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Mitochondria-targeted hydrogen sulfide donor AP39 improves neurological outcomes after cardiac arrest in mice

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ABSTRACT

Aims: Mitochondria-targeted hydrogen sulfide donor AP39, [(10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide], exhibits cytoprotective effects against oxidative stress *in vitro*. We examined whether or not AP39 improves the neurological function and long term survival in mice subjected to cardiac arrest (CA) and cardiopulmonary resuscitation (CPR).

Methods: Adult C57BL/6 male mice were subjected to 8 min of CA and subsequent CPR. We examined the effects of AP39 (10, 100, 1000 nmol kg^{-1}) or vehicle administered intravenously at 2 min before CPR (Experiment 1). Systemic oxidative stress levels, mitochondrial permeability transition, and histological brain injury were assessed. We also examined the effects of AP39 (10, 1000 nmol kg^{-1}) or vehicle administered intravenously at 1 min after return of spontaneous circulation (ROSC) (Experiment 2). ROSC was defined as the return of sinus rhythm with a mean arterial pressure >40 mm Hg lasting at least 10 seconds.

Results: Vehicle treated mice subjected to CA/CPR had poor neurological function and 10-day survival rate (Experiment 1; 15%, Experiment 2; 23%). Administration of AP39 (100 and 1000 nmol kg^{-1}) 2 min before CPR significantly improved the neurological function and 10-day survival rate (54% and 62%, respectively) after CA/CPR. Administration of AP39 before CPR attenuated *mitochondrial permeability transition* pore opening, reactive oxygen species generation, and neuronal degeneration after CA/CPR. Administration of AP39 1 min after ROSC at 10 nmol kg^{-1}, but not at 1000 nmol kg^{-1}, significantly improved the neurological function and 10-day survival rate (69%) after CA/CPR.

Conclusion: The current results suggest that administration of mitochondria-targeted sulfide donor AP39 at the time of CPR or after ROSC improves the neurological function and long term survival rates after CA/CPR by maintaining mitochondrial integrity and reducing oxidative stress.

1. Introduction

The overall survival rate of resuscitated patients after out-of-hospital CA is 7–10% in Europe and the United States [1] despite the advances in CPR method [2] and post CA care [3]. In addition, more than 50% of survivors have permanent neurological dysfunction of varying degrees [4,5]. Mitochondrial dysfunction following ischemia/reperfusion (I/R) injury including CA and CPR is characterized by an impairment of electron transport, generation of reactive oxygen species (ROS) and decreased mitochondrial membrane potential which leads to pro-apoptotic signaling and cell death [6–8]. No pharmacological agent has yet been found to improve clinical outcomes after CA.

Hydrogen sulfide (H_2S) mediates cytoprotective effects against I/R injury at least in part via preservation of mitochondrial integrity [9,10]. We previously reported that administration of sodium sulfide (Na_2S), a H_2S generating compound, 1 min before the initiation of CPR prevented neurological injury and improved survival in mice subjected to CA and CPR [11,12]. While the beneficial effects of H_2S after CA/CPR were later confirmed by several investigators [13,14], others failed to observe the protective effects of H_2S donor compounds [15,16]. Although reasons for the conflicting results are undoubtedly multifactorial, at least a part of the problem may relate to the use of Na_2S or sodium hydrosulfide (NaHS) as H_2S donor compounds in these studies. As these simple sulfide salts generate H_2S immediately in the solution, concentrations of H_2S in prepared "H_2S

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donor solution" are often unstable and unreliable [17]. Therefore, the H_2S concentrations in the target tissue (e.g., brain) are unpredictable after bolus or continuous infusion of sulfide salts [18]. It is imperative to develop H_2S donor compounds that are targeted to certain tissue or cellular organelle and release H_2S in a more controlled manner to translate the unique cytoprotective effects of H_2S into a useful drug. To this goal, we have recently developed a novel mitochondria-targeted H_2S donor, AP39. Mitochondria targeting is achieved using a triphenylphosphonium (TPP^+) conjugate, resulting in the compound being rapidly and extensively taken up by mitochondria due to the electric potential gradient [19]. AP39 is also highly lipophilic and is expected to readily permeate the cell membrane [20]. AP39 increased the abundance of mitochondrial H_2S and protected cultured brain endothelial cells from oxidative stress at doses less than 1/1000 of the conventional H_2S generating compounds (e.g, Na_2S, NaHS, or GYY4137) [21,22].

We hypothesized that AP39 would protect brain mitochondrial integrity *in vivo* and improve survival rate and neurological function after CA/CPR. To address this hypothesis, we examined the effect of AP39 in the well-established murine CA/CPR model. Here, we report that AP39, administered either before or after CPR, preserved brain mitochondrial integrity and improved long term outcomes after CA in mice.

2. Methods

2.1. Animals and synthesis of AP39

After being approved by the Massachusetts General Hospital Subcommittee on Research Animal Care, 8–10-week-old and weight-matched male C57BL/6J wild type mice were included in the study. To evaluate the effects of AP39, we assigned mice randomly to four groups (AP39 at 10, 100 or 1000 nmol kg^{-1}, and vehicle) in which the study drug was administered 2 min before the initiation of CPR (Experiment 1) and three groups (AP39 at 10 or 1000 nmol kg^{-1}, and vehicle) in which the study drug was administered 1 min after return of spontaneous circulation (ROSC) (Experiment 2). AP39 was synthesized in-house as described [23].

2.2. Animal preparation

Mice were anesthetized with 5% (v/v) isoflurane in 100% oxygen, intubated, ventilated mechanically and maintained with 1.5% (v/v) isoflurane. Temperature probe was inserted into the esophagus. Arterial blood pressure was measured *via* the left femoral artery. A microcatheter was inserted into the left femoral vein for drug administration. The electrocardiogram was monitored with subcutaneous needle electrodes.

2.3. Murine CA/CPR model

Cardiac arrest and CPR in mice were performed as previously described with minor modification [11,12]. Briefly, CA was induced by an administration of 0.08 mg g^{-1} body weight potassium chloride *via* the left femoral vein and mechanical ventilation was stopped. After 8 min of CA, chest compressions with a finger were delivered at a rate of 300–350 per minute. Mechanical ventilation with 100% oxygen and infusion of 0.6 μg min^{-1} adrenaline were initiated 30 seconds before CPR. Chest compressions were continued until ROSC was achieved when spontaneous heartbeat returns. Infusion of adrenaline was stopped at ROSC. Core body temperature was maintained at 37.2 ± 0.1 °C throughout the surgical procedure and 1 h after CPR. Mice were extubated and catheters were removed 1 h after CPR, then placed in a cage maintained at 30 °C by a heat lamp for the following 2 hours.

2.4. Drug administration

AP39 was dissolved in 20% (v/v) dimethyl sulfoxide (DMSO) and administered in the volume of 10 μl *via* the left femoral vein. Equivalent volume of 20% (v/v) DMSO was administered as vehicle. AP39 or vehicle was administered 2 min before CPR in Experiment 1 and 1 min after ROSC in Experiment 2, respectively. Synthesis and structure of AP39 was described elsewhere [23].

2.5. Assessment of neurological function

Neurological function at 48 h after CA/CPR was assessed by a previously reported scoring system with minor modifications [12,24]. The system has five categories: consciousness, corneal reflex, respiration, coordination, and movement/activity. Each category was scored as 0 (impaired), 1 (mild impaired), or 2 (normal). Total score was reported as neurological function score.

2.6. Measurement of serum peroxide levels

Blood samples were collected 5 min after the initiation of CPR or sham surgery and the concentrations of hydrogen peroxide in serum were measured with QuantiChrom Peroxide Assay Kit (BioAssay Systems, Hayward, CA, USA), as previously described [12]. Sham operated mice received the same procedure without CA.

2.7. Assessment of neuronal degeneration

Neuronal degeneration in the brain cortex and caudoputamen (CPu) 48 h after CA/CPR was evaluated with the Fluoro-Jade B staining, as previously described [25]. The number of Fluoro-Jade B positive cells per 1 mm^2 in the brain cortex and caudoputamen were calculated.

2.8. Assessment of mitochondria permeability transition pore (mPTP) opening in brain cortical cells

To estimate the impact of AP39 treatment on mitochondrial integrity after CA/CPR, mPTP was evaluated in dissociated cortical cells collected from mice 6 h after CA/CPR or naïve mice, using calcein–cobalt quenching method as described previously [14]. Relative fluorescence intensity was then expressed as the ratio to the control values obtained from naïve mice.

2.9. Measurement of H_2S, thiosulfate, cysteine, and homocysteine concentrations in the brain cortex

To determine the impact of CA and AP39 treatment on H_2S metabolism, concentrations of H_2S, thiosulfate, cysteine, and homocysteine were measured in the brain cortex collected from mice 30 min after CA/CPR or naïve mice using high performance liquid chromatography (HPLC), as described previously [26,27]. In brief, mice were euthanized and perfused with ice-cold Tris–HCl buffer (pH 9.5, 0.1 mM diethylenetriamine pentaacetic acid). Brain without cerebellum was extracted, homogenized and centrifuged to obtain supernatants. Then supernatants were derivatized with a fluorescent labeling reagent monobromobimane and analyzed by HPLC with a fluorescence detector (Waters 2475 Multi λ fluorescence detector, Waters, Milford, MA) equipped with Agilent 258 Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, CA, USA) at wavelength of λex = 390 nm and λem = 475 nm.

2.10. Immunoblots

Brain cortex and liver tissue were obtained from mice 6 h after CA/CPR or naïve mice. Protein levels of cystathionine c-lyase (CSE),

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3-mercapropyruvate sulfurtransferase (3MST), and cystathionine b-synthase (CBS) in brain and liver homogenates were determined using standard immunoblot techniques using primary antibodies against CSE (1:1,000; Proteintech, Chicago, IL, USA), 3MST (1:1000; Sigma-Aldrich, St. Louis, MO, USA), CBS (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and GAPDH (1:10,000; Cell Signaling Technology, Inc., Danvers, MA, USA). Bound antibody was detected with a horseradish peroxidase-linked antibody directed against rabbit IgG (1:10,000; Cell Signaling Technology, Inc.) and was visualized using chemiluminescence with ECL Advance kit (GE Healthcare Bioscience, Pittsburgh, PA, USA).

2.11. Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). Parametric data were analyzed by one-way analysis of variance (ANOVA) with Newman–Keuls multiple comparison post hoc test, Bonferroni's post hoc test or two-way repeated measures ANOVA with Bonferroni's post hoc test. Differences in survival rates were analyzed by log-rank test. Neurological function scores were analyzed by *Kruskal–Wallis* test with Dunn's post hoc test. Numbers of Fluoro-Jade B positive cells were analyzed by unpaired t-test with Welch's correction. Probability values less than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Administration of AP39 before CPR improves neurological function and survival rate after CA/CPR (Experiment 1)

Arterial blood pressure at ROSC in mice treated with AP39 1000 nmol kg^{-1} was higher than in mice treated with AP39 10 nmol kg^{-1} (Supplementary Table S1). No mice died on and after 8 days after CA/CPR. Administration of AP39 at 100 or 1000 nmol kg^{-1}, but not at 10 nmol kg^{-1} at 2 min before CPR significantly improved survival rate at 10 days after CA/CPR (Fig. 1A). The neurological function score 48 h after CA/CPR in mice treated with AP39 at 100 or 1000 nmol kg^{-1} before CPR was significantly better than mice treated with vehicle (Fig. 1B).

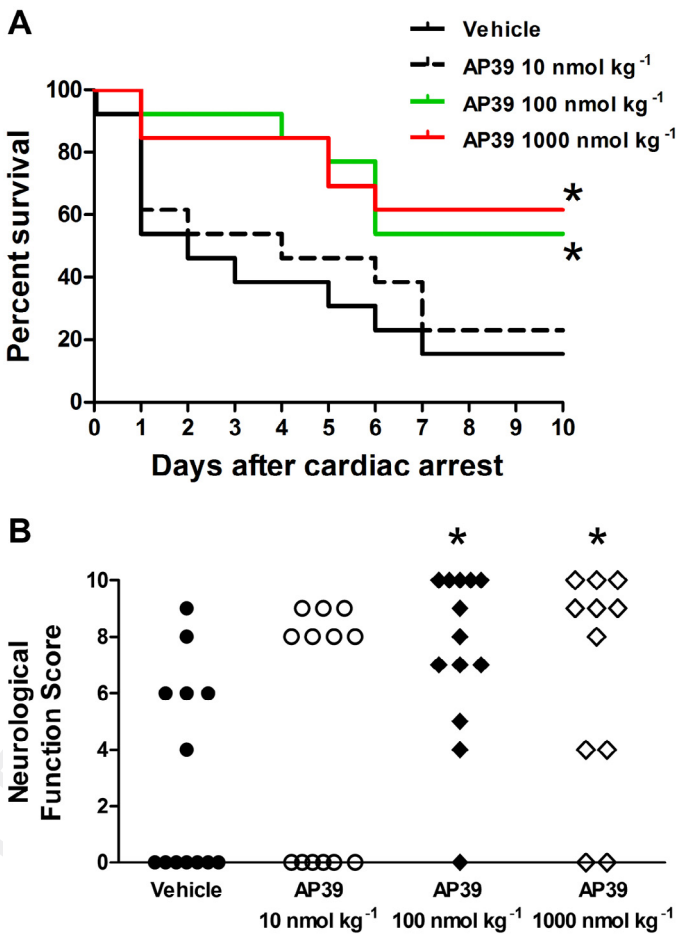

Fig. 1. Survival rates and neurological function scores in Experiment 1. n = 13 in each group. (A) Survival rate during the first 10 days after CA/CPR. *P = 0.01 versus Vehicle. (B) Neurological function scores at 48 h after CA/CPR. *P < 0.05 versus Vehicle. Vehicle = mice treated with vehicle 2 min before CPR. AP39 = mice treated with AP39 at 10, 100, or 1000 nmol kg^{-1} 2 min before CPR.

3.2. Administration of AP39 before CPR attenuates hydrogen peroxide generation in serum after CA/CPR

The concentrations of hydrogen peroxide in serum 5 min after CPR were increased in mice treated with Vehicle. Administration of 1000 nmol kg^{-1} AP39 before CPR significantly attenuated hydrogen peroxide levels in serum after CA/CPR (Fig. 2).

3.3. Administration of AP39 before CPR prevents neuronal degeneration in brain cortex and caudoputamen at 48 h after CA/CPR

The number of Fluoro-Jade B positive cells in the brain cortex and caudoputamen that indicates degenerated neurons were significantly increased in mice subjected to CA/CPR. Administration of AP39 at 1000 nmol kg^{-1} before CPR markedly decreased the number of Fluoro-Jade B positive cells compared to mice treated with vehicle (Fig. 3A and B).

3.4. Administration of AP39 before CPR inhibits mPTP opening in the brain mitochondria at 6 h after CA/CPR

Fluorescence intensity in mice treated with vehicle was significantly decreased 6 h after CA/CPR compared to naïve mice, suggesting that mPTP was opened after CA/CPR. In contrast, fluorescence intensity in mice treated with AP39 at 1000 nmol kg^{-1} before CPR was significantly increased compared to mice treated with vehicle, suggesting that AP39 inhibited mPTP opening after CA/CPR (Fig. 4).

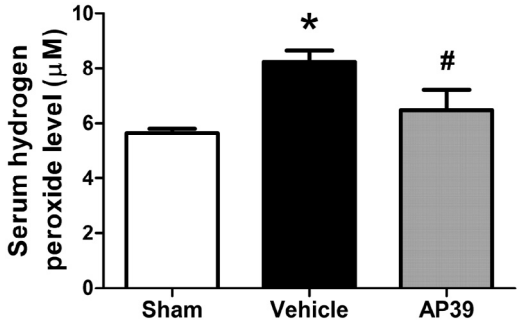

Fig. 2. Serum hydrogen peroxide levels 5 minutes after CPR. n = 6 in each group. *P < 0.01 versus Sham. #P < 0.05 versus Vehicle. Sham = mice received sham procedure without cardiac arrest. Vehicle = mice treated with vehicle 2 min before CPR. AP39 = mice treated with AP39 at 1000 nmol kg^{-1} 2 min before CPR.

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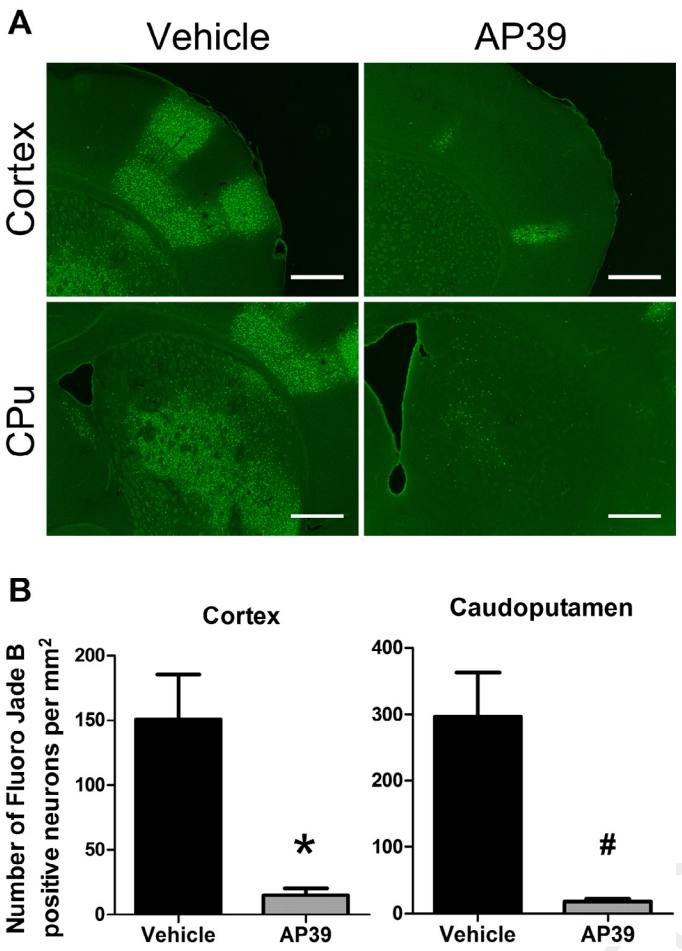

Fig. 3. (A) Comparison of Fluoro-Jade B positive cells in the brain cortex and caudoputamen 48 h after CA/CPR. Scale bar = 500 μm. (B) Number of Fluoro-Jade B positive cells per 1 mm^2 in the brain cortex and caudoputamen. n = 4 in each group. *P = 0.03 versus Vehicle. #P = 0.02 versus Vehicle. Vehicle = mice treated with vehicle 2 min before CPR. AP39 = mice treated with AP39 at 1000 nmol kg^{-1} 2 min before CPR.

3.5. Administration of AP39 after ROSC improves neurological function and survival rate after CA/CPR (Experiment 2)

Administration of AP39 at 10 nmol kg^{-1}, but not at 1000 nmol kg^{-1}, 1 min after ROSC significantly improved the survival rate at 10 days after CA/CPR (Fig. 5A). The neurological function score 48 h after CA/CPR in mice treated with 10 nmol kg^{-1} AP39 after ROSC was significantly better than mice treated with vehicle (Fig. 5B).

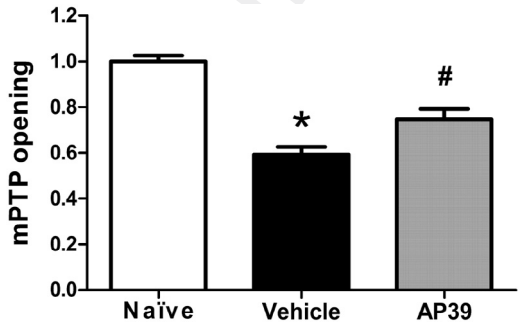

Fig. 4. mPTP opening in brain cortex 6 h after CA/CPR. n = 4 in each group. *P < 0.001 versus Naïve. #P < 0.05 versus Vehicle. Naïve = untreated naïve mice. Vehicle = mice treated with vehicle 2 min before CPR. AP39 = mice treated with AP39 at 1000 nmol kg^{-1} 2 min before CPR.

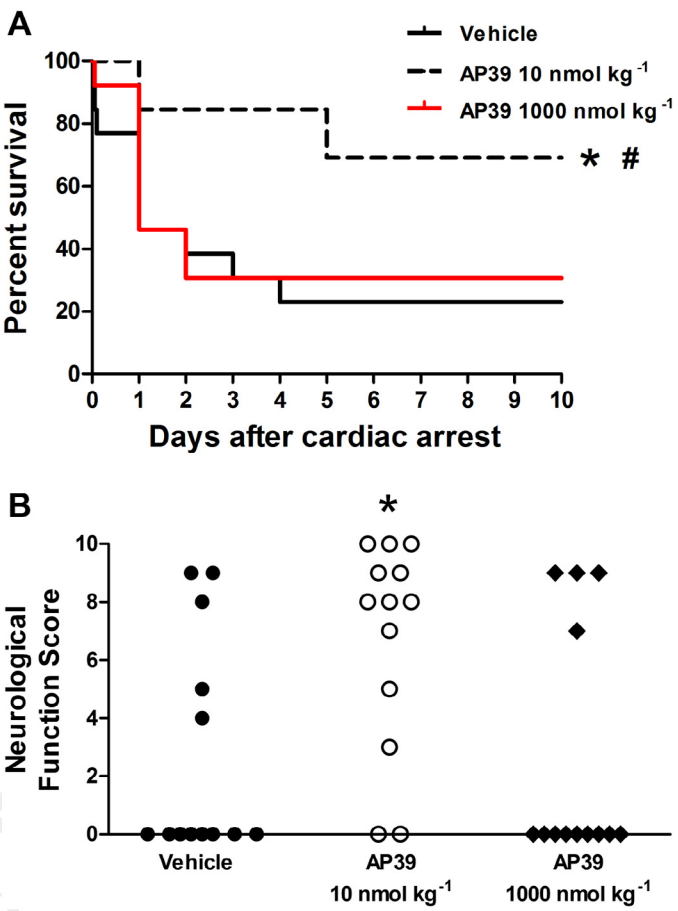

Fig. 5. Survival rates and neurological function scores in Experiment 2. n = 13 in each group. (A) Survival rate during the first 10 days after CA/CPR. *P = 0.006 versus Vehicle. #P = 0.03 versus AP39 1000 nmol kg^{-1}. (B) Neurological function scores 48 h after CA/CPR. *P < 0.05 versus Vehicle and AP39 1000 nmol kg^{-1}. Vehicle = mice treated with vehicle 1 min after ROSC. AP39 = mice treated with AP39 at 10 or 1000 nmol kg^{-1} 1 min after ROSC.

3.6. Brain H_2S concentration is decreased immediately after CA/CPR

H_2S and thiosulfate concentrations in the brain 30 min after CA/CPR were significantly decreased in mice treated with vehicle compared to naïve mice. Administration of AP39 at 1000 nmol kg^{-1} before CPR or 10 nmol kg^{-1} after ROSC restored brain H_2S concentration to the baseline levels observed in naïve mice (Fig. 6A). Similarly the brain thiosulfate levels were restored by administration of AP39 at 10 nmol kg^{-1} after ROSC and markedly increased by administration of AP39 at 1000 nmol kg^{-1} before CPR (Fig. 6B). Whereas CA/CPR without or with AP39 treatment did not affect the levels of cysteine and homocysteine (Fig. 6C and D).

3.7. Protein expression levels of CSE, 3MST, and CBS in the brain and liver 6 h after CA/CPR

To elucidate the mechanisms responsible for the altered sulfide metabolism, we examined the protein expression levels of CSE, 3MST, and CBS in the brain and liver at baseline and 6 h after CA/CPR. The protein expression levels of CSE, 3MST, and CBS in the brain and liver did not differ among naïve mice and CA/CPR mice treated with vehicle or AP39 (Supplementary Fig. S1).

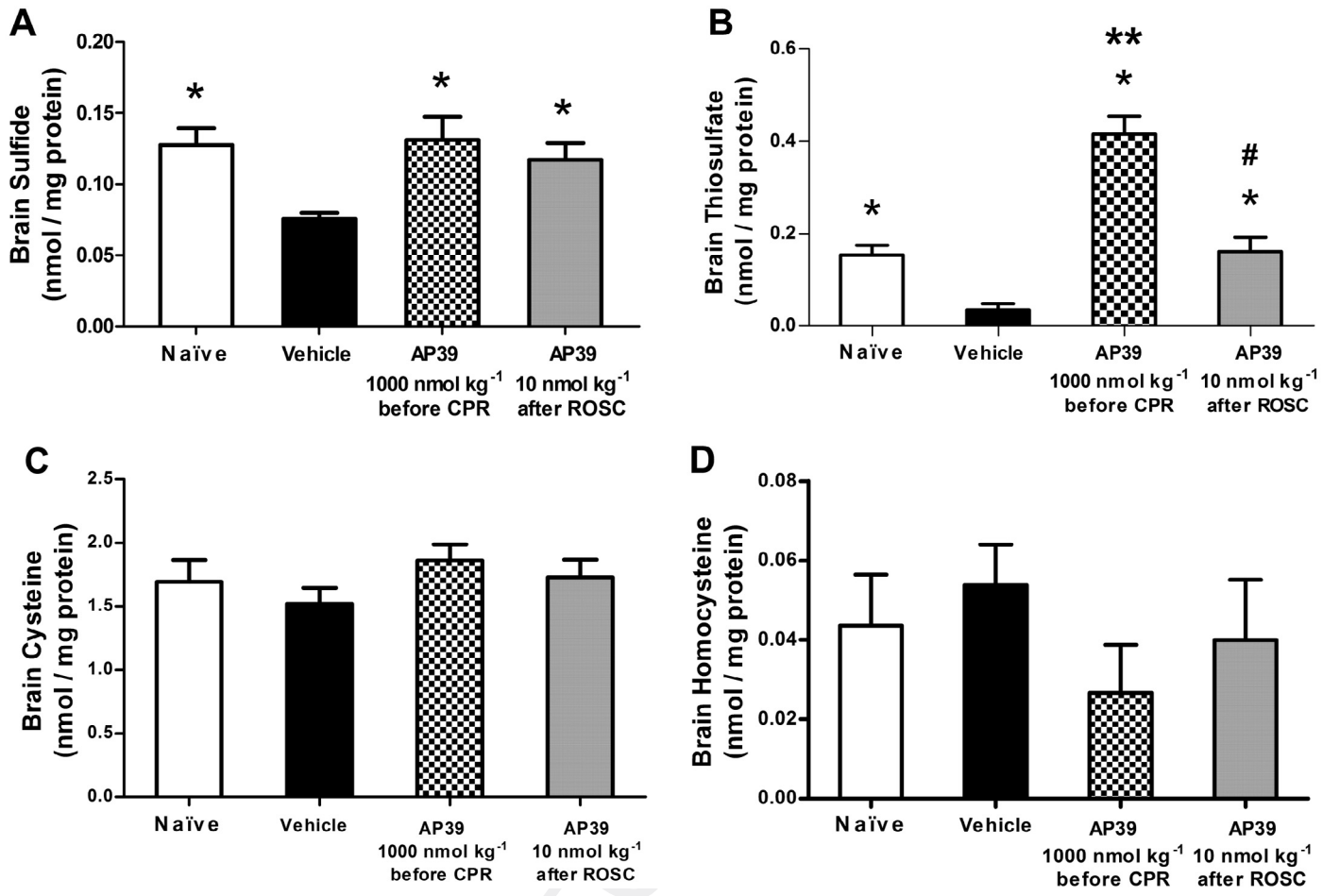

Fig. 6. Concentrations of H_2S (A), thiosulfate (B), cysteine (C), and homosysteine (D) in the brain cortex 30 min after CA/CPR. n = 7 in each group. *P < 0.05 versus Vehicle. **P < 0.05 versus Naïve. #P < 0.05 versus AP39 1000 nmol kg^{-1}. Naïve = untreated naïve mice. Vehicle = mice treated with vehicle 2 min before CPR. AP39 = mice treated with AP39 at 1000 nmol kg^{-1} 2 min before CPR or 10 nmol kg^{-1} 1 min after ROSC.

4. Discussion

The current study shows that administration of the mitochondrial-targeted H_2S donor compound AP39 exerts neuroprotective effect after CA/CPR. The effectiveness of H_2S on the outcome following CA/CPR is controversial. We previously reported that administration of Na_2S at 7 μmol kg^{-1} markedly improved the neurological outcomes and survival rate in mice subjected to CA/CPR [11,12]. In contrast Derwall et al. reported that administration of Na_2S at 3.8 μmol kg^{-1} bolus followed by infusion at 3.8 μmol kg^{-1} h^{-1} or at 12.8 μmol kg^{-1} bolus followed by infusion at 12.8 μmol kg^{-1} h^{-1} did not improve the survival rates and neurological function in swine [15]. Knapp and colleagues reported that administration of Na_2S at 6.4 μmol kg^{-1} bolus followed by infusion at 12.8 μmol kg^{-1} h^{-1} reduced the sensorimotor deficits 72 h after CA/CPR in rats without improving survival rate [16]. The discrepancy of these reports may be related to the differences in dosage of Na_2S, timing of administration, severity of ischemia, and animal species. In particular, markedly higher doses of Na_2S were used by the studies by Derwall (total dose 38 μmol kg^{-1}) and Knapp (total dose 83 μmol kg^{-1}) compared to our studies with Na_2S in mice (total dose 7 μmol kg^{-1}).

It is important to note that the doses of AP39 required to improve outcomes after CA/CPR were 3-orders of magnitude smaller than the doses of Na_2S found effective in the treatment of experimental I/R injury including CA/CPR [11,14]. Since one mole of both AP39 and Na_2S release one mole of H_2S, this translates that sulfide concentration in the target tissue (e.g., brain) is likely to be 3-orders of magnitude smaller after administration of AP39 than after Na_2S. It is well-known that high concentration of H_2S is neurotoxic [28]. Simple sulfide salts such as Na_2S spontaneously releases high concentrations of sulfide immediately upon administration. It is also of note that administration of low dose AP39 after ROSC markedly improved outcomes after CA/CPR, whereas post-CPR administration of Na_2S was ineffective [12]. From the standpoint of translation, effectiveness as a post treatment is highly clinically relevant.

Mitochondria generate ROS under hypoxic conditions [7,29] and are both an origin and a target of cytotoxicity in I/R injury. It has been reported that AP39 increased H_2S generation in the mitochondria, protected *mitochondrial membrane potential* and inhibited oxidative-stress induced cell death in cultured human endothelial cells [21,23]. In our experiments, administration of AP39 before CPR also attenuated the increase of hydrogen peroxide levels in serum after reperfusion (Fig. 2). These results suggest that AP39 treatment reduces neuronal damage through attenuation of oxidative stress *in vivo*, possibly *via* protection of mitochondrial integrity.

The mitochondrial permeability transition pore (mPTP) opening results in mitochondrial dysfunction and leads to apoptotic or necrotic cell death [30]. Although we previously found that Na_2S prevented mPTP opening of cardiac mitochondria after CA/CPR, Na_2S failed to protect the brain mitochondria [12]. The inhibitory effects of AP39 on the CA-induced mPTP opening in the brain mitochondria suggest that AP39 can permeate BBB after CA/CPR, although

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the permeability of AP39 across BBB remains to be formally examined.

It has been reported that myocardial H_2S levels were markedly reduced after myocardial ischemia and reperfusion injury [31]. Similarly, we found that brain levels of H_2S and thiosulfate, but not cysteine and homocysteine, were markedly decreased 30 min after CA/CPR in the current study. We also observed that protein expression levels of CSE, CBS, and 3MST were not altered in the brain and liver after CA/CPR. These results suggest that CA/CPR-induced reduction in H_2S levels is not due to increased oxidation of H_2S to thiosulfate or depressed H_2S synthesis via transsulfuration enzymes. Although the precise mechanism responsible for the reduction of brain H_2S levels after CA/CPR remains to be determined, maintaining physiological levels of H_2S may be critical for cell survival [31,32]. It is of note that the brain H_2S concentrations achieved by administration of AP39 at 1000 nmol kg^{-1} before CPR or 10 nmol kg^{-1} after ROSC were equivalent. The reason why two differing doses of AP39 achieved similar brain H_2S levels may be attributable to the organ distribution of TPP^+ conjugated compounds. It is likely that significant portion of AP39, especially administered before CPR, is rapidly taken up into other organs including kidney, liver, heart and fat before reaching the brain after intravenous injection during CA [33].

In mitochondrial respiratory chain, high concentrations of H_2S inhibit cytochrome oxidase, resulting in a shutdown of mitochondrial electron transport and adenosine triphosphate (ATP) generation. However, at lower concentrations, H_2S has been shown to accelerate ATP generation by acting as a stimulator of mitochondrial electron transport [34]. In brain microvascular endothelial cells, AP39 increased oxygen consumption ratio at 30 and 100 nM, but not at 300 nM, indicating the acceleration of electron transport at lower doses [21]. It is tempting to speculate that the better survival and neurological function after administration of AP39 at a low-dose, but not at high-dose, after CPR, were associated with an acceleration of mitochondrial ATP generation on demand after reoxygenation.

The current study was designed to determine whether or not AP39 improves neurological outcomes in a clinically relevant mouse model of CA/CPR. It is possible that AP39 directly protects neuronal integrity, as suggested by our current results, but it is also conceivable that AP39 prevents cardiovascular dysfunction after CA/CPR thereby improves neurological outcomes via improved cerebral perfusion. Further investigations are needed to establish the mechanisms accounting for the protective effects of AP39 after CA/CPR.

5. Conclusions

In summary, we observed potent neuroprotective effects of the mitochondrial-targeted H_2S donor compounds AP39 in a mouse model of CA/CPR. The dose of AP39 required to markedly improve survival after CA/CPR in mice was 3 orders of magnitude smaller than the dose of conventional H_2S generating compound, Na_2S. The beneficial effects of AP39 were associated with preservation of mitochondrial integrity and reduced oxidative stress. The effectiveness of AP39 administered after ROSC may be highly clinically relevant and warrants further investigation.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.niox.2015.05.001.

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