

Cardiopulmonary resuscitation may not stop glutamate release in the cerebral cortex

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Running head: Neuroprotective effects of CPR

Abstract

Background: Cardiopulmonary resuscitation (CPR) may not be sufficient to halt the progression of brain damage. Therefore, we quantitatively evaluated the degree of brain damage during resuscitation without return of spontaneous circulation (ROSC) using extracellular glutamate concentration.

Methods: Extracellular glutamate concentration in the cerebral cortex was measured every 2 min using microdialysis probes for 40 min after the onset of cardiac arrest in Sprague-Dawley rats. Cardiac arrest was induced using electrical stimulation through the esophagus for 6 min to prevent ROSC during the observation period. The rats were divided into three groups according to the treatment received. Artificial ventilation support without chest compression was provided for the ventilation (V) group during the 40-min observation period ($n = 7$). The ventilation+compression (VC) group received artificial ventilation and chest compression ($n = 7$). The ventilation+compression+hypothermia (VCH) group received artificial ventilation, chest compression, and hypothermia ($n = 7$). Chest compression (20 min) and hypothermia (observation period) were initiated after the electrical stimulation ended.

Results: Glutamate concentrations continued to increase in both V and VC groups. Although after the onset of chest compression, glutamate concentration showed a significant difference at 2 min and reached the maximum at 6 min (VC group; $284 \pm 48 \mu\text{mol/L}$ vs. V group $398 \pm 126 \mu\text{mol/L}$, $p=0.003$), there was no difference toward the end of chest compression ($513 \pm 61 \mu\text{mol/L}$ vs. $588 \pm 103 \mu\text{mol/L}$, $p=0.051$). Unlike in the VC group, glutamate concentrations did not increase in the VCH group.

Conclusions: Hypothermia minimizes glutamate release. CPR reduces the progression of brain damage for a limited period.

Keywords: Brain damage; Cardiopulmonary resuscitation; Glutamate; Hypothermia; Sprague-Dawley rats

Introduction

Cardiopulmonary resuscitation (CPR) is the gold standard for cardiac resuscitation.¹ However, if the time taken to achieve the return of spontaneous circulation (ROSC) exceeds 20 min, >50% of patients will have unfavorable neurological outcomes.^{2,3} These data indicate that CPR is required to initiate ROSC; however, CPR may be insufficient in ensuring neuroprotection, even if initiated immediately and continuously performed after the onset of cardiac arrest. Therefore, we hypothesized that CPR without ROSC may not be sufficient in halting the progression of brain damage.

It is well known that hypothermia is neuroprotective in cases of ischemia, and therapeutic hypothermia is performed during cardiac surgery that requires circulatory arrest.⁴ Although the neuroprotective effect of hypothermia during CPR has been reported,⁵ it has not been quantitatively evaluated.

Excess glutamate is released extracellularly during cerebral ischemia.^{6,7} Glutamate activates *N*-methyl-D-aspartate receptors and initiates a massive influx of Ca^{2+} into the intracellular space.⁸ Glutamate-induced Ca^{2+} influx triggers deleterious cascades, such as uncontrolled activation of phospholipases, proteases, and endonucleases, which leads to the release of arachidonic acid, hydrolysis of peptide chains, and fragmentation of genomic DNA, respectively.⁹ Since glutamate is a primary initiator of these destructive cascades, the extracellular glutamate concentration during cerebral ischemia is closely correlated with histological damage.¹⁰

We hypothesized that CPR may not be enough to prevent brain damage. Therefore, this study was designed to quantitatively evaluate the degree of protection against brain damage provided by CPR and hypothermia during resuscitation without ROSC using glutamate concentration as a marker for neuronal damage. Since the uptake of excess glutamate is performed by the gradient across the cell membrane,¹¹ we measured the

extracellular potential adjacent to the site of glutamate sampling in the right parietal cortex.

Materials and Methods

All experiments were performed in accordance with the National Institutes of Health animal care guidelines and the study was approved by the Animal Research Control Committee of Okayama University Medical School (approval number: OKU-2016146; approval date: April 1, 2016).

Preparation of animals

A total of 21 male Sprague-Dawley rats (Charles River Japan, Yokohama, Japan) weighing 289 ± 9 g (8-9 weeks old) were used in this study. Prior to the experimental procedures, the animals were fasted overnight, but given access to water *ad libitum*. The rats were anesthetized through inhalation of 4% isoflurane in oxygen before tracheal intubation and initiation of artificial ventilation using a respirator (SN-480-7, Shinano, Tokyo, Japan). Anesthesia was maintained using 1.5% isoflurane in 60% oxygen during preparation and maintained at 1.5% isoflurane in 100% oxygen during the intervention. During these procedures, the body temperature of the animals was monitored using a rectal probe and maintained at 37.0 ± 0.5 °C using a handmade water blanket. A PE50 polyethylene catheter (SP-45, Natsume Seisakusyo, Tokyo, Japan) was placed in the right femoral artery for continuous monitoring of arterial blood pressure. Blood gases were measured only once prior to ischemia using a hand-held blood analyzer (i-STAT 300F analyzer, Abbott Laboratories, Princeton, NJ, USA) and maintained within normal ranges. After placing the rats in a custom stainless-steel stereotaxic instrument (see Supplemental Digital Content 1) in the prone position (see Supplemental Digital Content 2), the skull was exposed, and burr holes were drilled into both parietal bones 3 mm posterior and 3 mm lateral to the bregma. The

microdialysis probe (membrane length, 1.0 mm; molecular weight cutoff, 50,000; outer diameter, 220 μm [A-I-4-01 probe, Eicom, Kyoto, Japan]) was placed in the fifth layer of the right cerebral cortex (3 mm to the right of the sagittal line, 3 mm posterior to the bregma, inserted 1,250 μm from the surface) to measure glutamate concentration every 2 min. A borosilicate glass electrode was placed adjacent to the microdialysis probe at a depth of 750 μm below the cortical surface to monitor the extracellular potential. The temperature of the brain was measured using a small thermocouple placed in the epidural space via a left burr hole and maintained at 37.0 ± 0.5 °C by using a continuous, gentle flow of warmed saline through a polyethylene cylinder (5 mm in height, 19 mm in inner diameter) that was placed on the surface of the skull.

Experimental groups

The rats were divided into three groups ($n = 7$ per group) according to the treatment received after cardiac arrest. None of the groups received electrical defibrillation. The V group was artificially ventilated without chest compression during the 40-min observation period. The VC group received artificial ventilation and 20 min of chest compression that was initiated after the end of electrical stimulation. The VCH group received artificial ventilation and 20 min of chest compression, and hypothermia was initiated after the end of electrical stimulation and continued during the observation period. The brain temperature dropped below 30 °C within 6 min of the onset of cooling; this temperature was maintained during the observation period. We commenced chest compression at 6 min after the onset of cardiac arrest based on the results of a preliminary study in rats that showed that cardiac activity resumed prematurely when chest compression was started earlier than this time point.

Cardiac arrest

Cardiac arrest was initiated using ventricular fibrillation induced by electrical stimulation (100 Hz, 50 mA) for 6 min through a pacing catheter inserted into the esophagus (SEN-3201 electronic stimulator and SS-102J isolator, Nihon Kohden, Tokyo, Japan). Cardiac arrest was confirmed based on the loss of arterial pulsation.

Chest compression

Chest compressions were performed with a custom-made compression device (the figure in Supplemental Digital Content 3 illustrates the chest compression device) to obtain a constant depth of compression. The middle of the sternum was depressed by 15 mm at a rate of 300 beats/min. Chest compression was stopped after 20 min to prevent pulmonary damage.

Nasopharyngeal cooling

Selective brain hypothermia was initiated by nasopharyngeal cooling.¹² This method was designed to allow heat exchange with both carotid arteries at the pharynx and to cool the entire brain hematogenously. Briefly, 20-gauge cannulas were inserted to a depth of 5 mm in both nasal cavities. Cold physiological saline (5 °C) was infused at a rate of 100 mL/min/kg body weight using a roller pump until the epidural temperature decreased to 30 °C. The cold saline flowed out of the oral cavity and was aspirated by a suction device to prevent the animal from becoming wet.

Glutamate assay

The microdialysis probe was perfused with Ringer's lactate solution at a flow rate of 2 µL/min using an infusion pump (ESP-32, Eicom). Measurements of glutamate concentrations were initiated after a 40-min stabilization period following the insertion of the microdialysis probe. This timing was based on a pilot study that showed that an increase in extracellular

glutamate levels caused by a mechanical injury during probe insertion resolved within 30 min.¹⁰ The dialysate was automatically collected every 2 min using a fraction collector (EFC-82, Eicom). The quantification of glutamate was performed using high-performance liquid chromatography with computerized controls (Nanospace instrument with Syscon-21 software, Osaka Soda, Osaka, Japan), as previously described.¹³ Briefly, glutamate was isolated from samples using a separation column (4.6×150 mm, GU-GEL, Eicom) and oxidized by an immobilized enzyme column (3.0×4.0 mm, E-ENZYM-PAC, Eicom) to hydrogen peroxide. The hydrogen peroxide was detected by the electrochemical detector. The mobile phase consisted of 250 mg/L cetrimonium bromide ($C_{19}H_{42}BrN$) and 0.050 mg/L EDTA sodium in 60 mmol/L NH_4Cl-NH_4OH solutions.

Statistical analysis

All data are expressed as means $\pm$ standard deviation and analyzed using Microsoft Excel (Microsoft Excel; Microsoft Corporation, Redmond, WA). The arterial blood pressure, epidural temperature, and glutamate concentration were analyzed using two-way repeated measures analysis of variance — followed by Fisher's post hoc test. All other statistical analyses were performed using Student's *t*-test. A *p*-value of < 0.05 was considered statistically significant for all tests. The sample size was estimated using a power analysis based on the results of a previous study that used the same animal model (actual power, 0.85; total sample size, 9).¹⁴

Results

The results of physiological measurements prior to ischemia were within normal limits. The extracellular potential showed a sudden negative shift 85 ± 20 s after the onset of ischemia, indicating a loss of membrane potential that did not recover during the observation periods in

176 all three groups.

177 Figure 1 shows the concentrations of extracellular glutamate in the three groups. The
 178 glutamate concentrations began to increase in all groups with the onset of ischemia. Two
 179 minutes after the onset of chest compression, the extracellular glutamate concentration was
 180 lower in the VC group ($182 \pm 53 \mu\text{mol/L}$) than in the V group ($271 \pm 118 \mu\text{mol/L}$) ($p = 0.02$).
 181 The difference in glutamate concentrations between the V and VC groups was greatest at 6
 182 min after the onset of chest compression; glutamate levels were $284 \pm 48 \mu\text{mol/L}$ and $398 \pm$
 183 $126 \mu\text{mol/L}$ in the VC and V groups, respectively ($p = 0.003$). At the end of the 20-min chest
 184 compression period, the difference in glutamate levels between the V and VC groups was no
 185 longer significant ($588 \pm 103 \mu\text{mol/L}$ and $513 \pm 61 \mu\text{mol/L}$, $p = 0.051$, V group vs. VC
 186 group). Two minutes after the onset of hypothermia in the VCH group, the increase in
 187 glutamate concentration was suddenly suppressed. At 6 min after the onset of cooling, a
 188 significant difference was observed in the glutamate concentration between the VC (284 ± 49
 189 $\mu\text{mol/L}$) and VCH ($208 \pm 58 \mu\text{mol/L}$) groups ($p = 0.048$). At the end of the 40-min
 190 observation period, the extracellular glutamate concentrations were $625 \pm 81 \mu\text{mol/L}$ for the
 191 VC and $287 \pm 75 \mu\text{mol/L}$ for the VCH groups ($p < 0.001$).

192 Figure 2 shows the arterial blood pressures in the three groups. The blood pressure
 193 increased with the onset of chest compressions. In the VC group, the blood pressure, during
 194 the first 2 min of chest compressions, was 56 ± 10 (systolic), 34 ± 6 (mean), and 21 ± 5
 195 (diastolic) mmHg. During chest compression (overall, 20 min), the maximum blood pressure
 196 values (systolic pressure: 68 ± 9 mmHg, diastolic pressure: 28 ± 6 mmHg, mean pressure: 41
 197 ± 6 mmHg) were noted at 94 ± 38 s after starting the compressions. The blood pressure
 198 gradually decreased, and the blood pressure during the last 2 min was 45 ± 15 mmHg
 199 (systolic), 28 ± 4 mmHg (mean) and 19 ± 2 mmHg (diastolic). The average blood pressure
 200 during 20 min of chest compressions was 31 ± 3 mmHg (mean).

The changes in the epidural temperatures are shown in Figure 3. In the VCH group, the epidural temperature declined to 30 °C after 4.8 ± 1 min of commencing brain cooling without a corresponding change in rectal temperature.

Discussion

To the best of our knowledge, this is the first study that quantitatively evaluated the changes in glutamate release during CPR and brain hypothermia using glutamate concentrations as an index of primary neuronal damage. In this study, glutamate concentrations were slightly lower in the VC group than in the V group, and the levels of glutamate continued to increase in both groups over time. These observations suggest that the suppression of glutamate release by CPR is limited, and neuronal damage due to glutamate release progresses even during CPR. However, CPR in combination with brain hypothermia induced a sustained decrease in glutamate concentrations in our resuscitation model.

A study that used rat model in which both common carotid arteries were occluded in the presence of hypotension, reported that 50% of the neurons were damaged when the extracellular concentration of glutamate reached 400 $\mu\text{mol/L}$.¹⁰ In the present study, CPR increased the time required for the extracellular concentration of glutamate in the brain to reach 400 $\mu\text{mol/L}$ by 5 min compared to that of the control group. In a study of prehospital resuscitation after cardiac arrest, CPR increased the number of patients who achieved ROSC by 11% after 10-15 min and by 12% after 15–20 min.³ Although there are some differences between the physiology of humans and that of rats, our data suggest that CPR after cardiac arrest may increase the therapeutic time window for ischemic brain damage.

The clearance of excess glutamate from the extracellular space is driven by glutamate transporters that use the electrochemical gradient of Na^+ across the cell membrane.¹¹ Another study reported that the thresholds of cerebral blood flow (CBF) for the loss and recovery of

membrane potential were 19% and 46%, respectively.¹⁴ Thus, after the release of glutamate, higher CBF may be required to restore membrane potential. Since the level of CBF provided by chest compression is 20%-30% of the normal level,^{15,16} CPR may not be able to initiate the recovery of the Na⁺ electrochemical gradient once the membrane potential has been lost, resulting in a lack of glutamate clearance.

In our study, the increase in extracellular glutamate concentration in the brain was strongly suppressed once the epidural temperature reached 33 °C–34 °C. Sato *et al.* reported that a target temperature of 32.6 °C was required to block glutamate release in a rat model used to study the effects of occlusion of both common carotid arteries and hypotension on the brain.¹⁷ Busto *et al.* reported that the optimal temperature for inhibiting glutamate release was 33 °C in a rat model of four-vessel occlusion and hypotension.¹⁸ In the present study, the temperature threshold for the suppression of glutamate release was consistent with those of previous studies.

Although the American Heart Association (AHA) recommended post-ROSC cooling in their 2005 and 2010 guidelines, the 2015 AHA guidelines on cardiopulmonary resuscitation (CPR) and emergency cardiopulmonary care recommend targeted temperature management in patients who remain comatose after cardiac arrest. Since this guideline is intended for post-ROSC, it describes what happens after ischemic recovery. In the present study, it was observed that hypothermia during ischemia strongly inhibited the release of glutamate. Castren *et al.* reported that intra-arrest transnasal cooling improved the discharge rates in subgroup analysis. Thus, previous studies suggest that hypothermia during ischemia has a strong brain-protective effect.

We used a chest compression device to obtain a constant depth and force. Table 1 summarizes the results of previous studies that have examined blood pressure during chest compressions in various species, including in humans. Regardless of the animal species (rat,

pig, dog and human), body weight (0.2–65 kg), or compression rate (60–240 compressions/min), blood pressures during chest compressions ranged from 30 to 39 mmHg in many studies.^{16,19-29} Therefore, the chest compressions in the present study were considered equally effective as those in the previous studies.

This study had some limitations. First, since all three groups did not show ROSC, we did not evaluate the histological data on neuronal damage. Second, humans and rats are obviously not the same. The data obtained in the animal study may not directly fit to the clinical situation in humans. Third, in this study, all treatments (i.e., CPR alone, and CPR and hypothermia) were initiated at 6 min after the onset of ischemia to avoid ROSC during CPR. Therefore, the membrane potential had already been lost when treatments were initiated. The neuroprotective effect of CPR may have been different if CPR had been initiated before the loss of membrane potential.

In conclusion, although CPR slightly attenuates glutamate release for a limited duration and does not last long, CPR may extend the therapeutic time window for ischemia-induced brain damage, while hypothermia in combination with CPR strongly suppressed the increase in glutamate levels. Timely CPR combined with brain hypothermia may be a good approach for neuroprotection, if it can be initiated within a short period in patients who undergo CPR without ROSC in the field.

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Figure Legends

Figure 1. Changes in the extracellular glutamate concentrations in the three groups. The filled circles, triangles, and squares indicate the levels of extracellular glutamate in the V, VC, and VCH groups, respectively. The asterisks indicate a significant difference in glutamate levels between the V and VC groups ($p < 0.05$). The section signs indicate a significant difference in glutamate levels between the V and VCH groups ($p < 0.05$). The daggers indicate a significant difference in glutamate levels between the VC and VCH groups ($p < 0.05$). Data are presented as mean $\pm$ standard deviation. V, ventilation; VC, ventilation+compression; VCH, ventilation+compression+hypothermia.

Figure 2. Changes in the mean arterial blood pressures in the three groups. The arterial blood pressures are shown as the mean values at 2-min intervals that correspond with the timing of each glutamate sample. The filled circles, triangles, and squares indicate the mean values for arterial blood pressure in the V, VC and VCH groups, respectively. The asterisks indicate a significant difference in the arterial blood pressure between the V and VC groups ($p < 0.05$). The section signs indicate a significant difference in the arterial blood pressure between the V and VCH groups ($p < 0.05$). Data are presented as mean $\pm$ standard deviation. V, ventilation; VC, ventilation+compression; VCH, ventilation+compression+hypothermia.

Figure 3. Changes in the epidural temperatures in the three groups. The temperatures are shown as the mean values at 2-min intervals and correspond to the period of each glutamate sample. The filled circles, triangles and squares indicate the epidural temperatures at each time point in the V, VC and VCH groups, respectively. The section signs indicate a significant difference in the epidural temperatures between the V and VCH groups ($p < 0.05$). The daggers indicate a significant difference in the epidural temperatures between the VC and

393 VCH groups ($p < 0.05$). Data are presented as mean $\pm$ standard deviation. V, ventilation; VC,
394 ventilation+compression; VCH, ventilation+compression+hypothermia.

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396 **List of Supplemental Digital Content**

397 **Supplemental Digital Content 1.** Supplemental Digital content 1.pptx (figure of the steel
398 stereotaxic instrument).

399

400 **Supplemental Digital Content 2.** Supplemental Digital content 2.pptx (figure of an
401 overview of the experimental setup).

402

403 **Supplemental Digital Content 3.** Supplemental Digital content 3.pptx (figure of the chest
404 compression device).