NADH fluorescence imaging and the histological impact of cortical spreading depolarization during the acute phase of subarachnoid hemorrhage in rats

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OBJECTIVE Although cortical spreading depolarization (CSD) has been observed during the early phase of subarachnoid hemorrhage (SAH) in clinical settings, the pathogenicity of CSD is unclear. The aim of this study is to elucidate the effects of loss of membrane potential on neuronal damage during the acute phase of SAH.

METHODS Twenty-four rats were subjected to SAH by the perforation method. The propagation of depolarization in the brain cortex was examined by using electrodes to monitor 2 direct-current (DC) potentials and obtaining NADH (reduced nicotinamide adenine dinucleotide) fluorescence images while exposing the parietal-temporal cortex to ultraviolet light. Cerebral blood flow (CBF) was monitored in the vicinity of the lateral electrode. Twenty-four hours after onset of SAH, histological damage was evaluated at the DC potential recording sites.

RESULTS Changes in DC potentials (n = 48 in total) were sorted into 3 types according to the appearance of ischemic depolarization in the entire hemisphere following induction of SAH. In Type 1 changes (n = 21), ischemic depolarization was not observed during a 1-hour observation period. In Type 2 changes (n = 13), the DC potential demonstrated ischemic depolarization on initiation of SAH and recovered 80% from the maximal DC deflection during a 1-hour observation period (33.3 ± 15.8 minutes). In Type 3 changes (n = 14), the DC potential displayed ischemic depolarization and did not recover during a 1-hour observation period. Histological evaluations at DC potential recording sites showed intact tissue at all sites in the Type 1 group, whereas in the Type 2 and Type 3 groups neuronal damage of varying severity was observed depending on the duration of ischemic depolarization. The duration of depolarization that causes injury to 50% of neurons (P50) was estimated to be 22.4 minutes (95% confidence intervals 17.0–30.3 minutes). CSD was observed in 3 rats at 6 sites in the Type 1 group 5.1 ± 2.2 minutes after initiation of SAH. On NADH fluorescence images CSD was initially observed in the anterior cortex; it propagated through the entire hemisphere in the direction of the occipital cortex at a rate of 3 mm/minute, with repolarization in 2.3 ± 1.2 minutes. DC potential recording sites that had undergone CSD were found to have intact tissue 24 hours later. Compared with depolarization that caused 50% neuronal damage, the duration of CSD was too short to cause histological damage.

CONCLUSIONS CSD was successfully visualized using NADH fluorescence. It propagated from the anterior to the posterior cortex along with an increase in CBF. The duration of depolarization in CSD (2.3 ± 1.2 minutes) was far shorter than that causing 50% neuronal damage (22.4 minutes) and was not associated with histological damage in the current experimental setting.

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KEY WORDS spreading depolarization; subarachnoid hemorrhage; histological evaluation; vascular disorders
EARLY brain injury has received attention as one of the important factors determining the prognosis of subarachnoid hemorrhage (SAH).1,2,22 Recently, it was reported that cortical spreading depolarization (CSD) was observed during the early phase of SAH in a clinical setting.4–6 CSD is a self-propagating wave of neuronal depolarization that travels at 2 to 5 mm/minute.14 Although CSD is not pathogenic in the intact brain, it is thought to be a key factor involved in expanding infarct volume in the penumbra area of stroke.10,15,16,18 To date, it is unclear whether CSD is pathogenic in the acute phase of SAH.1 The effects of membrane depolarization on neuronal damage in the acute phase of SAH must therefore be elucidated.

Oxidized nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide (NAD+/NADH) is an electron carrier in the mitochondrial respiratory chain. During membrane depolarization, NADH accumulates in mitochondria due to increased energy demand. Due to the unique behavior of NADH, which is excited by ultraviolet light (366 nm), the reduced form of this carrier (NADH) exhibits fluorescence (460 nm emission), while the oxidized form (NAD+) does not.7,11 We have used NADH fluorescence in our laboratory to visualize the 2D propagation of CSD in the intact brain and the stroke penumbra.8,17

The present study was designed to evaluate the impact of depolarization during the acute phase of SAH on histological outcome. The dynamics of the propagation of CSD in the rat cerebral cortex were examined using NADH fluorescence images and by measuring direct-current (DC) potentials. Intracranial pressure (ICP) and cerebral blood flow (CBF) were also monitored at the same time.

Methods

Animal Preparation

All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf) and were approved by the Animal Research Control Committee of Okayama University Medical School. Twenty-four male Sprague-Dawley rats (Charles River Laboratories Japan) with a mean weight of 306 ± 44 g were used. The animals were fed ad libitum and were deprived of food overnight prior to the experiments.

General Procedures

Anesthesia was induced with a mixture of 4% isoflurane in oxygen. After oral tracheal intubation, anesthesia was maintained by artificial ventilation (SN-480–7, Shimano) with 1.5% isoflurane in 50% oxygen balanced with nitrogen. A polyethylene catheter (PE50) was placed in the right femoral artery for continuous mean arterial blood pressure monitoring and blood sampling (i-STAT 300F, Abbott Point of Care). A perforator was inserted from the left external carotid artery to the left internal carotid artery, and the rat was placed into the stereotactic apparatus. To enable the observation of NADH fluorescence, a large cranial window (7 × 9 mm) was made in the left parietal-temporal bone by shaving the bone thinly until the brain surface became visible.

To monitor loss of membrane potential, 2 borosilicate glass DC electrodes with a tip diameter < 5 μm were inserted in the cranial window at a depth of 750 μm through dural incisions consisting of small bur holes. The positions of the electrodes were 4 mm posterior to the bregma and 2 mm and 5 mm lateral to the sagittal line; in this paper these electrodes are referred to as the medial and lateral DC electrode, respectively. The duration of depolarization was assessed from the start of a sudden negative shift in the DC potential to 80% recovery from the maximal DC deflection. To measure regional cerebral blood flow (CBF), a laser-Doppler flow probe (OmegaFlo FLO-C1, Omegawave) was placed adjacent to the lateral DC electrode. Each animal’s ICP was measured continuously using an ICP sensor (Codman MicroSensor, Codman & Shurtleff Inc.), which was inserted through a right temporal bur hole 4 mm posterior to the bregma and 7 mm lateral to the sagittal line (Fig. 1). Rectal temperature was monitored and maintained at 37.0° ± 0.5°C using a heated water blanket. Brain-surface temperature was maintained at 37.0° ± 0.5°C with a gentle flow of warmed saline (38.0° ± 0.5°C) that perfused over the skull surface.

Induction of SAH

SAH was initiated according to the perforation method originally described by Bederson and colleagues2 with a...
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Levels of DC potential measurement. To enable the identification of each DC potential recording site, the location was recorded in a photograph in which arteries and veins could be identified in each rat at the time of electrode insertion. Each DC potential recording site was identified after perfusion-fixation and marked using a 27-gauge needle with blue ink on the tip of the needle. All sections were stained with hematoxylin and eosin, examined, and photographed. At the sites of DC potential measurement, the number of injured pyramidal neurons in the 5th layer of the parietal-temporal cortex was counted by an observer who was blinded to the study conditions. Pyramidal neurons observed to have aggregated chromatin in the nucleus, shrinkage, or eosinophilic staining in the cytoplasm were considered injured. The percentage of neuronal damage was calculated as the number of damaged neurons divided by the total number of neurons in the visual field and then multiplied by 100.

Statistical Analysis
Experimental data are expressed in this paper as means ± standard deviation. A probit regression curve was used to evaluate the relationship between neuronal injury and the total duration of ischemic depolarization at the site of the DC electrode. This curve expresses the probability of occurrence and is generally used to identify the median lethal dose in toxicology. The probit curves were drawn using personal computer software (Origin 8, OriginLab Corporation). All statistical comparisons were performed using a Student t-test. A level of p < 0.05 was considered to be significant in all statistical tests.

Results
Table 1 shows the physiological parameters in each series of experiments. In all rats, mean arterial blood pressure, blood gases (partial pressure of carbon dioxide and partial pressure of oxygen), pH, blood glucose, and hematocrit were within normal ranges before induction of SAH.

Measurement of DC Potentials, ICP, and CBF
DC potentials were successfully measured at a total of 48 sites in 24 animals. As shown in Fig. 2, changes in DC potentials were divided into 3 types according to the appearance of ischemic depolarization following induction of SAH. In Type 1 (21 sites), ischemic depolarization was not observed during the 1-hour observation period. In Type 2 (13 sites), the DC potential demonstrated ischemic depolarization on initiation of SAH but recovered to 80% of the control level at 33.3 ± 15.8 minutes (minimum 4.7, maximum 51.3 minutes). In Type 3 (14 sites), the DC potential showed ischemic depolarization and did not recover during the 1-hour observation period. Changes in
ICP and CBF seen in each of these types are shown in Fig. 3. For all animals in this study, the mean control ICP was 9.0 ± 4.8 mm Hg. In the Type 1 group, CBF mildly decreased to 69.6% ± 9.5% of the control level due to the increase in ICP (40.1 ± 20.1 mm Hg), but this recovered to 80% of the control level 5.0 ± 5.2 minutes after onset of SAH. In the Type 2 group, CBF was moderately decreased to 36.3% ± 14.9% of the control level due to the increase in ICP (63.4 ± 23.3 mm Hg), but it recovered to 80% of the control level 24.0 ± 19.4 minutes after onset of SAH. In the Type 3 group, CBF was severely decreased to 24.6% ± 17.9% of the control level due to the increase in ICP (85.7 ± 12.1 mm Hg); at the end of the 1-hour observation period, CBF still had not recovered to 80% of the control level but rather remained at 49.7% ± 26.0%.

Observation of CSD
CSD was observed at a total of 6 sites in 3 rats, all in the Type 1 group, at 5.1 ± 2.2 minutes after initiation of SAH. CSD was initially observed via the medial DC electrode and was observed by means of the lateral DC electrode 0.95 ± 0.77 minutes later. Repolarization was observed in each electrode within 2.3 ± 1.2 minutes. No cases of multiple CSD during a single observation period were observed. Although CBF was 87.0% ± 6.5% of the control level before as well as during the passage of CSD (i.e., depolarization and repolarization), afterward CBF increased by 32.3% ± 12.8% compared with the control level but recovered to its pre-CSD level within 5.2 ± 0.7 minutes. ICP was 12.8 ± 5.5 mm Hg before the passage of CSD and remained unchanged during and after the passage of CSD.

Dynamic Changes in NADH Fluorescence
In the Type 1 group, fluorescence did not change with induction of SAH. In the Type 2 and Type 3 groups, however, the development of high-intensity fluorescence was observed in the entire hemisphere simultaneous with negative DC deflections (Fig. 4, A1–5). In the Type 1 group, during the passage of CSD, a wave of increased NADH fluorescence was observed simultaneously with a negative DC deflection (Fig. 4, B1–5). These waves of increased fluorescence were initially observed in the anterior cortex and propagated throughout the entire hemisphere in the direction of the occipital cortex at a rate of 3 mm/minute. After the passage of CSD, NADH fluorescence temporally decreased by 9.8% ± 7.6% (p = 0.02, compared with the control level) simultaneous with the increase in CBF. All CSDs propagated in this same manner (i.e., moving across the entire hemisphere in a rostral-to-caudal direction).

Histological Outcomes at DC Potential Recording Sites
Histological evaluation was successfully obtained at 26 sites in 13 rats (19 sites in the Type 1 group, 5 in the Type 2
group, and 2 in the Type 3 group). One of 7 rats in the Type 2 group and 4 of 7 rats in the Type 3 group died within 24 hours. In 6 rats (1 in the Type 1 group, 3 in the Type 2 group, and 2 in the Type 3 group), we failed to identify the DC potential recording sites during histological evaluation. In the Type 1 group all sites, including those sites undergoing CSD, showed intact tissue. In the Type 2 and Type 3 groups neuronal damage of varying severity was observed depending on the duration of ischemic depolarization. As shown in Fig. 5, the logistic regression curve shows a close relationship between the duration of depolarization and histological outcomes at DC potential recording sites (probit curve $r^2 = 0.701, p < 0.0001$). The duration of depolarization that induced ischemic cell changes in 50% of the pyramidal neurons was estimated to be 22.4 minutes along with a corresponding 95% confidence interval of 17.0–30.3 minutes.

**Discussion**

This is the first report to show the 2D propagation of CSD during the acute phase of SAH in rats. In our study, the passage of CSD was detected in the form of an increase in NADH fluorescence in the Type 1 group. CSD was observed to travel from the anterior cortex in the direction of the posterior cortex at rate of 3 mm/minute; depolarization began $5.1 \pm 2.2$ minutes after initiation of SAH, and repolarization followed within $2.3 \pm 1.2$ minutes. Beaulieu
The regression curve showed a high regression coefficient estimated to be 14.5, 22.4, and 30.9 minutes, respectively. Because the 5th layer of the parietal-temporal cortex were estimat-
ed for brain preservation. Further study will be required to confirm this hypothesis. The similarity between these 2 estimates implies that the duration of derangement of ion homeostasis is involved in a common mechanism that causes neuronal injury in focal ischemia and perforation models of SAH.

In this study, the Type 2 and Type 3 groups were found to have higher ICP and lower CBF values than the Type 1 group. The elevations of ICP appear to decrease cerebral perfusion pressure, resulting in a reduction of CBF. Since the duration of ischemic depolarization causing 50% neuronal damage was estimated to be 22.4 minutes, the Type 3 group, in which ischemic depolarization was sustained for more than 60 minutes, may be untreatable. In accordance with differences between the Type 2 and Type 3 groups, controlling ICP during the acute phase of SAH, especially during the 1st hour, is the most important factor for brain preservation. Further study will be required to confirm this hypothesis.

One limitation of the present study is the fact that CSD was detected in only 3 (12.5%) of 24 rats during an observation period lasting only 1 hour after onset of SAH. In the clinical setting, electrocorticography detected CSDs in 13 (72%) of 18 patients with SAH. CSDs in the clinical setting were observed up to 10 days after aneurysm surgery. CSDs observed during the period of delayed cerebral ischemia may have a pathogenic feature that differs from those observed in the present study.

Another limitation of the present study is the fact that the histological evaluation was performed 24 hours after SAH. In the clinical setting, the incidence of CSD was higher in patients with delayed brain infarct Days 7–9 after SAH. In that study, the occurrence of clusters of CSDs was related to delayed neurological deficit. Since the present study was designed to evaluate the impact of depolarization during the acute phase of SAH on histological outcomes, histological evaluation was performed 24 hours after SAH. Further study would be needed to elucidate the pathogenic feature of CSDs observed during a period of delayed cerebral ischemia.

**Conclusions**

CSD was successfully visualized using NADH fluorescence and was observed to propagate from the anterior cortex in the direction of the posterior cortex along with an increase in CBF. The duration of ischemic depolarization that causes damage in 50% of neurons was estimated to be 22.4 minutes. CSD was observed only in the mildest case within the 1st hour after onset of SAH and was not associated with histological damage.

**References**

2. Bederson JB, Germano IM, Guarino L: Cortical blood flow...

Disclosures
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions
Acquisition of data: Shimizu. Analysis and interpretation of data: Shimizu. Drafting the article: Shimizu. Administrative/technical/material support: Nishihiro, Shinji, Takasugi, Hiramatsu, Kawase, Sato, Mizoue. Study supervision: Takeda, Hishikawa, Sugiu, Morimatsu, Date.

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