PD98059 Protects Cerebral CortexMitochondrial Structure and Function at 48 h Post-Resuscitation in a Rat Model of Cardiac Arrest

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Background: Mitochondria play a critical role as effectors and targets of brain injury in the post-resuscitation period. Although we found previously that the extracellular signal-regulated kinase (ERK)1/2 inhibitor PD98059 (PD) protects the brain against mitochondrial-mediated cell death at 24 h post-resuscitation in rats subjected to cardiac arrest/cardiopulmonary resuscitation (CA/CPR), it is not clear whether PD also exerts mitochondrial protective effect for a lasting time. Therefore, we examined the effect of PD on brain mitochondria at 48 h post-resuscitation to evaluate the time-effect of PD in the current study.

Methods: Experimental rats were divided randomly into 5 groups: Sham, CA, dimethylsulfoxide (DMSO), 0.15 mg/kg PD and 0.3 mg/kg PD. Rats except for sham group were subjected to CA for 6 min followed by CPR. We detected survival rates and neurologic deficit scores, cerebral cortex mitochondrial function by evaluating adenosine triphosphate (ATP) levels, mitochondrial permeability transition pore (MPTP) opening, and the expression of mitofusin2 (Mfn2) and observing the ultrastructure by electron microscopy at 48 h post-resuscitation in a 6-min CA rat model.

Results: PD improved survival rates and neurologic deficit scores, alleviated cerebral cortex mitochondrial damage by reducing MPTP opening and increasing Mfn2 production at 48 h post-resuscitation in a 6-min CA rat model.

Conclusion: A single dose of PD improved 48 h post-resuscitation outcome and mitochondrial function, indicating the potential of the use of ERK inhibitors for the treatment of brain injury resulting from CA in the future.

Keywords: cardiac arrest, extracellular signal-regulated kinase, mitochondria, mitofusin2, mitochondrial permeability transition pore

Introduction

Brain injury is a major cause of high morbidity and mortality rates in the post-cardiac arrest period. 1 The mechanism of brain injury after resuscitation is global cerebral ischemia-reperfusion injury (IRI). A complex cascade of processes, which includes oxidative stress, calcium overload, excitotoxicity, cascade reactions of pathological proteases, and the activation of cell death signaling pathways, occurs after reperfusion. Mitochondria are involved in a myriad of complex signaling cascades that regulate cell death vs survival. Mitochondria play a critical role as effectors and targets of IRI. 2,3

The mitochondrial permeability transition pore (MPTP) is a nonspecific pore in the inner mitochondrial membrane. The MPTP is triggered by both cellular calcium
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Mitofusin2 (Mfn2), a mitochondrial
Based on the fine balance between mitochondrial fusion and fission in cells may be affected by I/R injury. An imbalance in mitochondrial dynamics has been shown to contribute to brain injury during I/R injury. Mitofusin2 (Mfn2), a mitochondrial outer membrane protein, is a key regulator of mitochondrial fusion and mitochondrial metabolism. Marked Mfn2 release observed during the early stages of reperfusion may thus represent an important mechanism of mitochondrial-mediated cell death at 24 h post-resuscitation in neurologic outcomes and protected the brain against mitochondrial dysfunction and regulating cell survival. In our previous research, we found that the ERK1/2 inhibitor PD98059 improved neurologic outcomes and protected the brain against mitochondrial-mediated cell death at 24 h post-resuscitation in rats subjected to CA/CPR.

Now, we detect PD98095 effects in 48 h post-resuscitation for a dose of PD98059 infection can last the brain protective effect longer, so that it is need to evaluate its time-effect. Now, we detect PD98095 effects in 48 h post-resuscitation rats, including survival rates, neurologic deficit scores, cerebral mitochondrial function and ultrastructure evaluated by adenosine triphosphate (ATP) levels, MPTP opening, and the expression of Mfn2 and electron microscopy.

Methods
Animal Preparation
Male Sprague-Dawley rats (210–270 g), aged 6 to 8 weeks and provided by the Experimental Animal Center of Guangxi Medical University (China, Nanning), were cared for according to the guidelines for the Care and Use of Laboratory Animals, and their use was approved by the animal ethics committee of Guangxi Medical University. The experiments were also conducted in a manner that addressed the key elements of the ARRIVE guidelines.

Animal Grouping
One hundred fifty-one rats were randomized into five groups: (1) the sham operation group (sham, n=11), which were subjected to the same procedures as the other groups except CA and CPR (2) the saline group (CA, n=35), which underwent 6 min of CA and received femoral vein injection of saline after resuscitation; (3) the dimethyl sulfoxide (DMSO) group (DMSO, n=35), which underwent 6 min of CA and received femoral vein injection of 5% DMSO after resuscitation; (4) the 0.15 mg/kg PD98059 group (PD0.15, n=35), which underwent 6 min of CA and received femoral vein injection of 0.15 mg/kg PD98059 after resuscitation; and (5) the 0.3 mg/kg PD98059 group (PD0.3, n=35), which underwent 6 min of CA and received femoral vein injection of 0.3 mg/kg PD98059 after resuscitation.

Animal CPR Model
CA was induced by alternating current (12 V) from a stimulator (Chengdu Technology & Market Co. Ltd., China) through a pacing electrode placed in the esophagus; this procedure was described in detail previously. Briefly, CA was defined as a mean aortic pressure (MAP) ≤ 20 mmHg. After 6 min of CA, CPR, including manual chest compression and mechanical ventilation with air, was started. After 1 min of CPR, one dose of epinephrine (20 μg/kg) was given through a left femoral vein catheter. Restoration of spontaneous circulation (ROSC) was defined as an organized cardiac rhythm with a mean aortic pressure (MAP) > 60 mmHg for ≥ 1 min. When there was a failure of ROSC after 3 min, resuscitation efforts were discontinued. After successful resuscitation, the animals were immediately injected with saline, 5% DMSO, 0.15 mg/kg PD98059 or 0.3 mg/kg PD98059. From anesthesia to awakening, the rectal temperature of the rats was monitored continuously and maintained by a heating lamp at 37.0±0.5°C. After the rats awoke, they were returned to their cages in an air-conditioned and peaceful room (room temperature, 27°C) and given free access to water and food.

Survival Observation and Neurological Evaluation
Survival rate and neurologic deficits were measured at 48 h post-resuscitation. Neurologic deficits were scored from 0 (death or brain death) to 80 (no observed neurologic deficit).

Brain Tissue Preparation
Eleven surviving rats in each group were anaesthetized using pentobarbital (60 mg/kg, i.p.) after the evaluation of neurologic deficits at 48 h post-resuscitation. The cerebral cortices of 8 rats were used to measure ATP, for

overload and oxidative stress, which occur during IR. Oxidative stress-induced MPTP opening leads to the destruction of the mitochondrial membrane potential, the inhibition of ATP synthesis, and ultimately cell death.
mitochondria isolation to detect MPTP opening and to determine protein content by immunoblotting. The remaining three surviving rats in each group were perfused with 2.5% glutaraldehyde and 4% paraformaldehyde through the left ventricle of the heart, and a fraction of the left cerebral cortex was extracted to observe the mitochondria ultrastructure by electron microscopy.

**Measurement of ATP Levels**

An ATP assay kit (Beyotime Biotechnology, China) was used to measure ATP levels in the cerebral cortex. Briefly, we harvested and homogenized cerebral cortices in an ice-cold ATP-releasing buffer and then centrifuged them at 12,000 × g for 5 min. After centrifugation, we transferred the supernatant to a new test tube to detect ATP levels. Then, we measured the luminescence of 60 μL of sample and 100 μL of ATP detection buffer with a luminometer (BIOTEK Synergy H1, USA) and normalized the protein concentration.

**Isolation of Mitochondria**

Mitochondria were isolated from the rat cerebral cortex with commercial kits (Genmed Scientific Inc., USA, 10006.2). Brain tissues were crushed on ice in 1.5 mL of ice-cold isolation medium and then manually homogenized using a glass homogenizer. The brain homogenate (10%) in the glass homogenizer was centrifuged at 1500 × g at 4°C for 10 min. The pellet was re-suspended and centrifuged at 10,000 × g at 4°C for 10 min, and the mitochondria were isolated. The mitochondrial protein concentration was detected by the Bradford method. Mitochondria were kept on ice to measure MPTP opening.

**MPTP Opening**

MPTP opening was monitored by coloading cells with calcein/AM and CoCl₂. Detailed instructions can be found in the MPTP assay kit (Genmed Scientific, Inc., Shanghai, P. R. China). Calcein fluorescence intensity was measured using a monochromator microplate reader with an excitation of 488 nm and emission of 505 nm (Safire II, Tecan, Switzerland).

**Observation of Mitochondrial Ultrastructure Using Electron Microscopy**

A fraction of the left cerebral cortex layer near the forehead was isolated and immediately fixed with 1% osmium tetroxide and then dehydrated and embedded in epoxy resin. According to the standard principle of three-dimensional localization, 100-nm sections were randomly cut regardless of their orientation, mounted on copper mesh, double stained with lead citrate and uranyl acetate, and then placed under a transmission electron microscope to observe the ultrastructure. Images were taken and examined under an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).

Mitochondrial injury was assessed blindly using a scoring system. Briefly, five grids from each group and four fields per grid were randomly selected. The standards for the evaluation of mitochondrial injury from grade 0 to 4 were as follows: grade 0, normal mitochondria with highly dense and well-organized cristae; grade 1, early tumid mitochondria with large amorphous matrix and linear densities and separation of cristae; grade 2, more marked tumid mitochondria with further clearing of matrix density and separation of cristae; grade 3, more extensive tumid mitochondria with disruption of cristae; grade 4, severe tumid mitochondria with disruption of cristae and rupture of the inner and outer mitochondrial membranes.

**Western Blot Analysis**

The prepared cortical area tissues were weighed and homogenized with a glass homogenizer in 1:10 (w/v) ice-cold whole-cell lysis buffer (Beyotime Biotechnology, China, P0013B). Soluble proteins were collected and centrifuged at 14,000 × g for 15 min at 4°C. The total protein concentration of the tissue was determined by a BCA Protein Assay Reagent kit (P0010). A total of 10 μL of total protein lysate of each tissue was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins on the gels were transferred to a PVDF membrane (Millipore, USA, 0.22 μm pore diameter). The membrane was blocked with 5% bovine serum albumin for 1 h and then incubated overnight at 4°C with corresponding primary antibodies. The primary antibodies were as follows: GAPDH (ab181602), ERK 1/2 (ab184699), phosphorylated ERK1/2 (purchased from Abcam Plc, Cambridge, UK) and Mfn2 (11925) (purchased from Cell Signaling Technology, Danvers, MA). Afterwards, the membrane was incubated with secondary antibody (Cell Signaling Technology, USA, #5151, 1:15,000) and quantified using a Western blotting detection system with a Li-cor Odyssey Scanner imaging densitometer. Finally, the detected bands were quantified with ImageJ software (v1.33, NIH, Bethesda, MD, USA).
Statistical Analysis

The data are presented as the mean ± standard deviation unless otherwise stated. Comparisons of the following indicators among multiple groups were made by using one-way ANOVA: baseline parameters, CPR duration, ATP levels, MPTP opening and the content of various proteins. The Kruskal–Wallis Test was used for comparisons of survival rate, neurologic deficit scores and mitochondrial injury scores among all groups. The statistical software SPSS 21.0 was used. A P value <0.05 was considered statistically significant.

Results

1. Baseline parameters.

There were no significant differences among the groups in baseline parameters, including body weight (BW), heart rate (HR), systolic pressure (SP), diastolic pressure (DP), and mean arterial pressure (MAP), before CA induction (P >0.05) (Table 1).

2. PD98059 improved survival rates and neurologic deficit scores (NDSs) at 48 h post-resuscitation.

As shown in Table 2, the survival rate in the PD groups was higher than that in the CA group at 48 h post-resuscitation (P<0.001). Furthermore, the NDSs in the PD groups, especially in the 0.3 mg/kg PD group, were higher than those in the CA group and DMSO group (CA, DMSO, P<0.001). However, there were no significant differences among the groups in CPR duration. This finding suggests that PD98059 does lessen the damage induced by CA/CPR in the experimental animals.

Table 1 Baseline Parameters

| Group   | n  | BW (g)           | HR (Beats/Min) | SP (mmHg) | DP (mmHg) | MAP (mmHg) |
|---------|----|-----------------|----------------|-----------|-----------|------------|
| Sham    | 11 | 238.59±17.93    | 419.95±21.04   | 121.64±9.78 | 94.41±7.73 | 99.88±9.68 |
| CPR     | 35 | 238.98±18.36    | 421.89±22.40   | 122.74±11.05 | 93.26±10.31 | 98.59±11.41 |
| DMSO    | 35 | 239.99±20.24    | 420.57±29.82   | 120.48±13.02 | 92.83±10.95 | 98.77±10.36 |
| 0.15 mg/kg PD | 35 | 240.12±15.85   | 423.17±26.21   | 123.50±11.37 | 93.63±9.84  | 99.35±10.86  |
| 0.3 mg/kg PD | 35 | 241.32±18.67   | 422.80±23.94   | 123.51±10.42 | 92.66±8.73  | 99±8.96     |

Note: All data above are presented as the mean ± standard deviation.
Abbreviations: BW, body weight; HR, heart rate; SP, systolic pressure; DP, diastolic pressure; MAP, mean arterial pressure.

Table 2 Survival Rates and NDSs at 48 h Post-Resuscitation

| Group   | CPR Duration (s) | Survival Rate (48 h) | NDS (48 h) |
|---------|-----------------|----------------------|------------|
| Sham    | –               | 11/11 (100%)         | 80 (79.80) (n=11) |
| CA      | 86.27±13.81 (n=11) | 11/35 (31.43%)***  | 69 (67.72)*** (n=11) |
| DMSO    | 83.72±12.63 (n=19) | 19/35 (54.29%)**    | 70 (68.72)** (n=19) |
| 0.15 mg/kg PD | 81.82±14.96 (n=26) | 26/35 (74.29%)**## | 74 (72.76)## (n=26) |
| 0.3 mg/kg PD | 84.75±11.28 (n=29) | 29/35 (82.86%)#### | 78 (78.80)###### (n=29) |

Notes: Survival rates are shown as ratios, NDSs are presented as the median± interquartile range, and CPR duration is presented as the mean ± standard deviation.
***P<0.01, **P<0.001 versus the sham group; *P<0.05 versus the CA group; @P<0.05 versus the DMSO group; #P<0.05 versus the PD0.15 group.
Abbreviations: CPR, cardiopulmonary resuscitation; NDSs, neurologic deficit scores.
3. PD98059 significantly elevated ATP in a dose-dependent manner at 48 h post-resuscitation in the cerebral cortex following CA/CPR.

As shown in Figure 1, the ATP level in the CA group was significantly lower than that in the sham group (P<0.01), and there were no significant differences in the ATP level between the PD0.15, DMSO and CA groups (P>0.05). However, the ATP level in the PD0.3 group was higher than that in the other treated groups (P<0.05). Therefore, ATP levels significantly increased in a dose-dependent manner upon PD98059 treatment.

4. Mitochondrial ultrastructure

As shown in Figure 2A, normal mitochondrial morphology with no evidence of swelling, outer membrane breaks, or intracristal dilation was observed in the cerebral cortex of

**Figure 2** Mitochondrial ultrastructure and injury scores in the cerebral cortex in each of the groups. (A) Representative electron photomicrographs of mitochondria from the brain cortex. Images were acquired with an electron microscope at 30000x (scale bar, 1 μm). A 70000x closeup of the image in the left panel is shown (scale bar, 500 nm). There was mitochondria normal ultrastructure in the sham control animals (a, b). Severe mitochondrial swelling, chondrolysis and vacuolization of cristae with a concomitant loss of membrane integrity were observed at 48 h post-resuscitation (c, d) in the CA group. More extensive tumid mitochondria with disrupted cristae were observed in the DMSO group (e, f) than in the PD0.15 group (g, h), but in the PD0.3 group, a range of small ultrastructural injuries were observed (i, j) (the arrows represent mitochondria). (B) Mitochondrial injury scores in brain cortex in each of the groups. The mitochondrial injury score in the CA group was significantly higher than that in the sham group (P<0.001), but in the PD0.3 group, the mitochondrial injury score was lower than that in the other treatment groups. All data above are presented as the median ± interquartile range (n=3 in each group). ###P<0.001 versus the sham group; ####P<0.001 versus the CA group; $$$P<0.001 versus the DMSO group; $$$$P<0.001 versus the PD0.15 group.
the sham group (a’, b’). In the CA group, disruption of cristae and rupture of the inner and outer membranes, which were the most serious injuries, were observed in the mitochondria (c’, d’). In the DMSO (e’, f’) and PD0.15 groups (g’, h’), compared to the sham group, there were more serious injuries with more extensive mitochondrial swelling. However, more mild mitochondrial ultrastructural damage was observed in the PD treatment groups, especially in the PD0.3 group (i, j).

These observations were confirmed by mitochondrial injury scores, which were determined based on mitochondrial ultrastructural analyses (Figure 2B) using the standards described in the Methods section.

5. PD98059 reduced the opening of the MPTP in a dose-dependent manner at 48 h post-resuscitation in the cortex of the rats following CA/CPR. As shown in Figure 3, the fluorescence intensity to which MPTP opening was inversely proportional in the CA and DMSO groups was significantly lower than that in the sham group (CA, DMSO, P<0.01). However, the fluorescence intensity in the PD0.3 group was significantly higher than that in the CA and DMSO groups (CA, DMSO, P<0.05), which suggested that MPTP opening in the PD0.3 group was decreased compared to that in the other treatment groups.

6. PD98059 significantly increased Mfn2 generation in a dose-dependent manner at 48 h post-resuscitation in the cerebral cortex following CA/CPR.

7. PD98059 decreased the phosphorylation of ERK1/2 (P-ERK1/2) in a dose-dependent manner, but the total protein levels of ERK1/2 (ERK) were not changed at 48 h post-resuscitation in the cerebral cortex following CA/CPR (Figure 5).

The phosphorylation level of ERK1/2 was significantly increased at 48 h post-resuscitation compared to that in the sham group (P<0.05), as determined by Western blot analysis, but the phosphorylation level of ERK1/2 was reduced in the PD98059-treatment groups, especially in the PD0.3 group, compared to the other treatment groups (CA, P<0.01; DMSO, P<0.01; PD0.15, P<0.05).

Discussion

We demonstrated that PD98059 improved post-resuscitation outcome, as well as cerebral cortex mitochondrial function and structure at 48 h CA/CPR as the previous study at 24 h,12 presenting a time-effect on a dose.

Mfn2 mediates mitochondrial fusion helping with energy generation. Mitochondria are highly dynamic organelles. Mitochondrial shape and function are highly regulated by mitochondrial dynamics, which involve sequential rounds of fusion and fission. Mfn2 is a central protein that is
located in the mitochondrial outer membrane and is involved in mitochondrial elongation of mitochondrial structures and leads to an increase in ATP production. In our study, it was confirmed that an increase in Mfn2 accompanying with an increase in ATP. Some previous studies have indicated that Mfn2-mediated mitochondrial fusion is associated with the ERK signaling pathway. Gan et al. revealed that the blockade of the mild cognitive impairment (MCI)-related stress-mediated activation of ERK signaling not only attenuates aberrant mitochondrial morphology and function but also restores mitochondrial fission and fusion balance, but the overexpression of Mfn2 is inhibited. Oppositely, in our study, the inhibition of the ERK signaling pathway improved mitochondrial function and structure and upregulated Mfn2 generation in the rat cerebral cortex at 48 h post-resuscitation. Other study also reported that the inhibition of the MEK-dependent signaling pathway elevates the Mfn2 protein level in differentiating muscle cells. From the above mentioned, the expression of Mfn2 is inconsistent after ERK inhibition. We consider the inconsistent results coming from different experimental models and pathological stages.

The MPTP is a critical mediator of cell death induced by IRI. Numerous studies have demonstrated that mitochondrial dysfunction results from the opening of the MPTP in I/R injury. MPTP is a nonselective conductance pore located in the inner mitochondrial membrane. The opening of the MPTP results in the impairment of mitochondrial function and structure, such as the collapse of the mitochondrial membrane potential (ΔΨm), the uncoupling of oxidative phosphorylation, mitochondrial swelling and outer membrane rupture. Finally, cell death occurs due to ATP depletion. An association between the activation of ERK1/2 pathways and the inhibition of MPTP opening was previously suggested. The activation of ERK1/2 signaling stimulates MPTP downstream, enhances cardiomyocyte survival and reduces mortality and mortality following I/R injury. These results indicate that MPTP is associated with the ERK signaling pathway. In our study, the inhibition of the ERK signaling pathway led to a decrease in MPTP opening. This suggests that the ERK signaling pathway has different effects on the MPTP in different pathological process. However, the specific reason is not clear. It has been reported that the actual mechanism through which the activation of ERK1/2 mediates MPTP inhibition is in part be due to downstream targets such as GSK-3β, the modulation of intracellular calcium regulation, eNOS, or changes in mitochondrial morphology. As mentioned above, the mechanism by which the ERK signaling pathway affects the MPTP is very complex, and this complexity leads to inconsistent effects in different disease models.

Mfn2 is associated with the opening of the MPTP. Neuspiel et al. found that the overexpression of Mfn2 prevents MPTP opening induced by free radicals in COS-7 cells. Ong et al. also reported that the overexpression of Mfn2 reduces MPTP opening induced by I/R injury in the HL-1 cardiac cell line. Cells with Mfn2 depletion show increased Ca²⁺ transfer from the ER to mitochondria. So, it is considered that Mfn2 accommodates mitochondrial calcium load to down-regulate MPTP opening. From that, PD98059 induced-Mfn2 expression promoted MPTP inhibition.
In our study, DMSO was used as a solvent for PD98059, and considering its pharmacological effects, we set up DMSO group to eliminate evaluation deviation. Compared to CA group, the survival rate and neurological function score of DMSO group were no significant differences. However, the survival rate and neurological function score of PD treated group is significantly higher than the CA group. Therefore, DMSO did not show obvious action, the improved outcomes were mainly depend on PD98059.

Although the present study demonstrates both Mfn2 and MPTP involved in the process of PD98059 improving cerebral cortex mitochondrial function and structure. However, we did detect the relating Mfn2 gene expression regulated by ERK inhibition, as well as, the Ca\(^{2+}\) load in mitochondria which may be the connection of Mfn2 and MPTP. It needs further studies to reveal.

In conclusion, a dose of PD98059 delivery improves 48 h post-resuscitation outcomes and mitochondrial functions, indicating the potential use of ERK inhibitors for the treatment of brain injury resulting from CA in the future.

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**Author Contributions**

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

**Disclosure**

The authors have no economic and noneconomic conflicts of interests to disclose.

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