Extracellular glutamate concentration increases linearly in proportion to decreases in residual cerebral blood flow after the loss of membrane potential in a rat model of ischemia.

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ABSTRACT

Background: Brain ischemia due to disruption of cerebral blood flow (CBF) results in increases in extracellular glutamate concentration and neuronal cell damage. However, the impact of CBF on glutamate dynamics after the loss of membrane potential remains unknown.

Methods: To determine this impact, we measured extracellular potential, CBF, and extracellular glutamate concentration at adjacent sites in the parietal cortex in male Sprague-Dawley rats (n=21). CBF was reduced via bilateral occlusion of the common carotid arteries and exsanguination until a loss of extracellular membrane potential was observed (low-flow group), or until CBF was further reduced by 5–10% of pre-ischemia levels (severe-low-flow group). CBF was promptly restored 10 minutes after the loss of membrane potential. Histological outcomes were evaluated 5 days later.

Results: Extracellular glutamate concentration in the low-flow group was significantly lower than that in the severe-low-flow group. Moreover, increases in extracellular glutamate concentration exhibited a linear relationship with decreases in CBF after the loss of membrane potential in the severe-low-flow group, and the percentage of damaged neurons exhibited a dose-response relationship with the extracellular glutamate concentration. The extracellular glutamate concentration required to cause 50% neuronal damage was estimated to be 387

μmol/L, at 8.7% of pre-ischemia CBF. Regression analyses revealed that extracellular glutamate concentration increased by 21 μmol/L with each 1% decrease in residual CBF, and that the percentage of damaged neurons increased by 2.6%.

Conclusions: Our results indicate that residual CBF is an important factor that determines the extracellular glutamate concentration after the loss of membrane potential, and residual CBF would be one of the important determinants of neuronal cell prognosis.

Keywords: brain ischemia, cardiopulmonary resuscitation, cerebral blood flow, glutamate, resuscitation

INTRODUCTION

The mechanisms of neurological damage after cardiac arrest involve primary and secondary injury. During primary injury, energy deprivation leads to reduced adenosine triphosphate production and sodium-potassium pump failure, which subsequently induces loss of membrane potential, sodium influx, and massive glutamate release.[1] Glutamate exacerbates the intracellular calcium overload, which contributes to secondary injury. Secondary injury is gradually initiated by several cellular processes after the return of spontaneous circulation (ROSC), including oxidative stress induced by free radicles, inflammation, and apoptosis.[2, 3] Several studies have demonstrated that glutamate plays a key role in primary injury.[4, 5, 6] Therefore, averting massive glutamate release via cardiopulmonary resuscitation (CPR) can be expected to improve neurological outcomes for cardiac arrest patients.

Chest compression is performed to supply adequate blood flow to vital organs, especially the brain.[7, 8] The level of CBF supplied by manual chest compressions is 20-40% of the normal level.[9, 10, 11] Since the CBF threshold for the loss of neuronal membrane potential is approximately 20% of the normal level,[12, 13, 14, 15] CBF supplied by manual chest compression is thought to be sufficient for maintaining this potential. Unlike patients with focal ischemia, who have gradients in extracellular glutamate level and residual cerebral blood flow (CBF), in patients with cardiopulmonary arrest, extracellular glutamate levels will not increase regardless

of the level of residual CBF, as long as neuronal membrane potentials are maintained by CPR. However, prolonged CPR will result in the loss of membrane potential, as manual chest compression cannot maintain membrane potential for a long period of time, resulting in massive glutamate release. It is unknown whether the degree of massive glutamate release after the loss of membrane potential is always same. Although the residual CBF supplied by CPR could have some effect on the degree of glutamate release, to our best knowledge, no previous studies have reported whether the level of residual CBF after loss of membrane potential has an impact on glutamate levels.

Accordingly, the present prospective animal study was designed to elucidate the impact of residual CBF on the cerebral extracellular glutamate concentration during the loss of membrane potential. The impact of residual CBF on the degree of neuronal damage was also examined.

MATERIALS AND METHODS

Experiments were performed in accordance with the National Institutes of Health animal care guidelines and were approved by the Animal Research Control Committee of Okayama University Medical School.

General Procedures

Male Sprague-Dawley rats (n = 21; Charles River Japan, Yokohama, Japan) weighing 307 ± 21 g were used for the present study. Animals were fasted overnight prior to the experiment but were allowed free access to water. Anesthesia was induced with 4% isoflurane in oxygen. Following tracheal intubation and initiation of artificial ventilation (SN-480-7; Shinano, Tokyo, Japan), anesthesia was maintained with 1.5% isoflurane in 60% oxygen. During the experiment, body temperature was monitored using a rectal probe and maintained at 37.0℃ ± 0.5℃ using a heated water blanket. PE50 polyethylene catheters (SP-45, Natsume Seisakusyo, Tokyo, Japan) were placed in the right femoral artery and vein to continuously monitor arterial blood pressure and permit blood withdrawal during controlled hemorrhagic hypotension, respectively. A loose ligature was placed around each common carotid artery. After placement of the animal in a stereotaxic instrument (Narishige, Tokyo, Japan), a borosilicate glass electrode (tip

106 diameter: 5-10 μm) filled with saline was placed in the 5th layer of the cerebral cortex (3 mm
107 to the right of the sagittal line, 3 mm posterior to the bregma, and 750 μm below the cortical
108 surface) through a dural incision to measure extracellular potential. A laser Doppler flow probe
109 (OmegaFlo FLO-C1, Omegawave, Tokyo, Japan) was placed on the surface of the thinly shaved
110 right temporal bone adjacent to the glass electrode to continuously monitor regional CBF. In
111 addition, a microdialysis probe (membrane length of 1mm, molecular weight cut off of 50,000,
112 OD of 220 μm; A-I-4-01, Eicom, Kyoto, Japan) was placed slightly posterior to the glass
113 electrode to measure extracellular glutamate concentration in the 5th layer of the cerebral cortex.
114 Brain temperature was measured using a small thermocouple (500 μm in diameter) placed in
115 the left epidural space and maintained at 37.0℃ ± 0.5℃ with a continuous gentle flow of
116 warmed saline into a polyethylene cylinder (5 mm in height, 19 mm in inner diameter) that had
117 been placed on the skull surface. After measuring the physiological variables, 100 U of heparin
118 was injected intravenously (IV) to prevent clotting of drainage blood. Baseline CBF values
119 were measured for 1 min prior to the start of ischemic insults. Following bilateral occlusion of
120 the common carotid artery, CBF was decreased continuously via exsanguination at a rate of
121 2.5% of the baseline level every 1 min until a sudden negative DC shift was observed (Figure
122 1).
123

Experimental Groups

The animals were divided into two groups according to the amount of residual CBF after the loss of membrane potential. Residual CBF was maintained at a slightly lower value than that observed after loss of membrane potential in the low-flow group, and further reduced by 5-10% of the pre-ischemia level in the severe-low-flow group. Ten minutes after the loss of membrane potential, CBF was promptly restored via reperfusion of the bilateral common carotid arteries and returning blood from the femoral vein (Figure 1). Three animals in the severe-low-flow group died before histological evaluation. In total nine animals in the low-flow group and 12 animals in the severe-low-flow group were analyzed.

Measurement of Extracellular Glutamate Concentration

The relative recovery rate of each probe was determined by performing microdialysis in a standard glutamate solution (100 μM) before and after the experiment. The probes were perfused with Ringer's solution at 2 μl/min using an infusion pump (ESP-32; Eicom). Based on a pilot study in which the increase in extracellular glutamate concentration caused by mechanical injury during probe insertion disappeared within 30 min, measurements of extracellular glutamate concentration were initiated after a 40 min stabilization period

following insertion of the microdialysis probe. The dialysate was automatically collected every 2 min using a fraction collector (EFC-82; Eicom) from 20min before to 80min after the start of the experiment.

Quantification of glutamate level was performed, via high-performance liquid chromatography with a computerized control (Nanospace Syscon 21; Shiseido, Tokyo, Japan), as originally described in our previous report.[16] Briefly, each dialysate (3 µl of the 4 µl collected) was injected into the chromatography column and glutamate was separated using an isolation column (4.6 × 150 mm, GU-GEL; Eicom). Isolated glutamate was converted to hydrogen peroxide using glutamate oxidase packed in an enzyme immobilized column (E-ENZYMPAC; Eicom). For electrochemical detection, oxidative potential was applied to a platinum electrode at 450 mV versus an Ag/AgCl reference electrode. The mobile phase (250 mg/L cetrimonium bromide [$C_{19}H_{42}BrN$] and 0.05 mg/L EDTA sodium in 60 mmol/L NH_4Cl-NH_4OH solution) was pumped using a microvolume plunger system (Nanospace SI-2 3001; Shiseido) at rate of 400 µl/min. The concentration of extracellular glutamate collected was calculated by dividing the concentration of glutamate in the dialysate by the relative recovery rate of each probe.

Histological Evaluation

158 After a 5 day survival period, all animals were anesthetized with 4% isoflurane in oxygen and
159 perfused with heparinized physiological saline (20U/ml) and 4% formaldehyde with buffer
160 solution (pH 7.4). The areas in which DC potential had been recorded were marked with blue
161 ink using a 21-gauge needle. Following brain removal and paraffin embedding, coronal tissue
162 samples were sectioned at 5 μm, including the site marked with blue-black ink, following which
163 sections were stained with hematoxylin and eosin. The areas in which DC potential had been
164 recorded were enlarged 200×, and the numbers of both damaged and intact pyramidal neurons
165 in the 5th layer of the parietal-temporal cortex were counted. In the current study, pyramidal
166 neurons exhibiting chromatin aggregation in the nucleus, shrinkage, or eosinophilic staining in
167 the cytoplasm were considered to have been injured. The number of injured pyramidal neurons
168 in the 5th layer of the parietal-temporal cortex was counted by an observer blinded to this study.

169

170 *Statistical Analyses*

171 Values are expressed as means ± SD. The probit curves were drawn using Microcal software
172 (Microcal Origin; Microcal Software, Northampton, MA, U.S.A.). All statistical comparisons
173 were performed using Student's t-test (Microsoft Excel; Microsoft Corporation, Redmond, WA,
174 U.S.A.). A P-value (*P*) of less than 0.05 was considered statistically significant.

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178 **RESULTS**

179 All physiological measurements obtained prior to ischemic insults were within normal ranges;
180 however, mean arterial blood pressure 10 min after loss of membrane potential was significantly
181 different between the two groups (severe-low-flow group: 30 ± 14 mmHg vs. low-flow group:
182 62 ± 19 mmHg, $P < 0.001$). The other physiological measurements are described in detail in
183 the supplementary material (Supplemental Digital Content 1). Representative traces of CBF
184 and extracellular potential during the experiment are shown in Figure 2. The degree of
185 maximum negative shift in extracellular potential shows no significant difference between the
186 two groups (severe-low-flow group: -21.0 ± 5.7 mV vs. low-flow group: -24.8 ± 3.4 mV, $P =$
187 0.09). The CBF threshold for the loss of membrane potential in all animals was 26 ± 14%. After
188 the onset of the loss of membrane potential, the mean CBF during depolarization was controlled
189 at 20 ± 5% of the pre-ischemia level in the low-flow group and decreased to 12 ± 4% of the
190 pre-ischemia level in the severe-low-flow group. The mean CBF during loss of the membrane
191 potential was controlled at a significantly higher level in the low-flow group than in the severe-
192 low-flow group ($P < 0.001$).

193 The extracellular glutamate concentration before ischemia was 61 ± 65 μmol/L in the severe-
194 low-flow group and 42 ± 25 μmol/L in the low-flow group, respectively. The extracellular
195 glutamate concentration 10 min after the loss of membrane potential was 58 ± 50 μmol/L in the

196 low-flow group and 300 ± 122 μmol/L in the severe-low-flow group. Extracellular glutamate
197 concentration in the low-flow group was significantly lower than that in the severe-low-flow
198 group ($P < 0.001$). Figure 3 shows changes in the extracellular glutamate concentration in the
199 two groups. In the severe-low-flow group, glutamate concentration began to increase 4 min
200 after the loss of membrane potential, reaching its maximum value 12 min after the loss of
201 membrane potential. However, glutamate concentration did not increase in the low-flow group
202 compared to the control level. Figure 4 shows the associations between mean residual CBF
203 during ischemic insult and extracellular glutamate concentration 10 min after the loss of
204 membrane potential in each group (severe-low-flow group: $R^2 = 0.30$, $P = 0.04$; low-flow
205 group: $R^2 = 0.08$, $P = 0.23$). There was a significant linear association between mean residual
206 CBF and extracellular glutamate concentration in the severe-low-flow group. As mean residual
207 CBF during ischemic insults increased, extracellular glutamate concentration 10 min after the
208 loss of membrane potential decreased in the severe-low-flow group. In contrast, there was no
209 significant association in the low-flow group. Furthermore, neuronal cells were significantly
210 more histologically damaged, as defined in the methods, in the severe-low-flow group (44 ±
211 9%) than in the low-flow group (15 ± 5%, $P < 0.001$). We also observed a dose-response
212 relationship between the maximum extracellular glutamate concentration and the percentage of
213 damaged neurons ($R^2 = 0.58$, $P < 0.001$; Figure 5). The extracellular glutamate concentration

214 required to damage 20%, 50% and 60% of neurons were estimated to be 110, 387 and 464
215 μmol/L, respectively. Representative histological slides of the groups are shown in Figure 5.

216 Mortality rate of the severe-low-flow group was 25% (3 of 12 animals), with three animals
217 dying on day 1, 2, and 4 of the experiment. In contrast, there were no dead animals in the low-
218 flow group, and no seizures within the scope of our observation in any animals.

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DISCUSSION

It is well known that extracellular glutamate concentration does not increase until residual CBF decreases under the threshold for the loss of membrane potential.[17] However, the impact of residual CBF on the release of extracellular glutamate after the loss of membrane potential remains unknown. Therefore, in the present study, we aimed to elucidate the impact of residual CBF on the release of extracellular glutamate after loss of membrane potential by comparing cerebral extracellular glutamate concentration between two groups: the low-flow group received residual CBF slightly less than the threshold for the loss of membrane potential, while the other severe-low-flow group received much lower residual CBF. Compared to the extracellular glutamate concentration in the severe-low-flow group, that in the low-flow group was significantly attenuated (58 ± 50 μmol/L vs. 300 ± 122 μmol/L, $P < 0.001$). In addition, extracellular glutamate concentration exhibited a linear correlation with residual CBF after the loss of membrane potential in the severe-low-flow group ($R^2 = 0.30$, $P = 0.04$).

Under normal conditions, glutamate is released via exocytosis, consuming ATP.[18] Released extracellular glutamate is then taken into glia and neurons by the high-affinity Na^+-dependent glutamate uptake system via sodium and potassium ion gradients.[19] Glutamate transporters take in extracellular glutamate with the influx of three sodium ions and efflux of one potassium ion.[19] Under ischemic conditions, exocytosis is suppressed due to the depletion of ATP. In addition,

240 the depletion of ATP reduces the activity of the Na^+-K^+-ATPase system and leads to the loss of
241 sodium and potassium ion gradients between the intracellular and extracellular spaces.[19]
242 Glutamate transporters operate in reverse under such conditions, increasing extracellular
243 glutamate concentration due to the passive release of glutamate from glia and neurons,[20, 21] until
244 a new equilibrium is reached.[22]

245 In the present study, the extracellular glutamate concentration increased linearly in proportion
246 to decreases in residual CBF after the loss of the membrane potential in the severe-low-flow
247 group. This finding indicates that residual CBF is involved with the released extracellular
248 concentration after the loss of membrane potential in severe low CBF state. Although the aim
249 of this study was not to elucidate the mechanism of glutamate dynamics, the slopes of the data
250 are different in each group, suggesting that the mechanisms of glutamate release differ between
251 the two groups. It has been reported that the glutamate concentration in response anoxia was
252 altered in a first phase by a mechanism involving exocytosis followed by a second phase
253 involving the reversed uptake of glutamate.[23] Taken together, the evidence suggests that the
254 second phase would not start until residual CBF decreases to a certain threshold.

255 It is also likely that mechanisms of glutamate decrease are affected by residual CBF. Firstly,
256 different CBF flow rates between the two groups would also contribute to the washout of
257 released glutamate from the extracellular space. In a relatively high CBF state, suppression of

extracellular glutamate concentration would be thought to be due to washout by more residual CBF. Secondly, previous studies have reported that glutamate transporters and Na^+-K^+-ATPases are part of the same macromolecular complexes, and that the uptake of glutamate depends on the activity of Na^+-K^+-ATPases.[24] Since more residual CBF would supply more oxygen and glucose, thereby preserving a certain level of Na^+-K^+-ATPase function, in the low-flow group, Na^+-K^+-ATPases and glutamate transporters may function using ATP produced by residual CBF to suppress the release of glutamate after the loss of membrane potential.

In the present study, neuronal cells were significantly more damaged in the severe-low-flow group than in the low-flow group. Furthermore, the logistic regression curve revealed a close association between extracellular glutamate concentration and neuronal damage. According to the regression analysis, in severe low flow state, extracellular glutamate concentration increases by 21 μmol/L with each 1% decrease in residual CBF, while the percentage of damaged neurons increases by 2.6%. These results suggest that residual CBF determines the degree of neuronal damage by controlling the release of glutamate after the loss of membrane potential. Therefore, as excess glutamate is a primary factor involved in neuronal cell injury during brain ischemia,[25] increased residual CBF would be an important factor which suppresses the release of glutamate to prevent more serious neuronal damage during brain ischemia.

While researchers believe that high-quality chest compression supplies sufficient CBF during

CPR in clinical situations, some recent clinical studies have indicated that it is difficult to achieve favorable neurological outcomes, if the duration of CPR is over 40 min.[26, 27] Such findings suggest that manual chest compression cannot always ensure adequate CBF for maintaining neuronal cell membrane potential throughout prolonged CPR. Patients with poor neurological outcomes thus lose the membrane potential, causing extracellular glutamate concentration to increase during CPR. The residual CBF in our study is thought to reflect the level of CBF supplied by CPR in clinical situations. Therefore, the current study indicates that, in patients with loss of membrane potential during CPR, persistent efforts to maintain CBF are critical for suppressing the release of glutamate and achieving better neurological outcomes.

In conclusion, although the loss of membrane potential in neurons triggers the release of glutamate, we observed that increases in extracellular glutamate concentration exhibited a linear correlation with decreases in residual CBF after the loss of membrane potential. Furthermore, the percentage of damaged neurons exhibited a concentration dependent relationship with the extracellular glutamate concentration, and the extracellular glutamate concentration required to damage 50% of neurons was 387 μmol/L. Our results indicate that, when neurons lose their membrane potential, increases in residual CBF are effective in inhibiting increases in extracellular glutamate concentration and alleviating neuronal cell damage.

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FIGURE LEGENDS

Figure 1. Experimental procedure and groups. Extracellular potential and percent change in cerebral blood flow (CBF) in the two groups. Bilateral common carotid arteries were occluded (2VO), and CBF was decreased by draining venous blood at a rate of 2.5% of the baseline value/min until a sudden negative shift in extracellular potential was observed. The CBF was maintained slightly below the level of membrane potential loss (low-flow group, n = 9), or further reduced by 5–10% of the pre-ischemia level (severe-low-flow group, n = 12). After 10 minutes of ischemic insult, CBF was promptly restored.

Figure 2. Representative changes in cerebral blood flow (CBF; black line) and extracellular potential (grey line) in the severe-low-flow group (A) and the low-flow group (B). CBF is shown as a percentage change from baseline CBF. The degree of maximum negative shift in extracellular potential shows no significant difference between the two groups (-21.0 ± 5.7 mV in the severe-low-flow group vs. -24.8 ± 3.4 mV in the low-flow; $P = 0.09$).

Figure 3. Changes in extracellular glutamate concentration. In the severe-low-flow group (solid line), glutamate concentration began to increase 4 minutes after the loss of membrane potential

379 and reached its maximum value 12 minutes after the loss of membrane potential. In contrast,
380 glutamate concentration did not increase in the low-flow group (dashed line) compared to the
381 control level.

382

383 Figure 4. Associations between mean residual cerebral blood flow (CBF) during ischemic
384 insults and extracellular glutamate concentration 10 minutes after the loss of membrane
385 potential in the two groups. The black filled circles and grey filled circles indicate alive animals
386 and dead animals in the severe-low-flow group, respectively. The open squares indicate the
387 low-flow group. We observed a close association between mean residual CBF and extracellular
388 glutamate concentration in the severe-low-flow group ($R^2 = 0.30$, $P = 0.04$). As mean residual
389 CBF during ischemic insults increased, extracellular glutamate concentration 10 minutes after
390 the loss of membrane potential decreased. In contrast, there was no significant association in
391 the low-flow group ($R^2 = 0.08$, $P = 0.23$).

392

393 Figure 5. Associations between maximum extracellular glutamate concentration and percentage
394 of histologically damaged pyramidal neurons, as defined in the method section, at the direct-
395 current (DC) recording site in the fifth layer of the parietal-temporal cortex in all experimental

396 animals and histological slides of severe-low-flow and low-flow group. The black filled circles
397 and the open squares indicate the severe-low-flow group and the low-flow group, respectively.
398 There was a dose-response relationship between maximum extracellular glutamate
399 concentration and the percentage of damaged neurons (R^2 = 0.58, $p < 0.001$). The arrow
400 indicates damaged neurons exhibiting shrinkage.

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403 Supplemental Digital Content: SDC1.power point file (physiological measurements during
404 experiment)

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