp during the procedures. Ventilation was adjusted to maintain an EtCO2 between 35 and 45mmHg (Micro-Capnometer, Columbus Instruments, Columbus, OH, USA), a respiratory rate (RR) of 40 per min, and a positive end-expiratory pressure (PEEP) of 1 cm H2O using a mechanical ventilator (Ventilator Model 683, Harvard Apparatus, Holliston, MA, USA). Anesthesia was maintained with 2% isoflurane to sustain a surgical depth of anesthesia. Temperatures were monitored using tympanic and rectal thermocouple probes and were maintained steadily at 37±0.5 ◦C with a heat pad and an incandescent heating lamp. This procedure takes ~ 40 min.

The left femoral artery was cannulated to measure arterial and the left femoral vein to measure central venous pressure. The right external jugular vein and the right femoral artery were cannulated for venous outflow and the arterial inflow of CPB fluid, respectively. After surgical preparation, isoflurane was decreased to 1% and waited for 15 min. Vecuronium bromide at 2 mg/kg was slowly administered through the left femoral vein for 4 min. After waiting for additional 3 min, asphyxial cardiac arrest was induced by switching off the ventilator. Isoflurane was discontinued thereafter. MAP below 20 mmHg was defined as CA, which was achieved in ~ 3 min.

After 30 min of asphyxia, resuscitation was started with the initiation of CPB flow and resumption of ventilation. The customized CPB circuit designed for rodents consists of a heat exchanger, an open venous reservoir, a membrane oxygenator, and a roller pump. For the oxygenator, 100% oxygen was used to saturate blood with oxygen. The open venous reservoir was filled with a 20 mL solution cocktail of plasma-lyte A (10 mL), 6% hetastarch (10 mL), 5% magnesium sulfate (0.8 mL), and 3.3 M THAM (0.3 mL). The initial CPB flow rate was 70 mL/min and gradually decreased to ~20 mL/min to meet venous outflow. During this, the complement solution containing plasma-lyte A (2.5 mL), 6% hetastarch (2.5 mL) and 10 mL blood from a donor rat was added to the reservoir as needed. After 60 min of CPB, animals were sacrificed for biochemical measurements.

Mitochondria isolation

General procedure

Each brain, heart, liver and kidney was immediately placed in mitochondrial isolation buffer (MESH) composed of 210 mM mannitol, 70 mM sucrose, 10 mM Hepes, 0.2 mM EGTA, pH 7.3. The tissues were trimmed in MESH buffer to remove spinal cord, extraventricular tissue, and fats, blot-dried on filter paper, weighed, and placed in MESH buffer freshly supplemented with 0.2% w/v fatty acid free-BSA. The tissues were homogenized with a teflon/glass motor-driven homogenizer (Glas Col LLC. Terre Haute, IN). A Beckman model J-301centrifuge (JA-30.50 rotor) was used with low speed centrifugation at 5600× g for 1 min and high speed centrifugation at 10000× g for 6 min. Mitochondria concentrations were determined by the BCA assay and expressed as mg mitochondrial protein/g tissue.

Brain mitochondria isolation

Minced brain tissue in MESH-BSA (10 mL/g tissue) was homogenized for 8 strokes at the setting of 40. The homogenates were centrifuged at low speed and the supernatant was poured into a polycarbonate tube. The pellet was homogenized and centrifuged as above and the pooled supernatant was centrifuged at high speed. The supernatant was poured out gently until synaptosomes layer reached the top. The remaining loose pellet was suspended with 20 ml of 12.5% percoll in MESH (v:v) and centrifuged at high speed. The supernatant was gently decanted with pipets without disturbing the mitochondria pellet (usually ~200 µL buffer remains). Finally, the pellet was resuspended in 20 mL of MESH buffer and centrifuged at high speed. The mitochondria pellet was suspended in 0.05 mL MESH per g tissue to yield ~20 mg/mL protein concentration.

Heart mitochondria isolation

Minced heart tissue in MESH-BSA (15 ml/g tissue) was homogenized for 12 strokes at the setting of 30 [14] The homogenates were centrifuged at low speed and the supernatant was

saved. The homogenization of the pellet and centrifugation was repeated two more times (The third homogenization was in 10 mL/g tissue). The pooled supernatant was centrifuged at high speed. The pellet was resuspended in 20 mL of MESH buffer and centrifuged at high speed again. The mitochondria pellet was suspended in 0.5mL MESH per g tissue to yield ~25 mg/mL protein concentration.

Kidney and liver mitochondria isolation

Minced kidney or liver tissue in MESH-BSA (10 ml/g tissue) was homogenized for 3 strokes at the setting of 30. The homogenates were centrifuged at low speed and the supernatant was centrifuged at high speed. The pellet was resuspended in 20 mL of MESH buffer and centrifuged at high speed. The mitochondria pellet was suspended in 0.4 mL MESH per g tissue to yield ~30 mg/mL protein concentration for kidney and ~45 mg/mL for liver mitochondria.

Table S1. Conditions used for assay of electron transport chain complexes

	Brain	Heart	Kidney	Liver
Complex I (340 nm)	50 µg mito 0.06 mM NADH 0.08 mM DUQ 0.25% BSA 0.05 µg AA 0.68 mM DDM	20 µg mito 0.14 mM NADH 0.08 mM DUQ 0.25% BSA 0.05 µg AA 0.68 mM DDM	50 µg mito 0.14 mM NADH 0.08 mM DUQ 0.25% BSA 0.05 µg AA 0.68 mM DDM	50 µg mito 0.14 mM NADH 0.08 mM DUQ 0.25% BSA 0.05 µg AA 0.68 mM DDM
Complex III (550 nm)	5 µg mito 0.04 mM DUQH 0.04 mM Cytc-ox 0.0005 mM NaN_3 0.68 mM DDM 0.25% BSA 0.5 µM rotenone	5 µg mito 0.032 mM DUQH 0.04 mM Cytc-ox 0.0005 mM NaN_3 0.45 mM DDM 0.25% BSA 0.5 µM rotenone	5 µg mito 0.032 mM DUQH 0.04 mM Cytc-ox 0.0005 mM NaN_3 0.45 mM DDM 0.25% BSA 0.5 µM rotenone	5 µg mito 0.032 mM DUQH 0.04 mM Cytc-ox 0.0005 mM NaN_3 0.45 mM DDM 0.25% BSA 0.5 µM rotenone
Complex IV (550 nm)	3 µg mito 0.03 mM cytc-red 0.45 mM DDM	1 µg mito 0.03 mM cytc-red 0.45 mM DDM	5 µg mito 0.03 mM cytc-red 0.45 mM DDM	5 µg mito 0.03 mM cytc-red 0.45 mM DDM
NADH Ferricyanide Reductase (340 nm)	10 µg mito 0.06 mM NADH 0.6 mM KFeCN 0.25% BSA 0.05 µg AA 0.68 mM DDM	10 µg mito 0.14 mM NADH 0.6 mM KFeCN 0.25% BSA 0.05 µg AA 0.68 mM DDM	20 µg mito 0.14 mM NADH 0.6 mM KFeCN 0.25% BSA 0.05 µg AA 0.68 mM DDM	20 µg mito 0.14 mM NADH 0.6 mM KFeCN 0.25% BSA 0.05 µg AA 0.68 mM DDM

Abbreviations: NADH, nicotinamide adenine dinucleotide; DUQ, decylubiquinone; BSA, bovine serum albumin; AA, antimycin a, DDM, n-Dodecyl β-D-maltoside; DUQH, decylubiquinol; cytc-oxi, oxidized cytochrome c; cytc-red, reduced cytochrome c; KFeCN, potassium ferricyanide

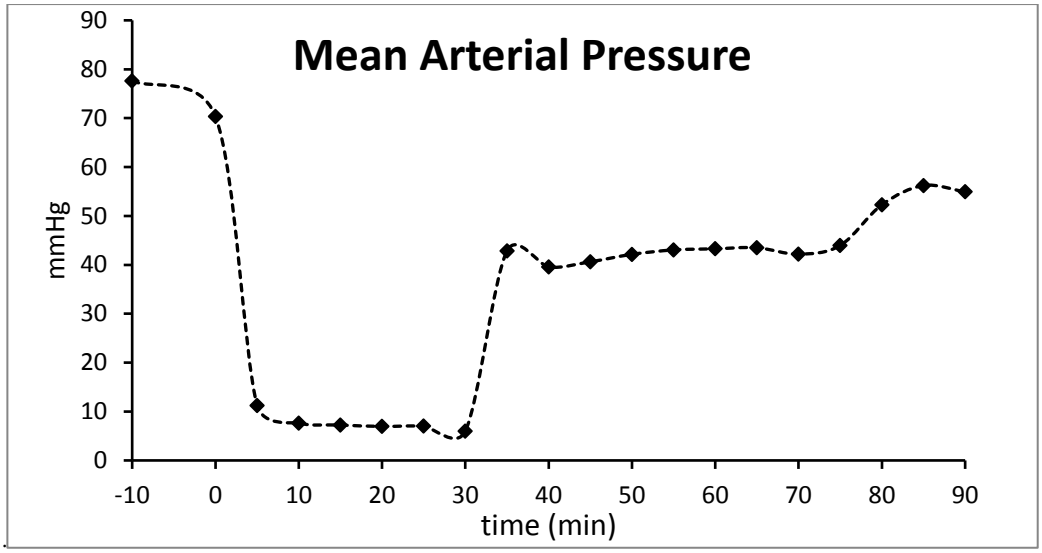

Fig. S1. Representative changes in mean arterial pressure following asphyxia and CPB resuscitation. (Time 0, beginning of asphyxia; time 30, beginning of CPB resuscitation)

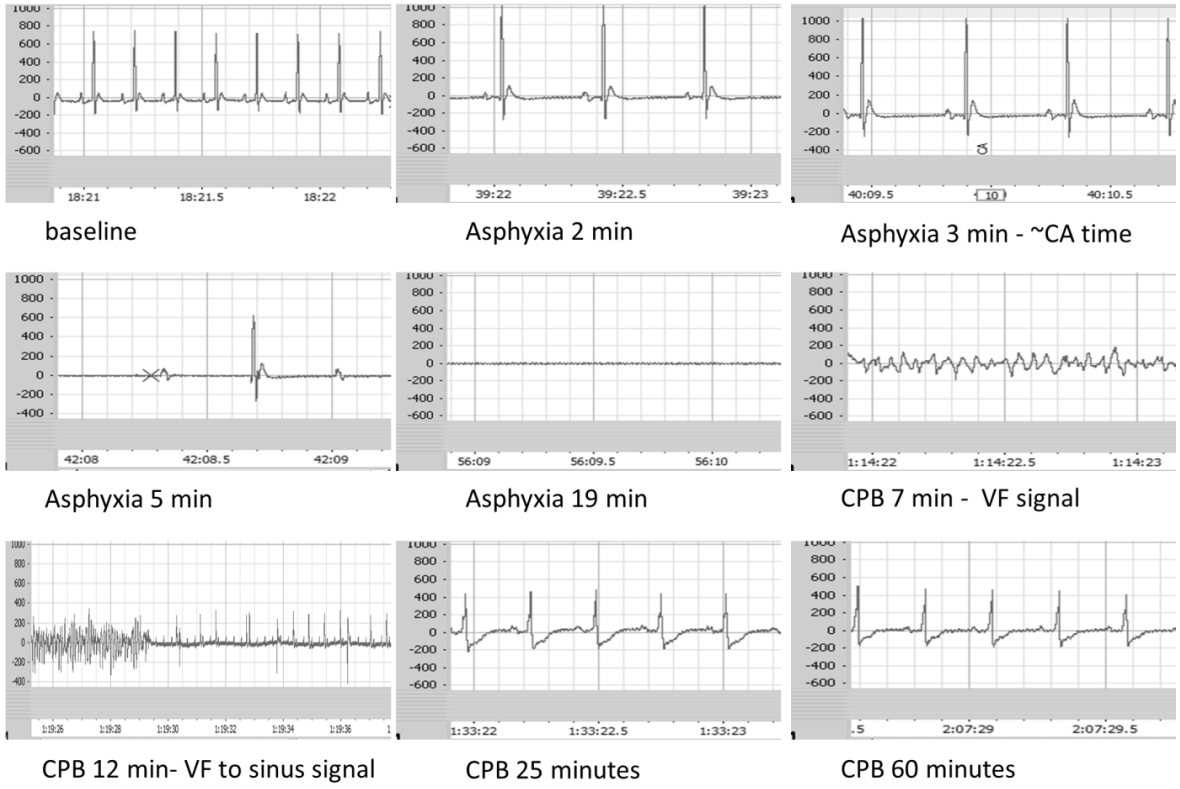

Fig. S2. Representative changes in electrocardiogram following asphyxia and CPB resuscitation. The time to reach cardiac arrest was ~3 min after the beginning of asphyxia and the time to achieved ROSC was ~8 min after the initiation of CPB resuscitation in this animal. Mean arterial pressure drops to ~10 mmHg shortly after initiation of asphyxia, indicating CA. At 30 minutes, CBP resuscitation is initiated and ROSC is marked by return of mean arterial pressure to ~40 mmHg. The electrocardiograms depict the evolution of cardiac dysfunction and recovery throughout the protocol, progressing from sinus to profound bradycardia shortly after initiation of asphyxia and subsequent development of ventricular fibrillation. Return to sinus—with residual ischemic changes—occurs shortly after initiation of CPB.

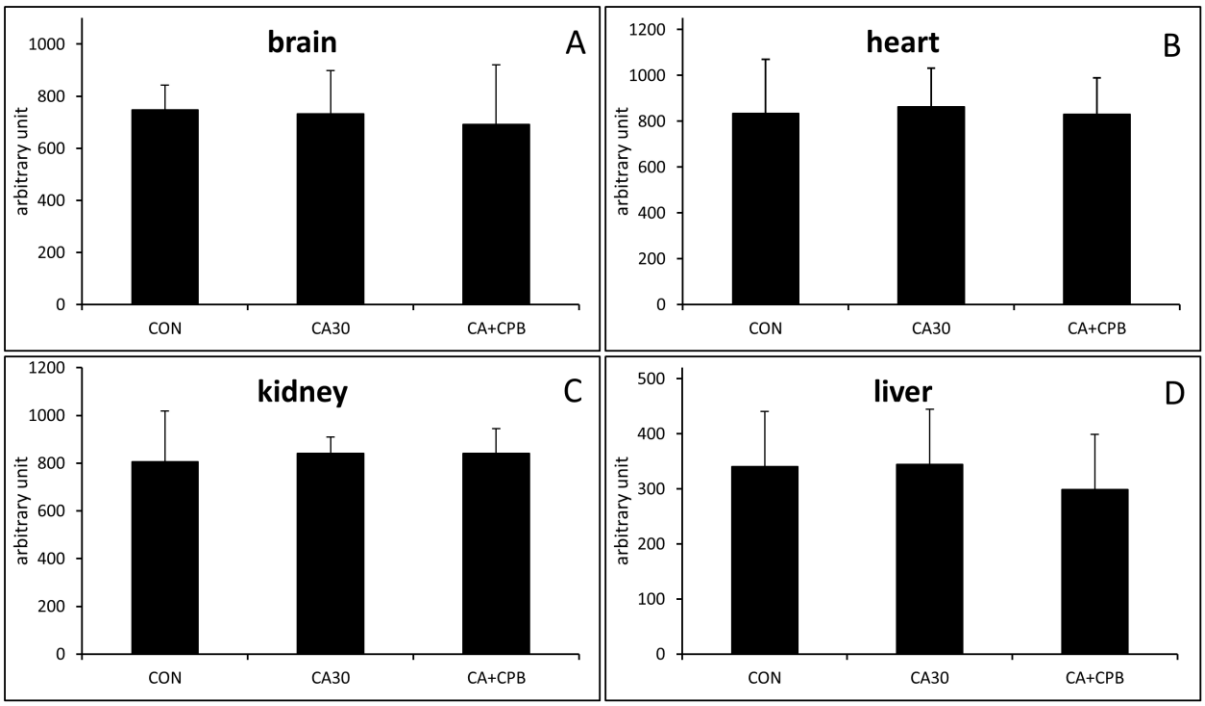

Fig. S3. Amounts of complex I following CA or CA with CPB resuscitation. No change in amount of complex I after CA or CA followed by CPB (CON, control; CA30, 30 min CA; CA+CPB, 30 min CA +60 min CPB resuscitation, n=5 for brain, n=6 for heart, kidney, and liver).