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Protective and biogenesis effects of sodium hydrosulfide on brain mitochondria after cardiac arrest and resuscitation

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ABSTRACT

Mitochondrial dysfunction plays a critical role in brain injury after cardiac arrest and cardiopulmonary resuscitation (CPR). Recent studies demonstrated that hydrogen sulfide (H_2S) donor compounds preserve mitochondrial morphology and function during ischemia-reperfusion injury. In this study, we sought to explore the effects of sodium hydrosulfide (NaHS) on brain mitochondria 24 h after cardiac arrest and resuscitation. Male Sprague-Dawley rats were subjected to 6 min cardiac arrest and then resuscitated successfully. Rats received NaHS (0.5 mg/kg) or vehicle (0.9% NaCl, 1.67 ml/kg) 1 min before the start of CPR intravenously, followed by a continuous infusion of NaHS (1.5 mg/kg/h) or vehicle (5 ml/kg/h) for 3 h. Neurological deficit was evaluated 24 h after resuscitation and then cortex was collected for assessments. As a result, we found that rats treated with NaHS revealed an improved neurological outcome and cortex mitochondrial morphology 24 h after resuscitation. We also observed that NaHS therapy reduced intracellular reactive oxygen species generation and calcium overload, inhibited mitochondrial permeability transition pores, preserved mitochondrial membrane potential, elevated ATP level and ameliorated the cytochrome c abnormal distribution. Further studies indicated that NaHS administration increased mitochondrial biogenesis in cortex at the same time. Our findings suggested that administration of NaHS 1 min prior CPR and followed by a continuous infusion ameliorated neurological dysfunction 24 h after resuscitation, possibly through mitochondria preservation as well as by promoting mitochondrial biogenesis.

1. Introduction

Despite numerous advances in laboratory and clinical research, neurological deficit after cardiac arrest remains a leading cause of high morbidity and mortality. The main contributors to brain injury after cardiac arrest and cardiopulmonary resuscitation (CPR) include disrupted calcium homeostasis, free radical formation and activation of cell death signaling pathways, which mostly through mitochondrial dysfunction (Ayoub et al., 2008). Preservation of mitochondrial function in brain seems to be a novel approach to neurological dysfunction after resuscitation.

Hydrogen sulfide (H_2S) has been known for centuries as a toxic gas due to its inhibition effect on cytochrome c oxidase (COX), the terminal enzyme in the respiratory electron transport chain of mitochondria. However, recent evidence revealed that H_2S exerts biological functions in mitochondria. At low concentration, H_2S mediates mitochondrial preservation during ischemia-reperfusion injury by inhibiting the activity of COX, increasing superoxide dismutase (SOD) activity and glutathione levels, decreasing reactive oxygen species content and stimulating ATP production in mitochondria (Nicholson and Calvert, 2010; Pun et al., 2010). These led us to hypothesize that H_2S might protect cerebral mitochondrial function after resuscitation. To test this hypothesis, in the current study, we used sodium hydrosulfide (NaHS), a H_2S donor, to investigate the effects of H_2S on brain mitochondria after resuscitation and the possible underlying mechanisms.

2. Materials and methods

2.1. Animal preparation

All animals were cared in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of the People's

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Republic of China. Experimental protocols were approved by the Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology.

Male Sprague-Dawley rats (weight 300–400 g, Animal Experimental Center of Tongji Medical College of Huazhong University of Science and Technology) were fasted except for water overnight. Anesthesia was performed by pentobarbital sodium (45 mg/kg, ip) and supplemented with additional doses of 10 mg/kg during the entire experimental procedures. The tracheas of the rats were orally intubated and mechanically ventilated (HX-300S, Chengdu Taimeng Technology Corp., China). Two polyethylene catheters (PE 50) were inserted into the left femoral artery and vein. Arterial blood pressure, heart rate and electrocardiogram were continuously recorded (RM6240BD, Chengdu Instrument Factory, China).

2.2. Cardiac arrest and CPR protocols

Rats were randomly divided into 3 groups: sham group, CPR group, and CPR+NaHS group. The sham group ($n=10$) received the same procedures except cardiac arrest. Rats in another two groups were randomized until 10 animals per group reached the predefined endpoint of 24 h of survival. Cardiac arrest was induced by transcutaneous electrical epicardium stimulation via two disposable acupuncture needles as previously described (Pan et al., 2013). A progressive increase in 60 Hz current to a maximum of 2 mA was delivered. The current flow was continued for 3 min to prevent spontaneous defibrillation. Cardiac arrest was confirmed by mean arterial pressure less than 20 mmHg and ventricular fibrillation (VF), pulseless electrical activity (PEA) or asystole on ECG when electrical stimulation stopped. After 6 min of untreated cardiac arrest, mechanical ventilation begun at a tidal volume of 0.65 ml/100 g with a frequency of 100 breaths/min ($FiO_2=1.0$). Coincident with the start of ventilation, chest compressions at a rate of 200/min were delivered and adrenaline (20 μg/kg) was administered. Restoration of spontaneous circulation (ROSC) was defined as an organized rhythm with a mean aortic pressure (MAP) ≥ 60 mmHg for a minimum of 10 min. After ROSC, mechanical ventilation was continued with 21% oxygen for the next 3 h. All catheters were removed at the end of 3 h infusion. The rats were then returned to the cages with close observation.

NaHS (Sigma-Aldrich, USA) was freshly diluted in 0.9% NaCl to the desired concentration (0.3 mg/ml) before administration. Vehicle placebo and all doses of NaHS were reconstituted in a standard volume (5 ml) blindly by a laboratory technician who did not participated in the study. A bolus of NaHS (0.5 mg/kg) or vehicle placebo (0.9% NaCl, 1.67 ml/kg) was injected intravenously 1 min before start of CPR. Concomitant to the start of CPR, the infusion of NaHS (1.5 mg/kg/h) or vehicle placebo (5 ml/kg/h) was started at 3 h. The NaHS dosage was based on a previous study with minor modification (Knapp et al., 2011; Kida et al., 2012).

At 24 h after ROSC, neurological outcome was assessed in CPR and CPR+NaHS groups. Rats were then euthanized by anesthesia overdose and cortex was carefully separated on the ice under a dissecting microscope within 5 min. Fresh frontal cortex was used for ultrastructure examination, mitochondrial function investigation, flow cytometry study and ATP level evaluation immediately. Other part of frontal cortex was kept into liquid nitrogen for freezing and stored at −80 °C for later use.

2.3. Assessment of neurological outcome

Neurological outcome was assessed using neurological deficit scoring (NDS) system (Neumar et al., 1995). The score is composed of 6 items representing the level of consciousness, respiration, cranial nerves, motor, sensory and coordination. The scoring system ranged from 0 to 100 (normal: 100; brain death: 0). The scorer was blinded to the three groups.

2.4. Transmission electron microscopy (TEM) for brain mitochondria

To study the mitochondrial damage between groups, we examined the ultrastructure of cortex by transmission electron microscopy. Briefly, fresh frontal cortex was fixed in cold 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide, dehydrated through graded ethanol and embedded in epoxy resin. The specimen were cut into ultrathin sections (60–80 nm), stained with 2% aqueous uranyl acetate and lead citrate and viewed with a transmission electron microscope (FEI Tecnai G2 12, Netherlands) equipped with a Gatan 832 CCD camera (Gatan, Pleasanton, CA, USA) at a final magnification of 26,500×.

2.5. Isolation of cortex mitochondria

50 mg fresh cortex pieces obtained from the three groups were homogenized in cold isolation buffer containing 20 mM HEPES-KOH, pH 7.4, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.7% protease and phosphatase inhibitor. The lysates were centrifuged at 750*g* for 15 min at 4 °C, and then at 8000*g* for 20 min at 4 °C. The precipitates were collected as mitochondrial fraction for mPTP determination and Western blot analysis. The supernatant was further centrifuged at 16000*g* for 25 min at 4 °C. The supernatant was collected as cytosolic fraction for Western blot analysis.

2.6. Mitochondrial permeability transition pore (mPTP) opening determination

Opened mPTPs of cortex mitochondria were detected employing calcein–cobalt with a mPTP assay kit (Genmed Scientifics Inc., USA) according to the manufacturer's directions. Calcein-AM can diffuse into cell and mitochondrial membranes freely, whereas cobalt chloride is restricted to the cytosol. Cobalt chloride quenches calcein fluorescence in all cellular compartments except the mitochondrial matrix. During mPTP opening, calcein is released into cytosol where its fluorescence is quenched by colbalt chloride; thus, the changes of calcein fluorescence in mitochondrial matrix can reflex the degree of mPTP opening. Fluorescence intensity was measured using a monochromator microplate reader with excitation at 488 nm and emission at 505 nm. Results were expressed as RFU (U/mg protein). The mitochondrial protein concentrations were measured using the BCA protein assay.

2.7. Preparation of single cell suspension from fresh cortex

Cortex cell dissociation was prepared according to Sung et al. (2010) with minor modification. After removal of surface membranes and blood vessels, 100 mg fresh cortex was minced with scalpels and incubated in 0.25% trypsin (Invitrogen, USA) in Hanks' balanced salt solution (HBSS) in a conical tube at 37 °C for 15 min. The tissue was triturated with a 10 ml pipet and then filtered through a 40 μm cell strainer (Falcon, Heidelberg, Germany). The cells were collected by centrifugation at 200*g* for 5 min and then the pellet was resuspended in DMEM/F12 (Invitrogen, USA). The cell viability (approximately 98%) was assessed by typan blue dye exclusion. Immediately after the cell dissociation procedure, the cell suspension was incubated with different agents for the following experiments.