Supplementary Materials

Materials

Tricarbonyldichlororuthenium (II) dimer (carbon monoxide releasing molecule-2, CORM-2, Chemical structure shown in Supplementary Fig 1) was obtained from Sigma Aldrichc (St Louis, Mo., USA) and solubilized in dimethyl sulfoxide (DMSO) to obtain a 40 mmol/L stock. Inactive form of CORM-2 (negative controls) was used in some experiments and prepared as follows: CORM-2 was inactivated (iCORM-2) by leaving the stock of CORM-2 at 37°C in a 5% CO_2 humidified atmosphere for 24h to liberate CO. The iCORM-2 solution was finally bubbled with nitrogen to remove the residual CO present in the solution. Interleukin-1 beta (IL-1β) and Tumor necrosis factor-alpha (TNF-α) ELISA kits were purchased from eBioscience (San Diego, Calif., USA). 2',7'-dichlorofluorescein diacetate (DCFH-DA), 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1), Adenosine Triphosphate (ATP) assay kit and bicinchoninic acid (BCA) protein assay kit was obtained from Beyotime (Nantong, Jiangsu, China). Lactate assay kit was obtained from Jiancheng (Nanjing, Jiangsu, China). Cell culture reagents were obtained from Gibco (Grand Island, NY., USA) and culture supplies from Corning (Corning, NY., USA) and Falcon (Lincoln Park, NJ., USA). All the other chemicals were of reagent grade and obtained from Sigma unless otherwise stated.

Animal and CLP protocol

Total of two hundred and seventy eight male C57BL/6 mice (body weight 20 ± 2 g, Experimental Animal Center of the Jiangsu University, Zhenjiang, Jiangsu, China) were given free access to normal mouse diet and tap water. All mice were randomly assigned to 4 groups according to the different design of each experiment: Sham group; CLP group; CLP + CORM-2 group; and CLP +

iCORM-2 group. In each experiment, mice in sham group underwent sham procedure, whereas mice in CLP group received cecal ligation and puncture surgery, mice in CLP + CORM-2 group and CLP + iCORM-2 group were subjected to the same injury with immediate administration of CORM-2 (8 mg/kg, *i.v.*) and iCORM-2 (8 mg/kg, *i.v.*), respectively. The concentration of CORM-2 used in the present study was based on a previous report and the preliminary experiments in our lab [1].

Acute septic peritonitis was induced by CLP. Mice were anesthetized with 2% isoflurane in oxygen via a face mask. A 2-cm midline incision was made through the abdominal wall; the cecal was identified and ligated with a 3-0 silk tie 1 cm from the tip. Care was taken not to cause bowel obstruction. A single puncture of the cecal wall was performed with a 20-gauge needle. The cecal was lightly squeezed to express a small amount of stool from the puncture site to assure a full thickness perforation. Great care was taken to preserve the continuity of flow between the small and large bowels. The cecal was returned to the abdominal cavity and the incision was closed with surgiclips. Sham mice underwent midline laparotomy under anesthesia; the cecal was exteriorized and returned to the abdomen, and the incision was closed with surgiclips.

Primary neonatal rat ventricular myocyte cultures (NRVMCs)

NRVMCs were prepared as previously described [2]. Animals were anaesthetized with 2% isoflurane and sacrificed by cervical dislocation before removing tissues. Small scissor was used to open the chest of One-day-old SD rats, forcep was used to remove the heart. Heart was transferred to a dish containing ice-cold PBS to squeeze the residual blood and then remove atrium and great vessels tissues. Total 10 hearts had been processed; 0.5 mm pieces were carefully minced with a sterile small scissor and then transferred to a 50mL centrifuge tube. 5mL 0.06%

pancreatin was added to the centrifuge tube within a 37°C water bath for 5min. Removed the suspension, 5mL 0.06% type Ⅱ collagenase was used for further digestion. After a gentle vibrate in a 37°C water bath for 5min, suspension was pipetted out to DMEN/F12 (1:1) with 20% fetal bovine serum (FBS) to stop digestion. This step was repeated 4-5 times for a complete digestion of the remaining tissue fragments. Then cells were collected by centrifugation (1800 g for 10 min). The cells were resuspended in DMEN/F12 (1:1) with 20% FBS, penicillin (100 IU/mL), streptomycin (100 μg/mL) and maintained for 1 h in a humidified atmosphere with 5% CO_2 and 95% air. Then the supernatant containing the ventricular myocytes was harvested gently, counted, and plated out to an amount of $1\times10^5/cm^3$ in a 6-, 24-, or 96-well plate. Forty-eight hours after planting, NRVMCs were washed with medium and incubated with lipopolysaccharide (LPS, 10 μg/mL) in the absence or presence of CORM-2 (50 μmol/L) for 12 h.

Survival

In order to confirm the protective effect of CORM-2 against sepsis, a total of 53 C57BL/6 male mice were randomly assigned into 4 groups: Sham group (n=8), CLP group (n=15), CLP + CORM-2 group (n=15), CLP + iCORM-2 group (n=15). All mice had normal access for water and food, and were monitored every 6 h for 72 h.

Biochemical assays

Mice were anesthetized with 2% isoflurane and blood samples were collected from the heart at 6, 12, 24 h after CLP. Serum levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase (CK) and CK myocardial isoform (CK-MB) were determined by a serum autoanalyzer (AU2700, Olympus, Tokyo, Japan). The released LDH in the supernatants of NRVMCs was calculated according the following equation: LDH released = LDH in the

supernatant - LDH in the cell-free medium.

Quantitation of apoptosis

Animals were anaesthetized with 2% isoflurane and sacrificed by cervical dislocation before removing tissues. 24 h after CLP surgery, hearts were harvested and apoptosis was quantified by flow cytometry (FC). Specimens were minced into small pieces, and then digested for 10 min at 37°C with trypsin solution. Cells were collected and washed twice with cold PBS. Annexin V and PI were added to the cells and gently vortexed. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS, and binds to exposed apoptotic cell surface PS. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Cells that were positive for Annexin V but negative for PI were considered undergoing apoptosis. Incubated for 15 min at room temperature in the dark, then analyzed by FC within 1 h. The apoptotic rate was calculated as the percentage of Annexin V-positive and PI-negative cells divided by the total number of cells in the gated region.

Measurement of IL-1β and TNF-α

Blood samples were collected from the heart 24 h after surgery. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to measure serum concentrations of IL-1β and TNF-α following the manufacturer's recommended procedures.

Echocardiography

Echocardiography was performed as previously described [3]. Transthoracic echocardiography was performed using Agilent Sonos 5500 ultrasound systems (Agilent Technologies Inc,

Lexington, MA., USA). 24 h after CLP mice were anesthetized with 2% inhaled isoflurane and placed on a temperature-controlled platform. Preheated ultrasound transmission gel was applied to the left thorax and a probe was stroked upon the chest. After tracing the two-dimensional-guided M-mode image at the level of left ventricular short-axis, the following parameters were detected digitally using the leading-edge technique: left ventricular end-diastolic dimensions (LVDd) and left ventricular end-systolic dimensions (LVDs), and the manufacturer software automatically computed left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) using manufacturer software according to Teichholz Formula [4]. All date were collected from 10 consecutive cardiac cycles and averaged. Analysis of the data was performed with software provided by VisualSonics (Toronto, Ont., Canada).

Mitochondria isolation

Mitochondria isolation was performed by gradient centrifugation as described previously [5]. Briefly, 6, 12, 24 h mice after CLP, fresh heart tissues (100mg) were homogenized with 1 mL of isolation buffer containing 220 mmol/L d-mannitol, 70 mmol/L sucrose, 10 mmol/L Tris-HCL, 1 mmol/L EGTA and 0.4% bovine serum albumin (pH 7.4). The homogenates were centrifuged at 850 g for 10 min to collect supernatants, followed by centrifuging at 10,000 g for additional 10 min. The mitochondrial pellet was then resuspended in a final washing buffer containing 220 mmol/L d-mannitol, 70 mmol/L sucrose and 10 mmol/L Tris-HCL (pH 7.4). Protein concentration was determined by BCA protein assay kit.

Determination of mitochondrial ROS

Mitochondrial ROS was measured with a nonfluorescent probe DCFH-DA. All groups of isolated cardiac mitochondria were incubated with DCFH-DA at 37°C for 30 min. ROS levels were

determined via a fluorescence spectrometry at an excitation wavelength of 488 nm and an emission wavelength of 535 nm. Intracellular ROS in NRVMCs were detected with both fluorescence spectrometry and fluorescence microscope after being incubated with DCFH-DA for 30 min.

Determination of cardiac mitochondrial membrane potential (MMP)

MMP was measured with a fluorescent dye, JC-1. After treatment, all groups of isolated cardiac mitochondria were stained with JC-1 at 37°C for 30 min. MMP was shown as fluorescence intensity via the use of a fluorescence spectrometry at an excitation wavelength of 488 nm and an emission wavelength of 595 nm.

Determination of mitochondrial swelling

Mitochondrial swelling was assessed by measuring the absorbance of their suspension at 540 nm. Cardiac mitochondria were prepared in the assay buffer containing 125 mmol/L sucrose, 50 mmol/L KCl, 2 mmol/L KH_2PO_4, 5 μmol/L rotenone, 10 mmol/L Hepes, and 5 mmol/L succinate. The extent of mitochondrial swelling was assayed by measuring the decrease in absorbance (A540) every 30 sec for 30 min after the addition of 50 μmol/L Ca^{2+} at 37°C. Results were normalized to sham group.

RNA Extraction and Real-time quantitative PCR (qPCR)

Total RNA from each heart was extracted using the TRIzol Reagent. First-strand complimentary DNA (cDNA) was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo, Waltham, MA., USA). Real-time qPCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo, Waltham, MA., USA). Gene special primers were listed in Table 1. The relative level of gene expression was normalized to the level of GAPDH and calculated using the

$2^{-\Delta\Delta CT}$ method.

Confocal microscopy

Confocal microscopy examination of mitochondrial staining in heart sections was performed as previous described [6]. Hearts were harvested at 24 h after CLP. Placed in a cryomold, then heart was covered in Optimal Cutting Temperature medium (OCT), and frozen on an aluminum block partially submerged in liquid nitrogen. Within 30 min after harvest, samples were sectioned at 7 μm via cryotome and kept frozen within the chamber until all slides had been cut. Upon removal from the chamber samples were immediately exposed to 100 nM Mitotracker Green FM in PBS, pH 7.4 and placed in an incubator for 30 mi at 37°C. Slides were gently rinsed with PBS and cover slips placed without fixation and immediately imaged with a Leica TCS SP5 II confocal microscope. Mean fluorescence intensities from at least four individual hearts were measured using ImageJ and the means of these images were used for further statistical analysis.

Small-animal Positron Emission Tomography (PET) imaging

Small-animal PET imaging was performed as previously described [7]. All PET scans were performed with an Inveon Dedicated PET scanner (Siemens Medical Solutions, Knoxville, Tenn., USA). The device has a ring diameter of 26 cm and a 7.8-cm axial field of view. The intrinsic spatial resolution ranges from 1.56 to 2.01 mm, with a mean of 1.75 mm. The reconstructed resolution is 1.8-mm full width at half maximum in the center of the field of view and 3 mm at 4 cm radial offset. ^{18}F-fluorodeoxyglucose (FDG) is a sugar molecule labeled with the positron emitting radionucleotide ^{18}F. Taken up by cells, FDG is phosphorylated but cannot be metabolized any further, thus providing a method to measure the glucose uptake by cells. 24 h after CLP, mice were maintained under 2% isoflurane anesthesia throughout the scanning period. Mice were

positioned in a prone position and kept warm with a heating pad so as to maintain body temperature. 60 min after intravenous administration of FDG (18.9 ± 1.6 MBq), cardiac FDG uptake was monitored for a span of 10min. A transmission scan was performed after each emission study. The PET images were reconstructed using the Inveon Acquisition Workplace software. Cardiac FDG uptake was calculated as standardized uptake value (SUV).

Determination of ATP and lactate content

Cardiac ATP and lactate content were assessed in homogenized tissue samples. Briefly, 24 h after CLP heart samples (100 mg) were suspended in 1 mL phosphate buffered saline and sonicated on ice. Homogenates were centrifuged at 2500 g at 4°C and the supernatants were harvested. Then, ATP and lactate content in the homogenates were measured using assay kits following the manufacturer's instructions. Protein concentration was determined by BCA protein assay kit.

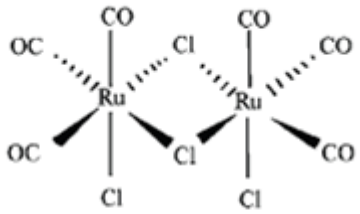
Measurement of oxygen consumption

NRVMCs were harvested from culture, resuspended in 3 mL of complete medium. Out of this suspension, 2 mL was transferred into a sealed chamber connected to a Clark-type electrode (Hansatech, King's Lynn, Norfolk, UK), and maintained at 37°C. Oxygen consumption was recorded as the rate of decrease in oxygen tension within the chamber over the first 180 sec. Results were corrected for spontaneous drift (oxygen used by the electrode itself) and cell count.

Statistical Analyses

Performed as described in the manuscript.

Supplementary Figures and Figure Legends:



Tricarbonyldichloro ruthenium (II) dimer
$[Ru(CO)_3Cl_2]_2$

Supplementary Fig 1. Chemical structure of Tricarbonyldichlororuthenium (II) dimer (CORM-2).

Supplementary Tables

Supplementary Table 1. Sequences of the used primers

Genes	Forward primer	Reverse primer
PGC-1α	ctacagacaccgcacacacc	tctgtttggccctttcagac
NRF-1	cacgtttgcttcggaaactc	actttcgcaccacattctcc
NRF-2	cctggacgggactattgaag	ggccgttctgtttgacactt
Tfam	caaaggatgattcggctcag	cccaatgacaactccgtctt
GAPDH	caccccatttgatgttagtg	ccatttgcagtggcaaag
mtCOII	aaccatagggcaccaatgatac	ggatggcatcagttttaagtcc-
18S	aggctgctttaagacctaccg	gccacacaagcatcaaagaa

Supplementary Table 2. Effect of carbon monoxide releasing molecule-2 (CORM-2) on cardiac function of septic mice

Parameter	Sham	CLP	CLP + CORM-2	CLP + iCORM-2
LVDd	4.44±0.32	4.32±0.37	4.96±0.29	4.29±0.29
LVDs	2.22±0.28	2.81±0.28	2.8±0.17	2.76±0.35
LVEF (%)	85.88±3.31	71.21±1.37***	80.26±2.37$^{\#\#\#}$	73.25±3.9***
LVFS (%)	48.01±3.84	34.93±1.28***	43.52±1.92$^{\#\#\#}$	35.49±1.52***

Data were shown as mean±SD, $^{***}P < 0.001$ compared with sham, $^{\#\#\#}P < 0.001$ compared with CLP.

Supplemental References:

1. Sun B, Sun H, Liu C, Shen J, Chen Z, Chen X: **Role of CO-releasing molecules liberated CO in attenuating leukocytes sequestration and inflammatory responses in the lung of thermally injured mice**. *The Journal of surgical research* 2007, **139**(1):128-135.
2. Golden HB, Gollapudi D, Gerilechaogetu F, Li J, Cristales RJ, Peng X, Dostal DE: **Isolation of cardiac myocytes and fibroblasts from neonatal rat pups**. *Methods Mol Biol* 2012, **843**:205-214.
3. Amundsen BH, Ericsson M, Seland JG, Pavlin T, Ellingsen O, Brekken C: **A comparison of retrospectively self-gated magnetic resonance imaging and high-frequency echocardiography for characterization of left ventricular function in mice**. *Laboratory Animals* 2011, **45**(1):31-37.
4. Teichholz LE, Kreulen T, Herman MV, Gorlin R: **Problems in echocardiographic volume determinations: echocardiographic-angiographic correlations in the presence of absence of asynergy**. *The American journal of cardiology* 1976, **37**(1):7-11.
5. Frezza C, Cipolat S, Scorrano L: **Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts**. *Nature protocols* 2007, **2**(2):287-295.
6. Coleman MC, Olivier AK, Jacobus JA, Mapuskar KA, Mao G, Martin SM, Riley DP, Gius D, Spitz DRD: **Superoxide mediates acute liver injury in irradiated mice lacking Sirtuin 3**. *Antioxidants & redox signaling* 2013.
7. Brunner S, Todica A, Boning G, Nekolla SG, Wildgruber M, Lehner S, Sauter M, Ubleis C, Klingel K, Cumming P *et al*: **Left ventricular functional assessment in murine models of ischemic and dilated cardiomyopathy using [18 F]FDG-PET: comparison with cardiac MRI and monitoring erythropoietin therapy**. *EJNMMI Res* 2012, **2**(1):43.