ORIGINAL ARTICLE

Fluorometric Measurement of Intracellular pH *in vivo* in Feline Cerebral Cortex during Ischemia and Reperfusion

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Abstract. Intracellular acidosis has been considered to play a pivotal role in the progression of neuronal damage after cerebral ischemia. However, continuous measurement of the intracellular potential of hydrogen (pH) has not been done during and after ischemia. We measured temporal changes in intracellular pH in the feline cerebral cortex *in vivo* during and after ischemia using a novel fluorescent pH probe, 2',7'-biscarboxyethyl carboxyfluorescein (BCECF). A closed cranial window was installed in the left temporal skull. BCECF acetoxymethyl ester was superfused over the cortex, hydrolyzed and trapped in cortical cells. Intracellular pH was measured utilizing excitation light at 507 nm and fluorescent light at 550.5 nm. Focal cerebral ischemia for 60 minutes was induced by means of middle cerebral artery occlusion. Intracellular pH in the severely ischemic group became significantly acidic ($p < 0.01$) during ischemia and the acidosis persisted for at least 30 minutes after recirculation. The pH change was not significant in the mildly ischemic group. The severity of ischemia was determined based on the mean transit time, which was calculated from the hemodilution curve obtained by bolus injection of saline. The extent of ischemia was further confirmed pathologically ($p < 0.01$). The above results suggest that intracellular acidosis resulting from severe ischemia persists even after recirculation. (Keio J Med 47 (1): 37–41, March 1998)

Key words: acidosis, BCECF, focal cerebral ischemia, fluorescence, cranial window

Introduction

Intracellular acidosis has been considered to play a pivotal role in the progression of neuronal damage after cerebral ischemia.^{1–6} Mitochondrial damage,⁵ protein degeneration,³ synaptic suppression,⁴ inhibition of glucose utilization,⁶ free radical formation,² and lysosomal activation¹ have been reported as mechanisms underlying cellular damage in tissue acidosis. Several methods have been developed to measure the intracellular potential of hydrogen (pH) during cerebral ischemia including autoradiography,⁷ microelectrode⁸ and magnetic resonance.⁹ However, these methods do not provide continuous information on changes in intracellular pH. In addition, simultaneous estimation of cerebral blood flow (CBF) and intracellular pH during ischemia has been impossible. The purpose of the present study was to investigate the time-course of changes in

intracellular pH during and after ischemia by a fluorometric method. For the first time, we employed the fluorescent dye 2',7'-biscarboxyethyl carboxyfluorescein (BCECF),¹⁰ which can easily be trapped inside cells when superfused in the form of acetoxymethyl ester, to measure intracellular pH *in vivo*.

Materials and Methods

Animal preparation

Eighteen adult cats, weighing 2.5 to 3.5 kg, were divided into an ischemic group ($n = 14$) and a sham operation group ($n = 4$). They were anesthetized by intraperitoneal injection of 50 mg/kg α -chloralose (Sigma) and 500 mg/kg urethane (Sigma). After tracheotomy, they were ventilated with a respirator (Harvard Respirator, Model 662). The femoral and lingual arteries

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were catheterized to measure blood pressure and to inject saline into the cerebral circulation, respectively. The head was fixed in a stereotactic holder (Narishige, Tokyo). Following craniotomy at the left temporal skull, the dura matter was excised. A closed cranial window made of quartz, 10 mm in diameter with inlet and outlet tubes was installed. Artificial cerebrospinal fluid (ACSF) was superfused beneath the window throughout the experiments.

Fluorometric measurement of intracellular pH

ACSF containing 20 mM BCECF acetoxymethyl ester (Dojindo, Japan) was superfused at a rate of 5 ml/hr. This compound is hydrolyzed and becomes membrane-impermeable BCECF inside the cortical cells. After the loading of dye for sixty minutes, residual extracellular dye was washed out. Excitation light at 507 nm from a xenon arc lamp with a spectroscope (Nihonbunko, Japan) was focused onto the cerebral cortex and 550.5 nm BCECF fluorescent light emitted from the cortex was measured using a fluoromicroscope equipped with two photomultiplier tubes (Hamamatsu Photonics, Japan) (Fig 1).

Rink *et al*¹⁰ reported that intracellular pH and the fluorescence intensity of BCECF correlate positively at this wavelength. For example, relative fluorescence intensity is 15 arbitrary units at pH 6.4, 45 arbitrary units at pH 7.5 and 55 arbitrary units at pH 8.1. At least

in the range from pH 3.56 to pH 9.15, the more the acidosis of the specimen progresses, the lower the fluorescent intensity becomes.

The reflectance at 507 nm was also measured to correct apparent fluorescence intensity according to the method described below.

Mean transit time and influence of cerebral blood volume

During ischemia (10, 30 and 60 minutes after the start of ischemia) and recirculation (10 and 60 minutes after the start of recirculation), 0.2 ml of saline was repeatedly injected through the lingual artery such that the hemodilution induced caused a transient increase in reflectance and apparent fluorescence. Mean transit time (MTT), which is considered to be an indicator of CBF, was calculated from the recorded dilution curve based on Zieler's area-over-height method.¹¹

The dilution curve before ischemia was also utilized for the correction of apparent fluorescence intensity. The method was previously described elsewhere¹². The apparent fluorescence and reflectance intensity are influenced by changes in cerebral blood volume (CBV), as increased red blood cells absorb the light and lessen both intensities. Since a linear correlation exists between changes in fluorescence and reflectance, k factor and real fluorescence change can be expressed as follows:

$$k = DF/DR$$

DF: [(peak intensity) – (basal intensity)] of apparent fluorescence during hemodilution
DR: [(peak intensity) – (basal intensity)] of reflectance during hemodilution

$$CF = F - k \times R$$

CF: changes in corrected fluorescence intensity
F: changes in recorded fluorescence intensity
R: changes in reflectance intensity

Focal cerebral ischemia was induced by 60 minutes of middle cerebral artery (MCA) occlusion *via* the transorbital approach and intracellular pH was measured throughout the ischemic period and for 30 minutes after reperfusion. Depending on the MTT at 30 minutes after MCA occlusion, the ischemic group was further subdivided into mild (MTT < 10 sec) and severe (MTT > 10 sec) ischemia subgroups.¹³

Three hours after recirculation, the animals were sacrificed and the brains were formalin-fixed for pathological examination. The brain slices at the point of pH measurement were stained with hematoxylin-eosin for microscopic examination. The total area of infarction and its ratio to the hemisphere were calculated and

Fig 1 The *in vivo* fluorometric system. Excitation light at 507 nm from a xenon arc lamp with a spectroscope was focused onto the cerebral cortex and BCECF fluorescent light at 550.5 nm emitted from the cortex was measured using a fluoromicroscope equipped with two photomultiplier tubes. The reflectance at 507 nm was also measured to correct apparent fluorescence intensity. PMT: photomultiplier tube, BF barrier filter

compared between the groups.

For the statistical analysis of pH intensity and MTT, one-way ANOVA was applied to elucidate significant differences between the groups at each observation time. When significance was noted, the data of each group were further compared with those of the other group using Scheffe's multiple comparison. To compare the infarction area between the two groups, one-way ANOVA with Scheffe's multiple comparison was used.

Results

Intracellular pH decreased immediately after the induction of ischemia. One of the actual recordings of BCECF fluorescence and reflectance intensity is shown in Fig 2. Following MCA occlusion, fluorescence and the reflectance signal increased simultaneously reflecting decreased CBV. Within 3 seconds, fluorescence intensity decreased despite the unchanged reflectance intensity indicating that the tissue became acidic and the acidosis progressed. The acidosis persisted during ischemia. Following recirculation, fluorescence intensity increased whereas reflectance decreased, suggesting recovery from the acidosis.

MTT changes during the procedure are shown in Fig 3. Ischemic groups showed prolonged MTT, suggesting reduced CBF. The ischemic group was further subdivided into two subgroups, according to the criteria above. In the severe ischemia subgroup, MTT was significantly prolonged in comparison with the sham group ($p < 0.05$ at 10 and 60 minutes and $p < 0.01$ at 30 minutes). After recirculation, MTT recovered to the previous level.

The corrected fluorescence intensity is shown in Fig 4. In the severe ischemia subgroup, the intensity

Fig 2 The actual recording of BCECF fluorescence and reflectance intensity. Following MCA occlusion, fluorescence and the reflectance signal increased simultaneously reflecting decreased CBV. Within 3 seconds, fluorescence intensity decreased despite the unchanged reflectance intensity indicating that the tissue became acidic and the acidosis progressed. Following recirculation, fluorescence intensity increased whereas the reflectance decreased, suggesting recovery from acidosis. F: recorded fluorescence intensity, R: reflectance intensity

decreased rapidly after MCA occlusion indicating intracellular acidosis. Decreased fluorescence intensity was observed throughout the ischemic period. After recirculation, fluorescence intensity increased indicating recovery of pH, but the change was transient. In the mild ischemia subgroup, decreased fluorescence inten-

Fig 3 MTT changes of (mean $\pm$ SD). Ischemic groups showed prolonged MTT, suggesting reduced CBF. After recirculation, MTT recovered to the previous level.

pH Signal (% of control)

Fig 4 Changes in cortical intracellular pH (mean $\pm$ SD). In the severe ischemia subgroup, the tissue rapidly became acidic after MCA occlusion and the acidosis persisted throughout the ischemic period. After recirculation, a mild tendency for recovery from acidosis was observed but was transient. In the mild ischemia subgroup, the degree of acidosis was mild. Among the three groups, the intracellular pH change during and after ischemia in the severe ischemia subgroup was statistically significant in comparison to those in the mild ischemia subgroup ($p < 0.01$) and the sham group ($p < 0.01$).

Fig 5 Ratio of total infarction area to that of the affected hemisphere (mean $\pm$ SD). The calculated ratios were $18.0 \pm 14.7\%$ (severe ischemia subgroup), $2.6 \pm 3.2\%$ (mild ischemia subgroup) and 0% (sham group). The area of infarction in the severe ischemia subgroup was significantly larger than that of the other two groups ($p < 0.05$).

sity was also observed after MCA occlusion but the extent of acidosis was mild. Among the three groups, the reduced fluorescence intensity during and after ischemia in the severe ischemia subgroup was statistically significant in comparison to that in the mild ischemia subgroup ($p < 0.01$) and that in the sham group ($p < 0.01$).

Pathologically, the total area of infarction and the area of the hemisphere in the cross-section were measured. The calculated ratios of infarction to the hemisphere were $18.0 \pm 14.7\%$ (severe ischemia subgroup), $2.6 \pm 3.2\%$ (mild ischemia subgroup) and 0% (sham group) (Fig 5). The infarcted area in the severe ischemia subgroup was significantly larger than those of the mild ischemia subgroup and the sham group ($p < 0.05$).

Discussion

In this study, intracellular acidosis in the cerebral cortex was present during ischemia and persisted after recirculation in cases with severe ischemia. The severity of ischemia was determined based on MTT and pathologically confirmed as the larger infarcted area.

Intracellular pH during ischemia has been reported

by several authors. Using autoradiography with dimethylxazolidinedione, Hakim *et al*¹⁴ measured intracellular pH during 4 hour MCA occlusion, which decreased from the normal level (pH 7.0) to pH 6.7. However, the temporal profile of pH change and the relation to CBF were not determined. Komatsumoto *et al*⁹ used nuclear magnetic resonance for the measurement of intracellular pH in a feline MCA occlusion model and found that acidosis remained after reperfusion in severe ischemia. Their results correspond well with ours. By means of a microelectrode, Kraig *et al*⁸ assessed intracellular pH in astrocytes of the cerebral cortex *in vivo*. They reported that the most acidic locus (pH 4.3) appeared at 3 to 7 minutes after cardiac arrest. Smith *et al*¹⁵ calculated intracellular pH from tissue CO_2 tension and bicarbonate and found transient acidosis (pH 5.9) during cerebral ischemia. Mabe *et al*¹⁶ also assessed intracellular pH using the $\text{HCO}_3^-/\text{H}_2\text{CO}_3$ method and compared it with pH derived from creatine kinase equilibrium. Transient acidosis and recovery at 15 minutes into recirculation were shown by both methods. They all agreed on the point that intracellular acidosis was induced during cerebral ischemia, but the changes after reperfusion varied, presumably due to difficulty measuring intracellular pH continuously along with CBF. Our methods allow the repeated and simultaneous assessment of intracellular pH and cerebral circulation (MTT).

For the measurement of intracellular pH in brain slices using a fluorometric procedure, umbelliferon¹⁷ and neutral red¹⁸ have been used. In the present study, we used a fluorescent dye, BCECF. BCECF-AM easily permeates cells and becomes hydrophilic intracellularly. This dye is known to be loaded most efficiently among all of the fluorometric indicators¹⁰. Fujiwara *et al*¹⁹ utilized BCECF to measure the intracellular pH of brain slices during hypoxia. They reported biphasic acidosis during hypoxia and reperfusion, which is consistent with our results. They also pointed out a tendency for dye leakage. In our study, fluorescence intensity in the sham group decreased slightly with the passage of time, suggesting dye leakage or photo bleaching. However, the reduction in fluorescence intensity was clearly much larger in the severe ischemia subgroup than in the sham group.

In this study, the absolute pH value could not be determined, since the measurement of absolute pH requires either two wavelengths of excitation or two wavelengths of fluorescence. However, we had to correct the effect of CBV during the procedure by measuring reflectance at another wavelength and the correction of CBV is essential for proper assessment of cerebral pH *in vivo*. We avoided complicated and erroneous correction by using three wavelengths.

The most acidic cell in the cortex during ischemia is

believed to be the astrocyte⁸. Using a CCD camera or confocal laser microscopy, spatial resolution as well as time resolution is anticipated in further studies.

In conclusion, this study is the first to directly and continuously measure changes in intracellular acidosis in the cerebral cortex during and after cerebral ischemia. Though the results are comparable to those of previous reports, our fluorometric system elucidated for the first time that intracellular acidosis persists after recirculation in the setting of severe ischemia.

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