

## Alterations in Lipid and Calcium Metabolism Associated with Seizure Activity in the Postischemic Brain

Ken-ichiro Katsura, \*Elena B. Rodriguez de Turco, †Tibor Kristián, ‡Jaroslava Folbergrová, \*Nicolas G. Bazan, and †Bo K. Siesjö

Second Department of Internal Medicine, Nippon Medical School, Tokyo, Japan; \*Louisiana State University Eye Center and Neuroscience Center, New Orleans, Louisiana; †Center for the Study of Neurological Disease, Queen's Medical Center, Honolulu, Hawaii, U.S.A.; and ‡Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

**Abstract:** Transient ischemia is known to lead to a long-lasting depression of cerebral metabolic rate and blood flow and to an attenuated metabolic and circulatory response to physiological stimuli. However, the corresponding responses to induced seizures are retained, demonstrating preserved metabolic and circulatory capacity. The objective of the present study was to explore how a preceding period of ischemia (15 min) alters the release of free fatty acids (FFAs) and diacylglycerides (DAGs), the formation of cyclic nucleotides, and the influx/efflux of  $\text{Ca}^{2+}$ , following intense neuronal stimulation. For that purpose, seizure activity was induced with bicuculline for 30 s or 5 min at 6 h after the ischemia. Extracellular  $\text{Ca}^{2+}$  concentration ( $\text{Ca}^{2+}_e$ ) was recorded, and the tissue was frozen in situ for measurements of levels of FFAs, DAGs, and cyclic nucleotides. Six hours after ischemia, the FFA concentrations were normalized, but there was a lowering of the content of 20:4 in the DAG fraction. Cyclic AMP levels returned to normal values, but cyclic GMP content was reduced. Seizures induced in postischemic animals showed similar changes in  $\text{Ca}^{2+}_e$ , as well as in levels of FFAs, DAGs, and cyclic nucleotides, as did seizures induced in nonischemic control animals, with the exception of an attenuated rise in 20:4 content in the DAG fraction. We conclude that, at least in the neocortex, seizure-induced phospholipid hydrolysis and cyclic cAMP/cyclic GMP formation are not altered by a preceding period of ischemia, nor is there a change in the influx/efflux of  $\text{Ca}^{2+}$  during seizure discharge or in associated spreading depression. **Key Words:** Postischemia—Free fatty acids—Diacylglycerides—Calcium—Seizure.

*J. Neurochem.* **75**, 2521–2527 (2000).

electrical activity (Pulsinelli and Duffy, 1983; Ueki et al., 1988). However, the metabolic rate and blood flow remain depressed for hours to days (Pulsinelli et al., 1982b; Kozuka et al., 1989), suggesting a sustained disturbance of membrane function. In support of these proposals, physiological stimulations fail to give a normal metabolic and circulatory response (Dietrich et al., 1986; Ueki et al., 1988). Alterations in metabolic functions are also reflected in a sustained decrease of pyruvate dehydrogenase complex activity (Cardell et al., 1989). These changes probably reflect a disturbance of the stimulus–response–metabolism coupling, rather than overt damage to plasma membranes and/or mitochondria. Thus, when postischemic tissues are challenged with bicuculline-induced seizures, they respond with a brisk increase in metabolic rate and blood flow (Katsura et al., 1994). This response indicates that the metabolic capacity of postischemic tissues, at least those of neocortical origin, is maintained in the period when the metabolic rate is markedly reduced and functions related to physiological stimulations are depressed.

These findings give rise to questions regarding the molecular mechanisms involved in the disturbed stimulus–response–metabolism coupling during the postischemic period. Furthermore, these mechanisms may also be responsible for the delayed cell death. To elucidate these mechanisms, we investigated the metabolic response of postischemic brain to seizures, particularly the release of free fatty acids (FFAs) and diacylglycerides (DAGs), the release/reuptake of calcium, and the

Transient ischemia of brief to intermediate duration is known to result in neuronal damage that is often conspicuously delayed (Kirino, 1982; Pulsinelli et al., 1982a). In the “free” interval between the initial insult and the ultimate tissue damage, cells, e.g., those in the CA1 sector of the hippocampus, resume both oxidative phosphorylation and spontaneous, as well as evoked,

Received February 29, 2000; revised manuscript received July 20, 2000; accepted July 20, 2000.

Address correspondence and reprint requests to Dr. K. Katsura at Second Department of Internal Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-Ku, Tokyo 113-8603, Japan. E-mail: k-katsur@nms.ac.jp

*Abbreviations used:*  $\text{Ca}^{2+}_e$ , extracellular  $\text{Ca}^{2+}$  concentration; cAMP, cyclic AMP; cGMP, cyclic GMP; DAG, diacylglyceride; FFA, free fatty acid.

changes in levels of cyclic AMP (cAMP)/cyclic GMP (cGMP).

Substantial amounts of the FFAs that accumulate in the brain during seizures and ischemia, particularly during the initial periods, originate from inositol phospholipids enriched in 18:0 and 20:4 (Ikeda et al., 1986; Yoshida et al., 1986; Abe et al., 1987; Reddy and Bazan, 1987; Sun et al., 1990). Activation of phospholipase C is followed by the release of phosphoinositide-derived DAGs and their subsequent degradation to FFAs and glycerol, which is mediated by DAG-lipase and monoacylglyceride-lipase. However, in the early phase of FFA accumulation triggered by ischemia and seizures, a significant contribution from phospholipase A<sub>2</sub> cannot be excluded (Bazan et al., 1993; Katsura et al., 1993). In fact, phenylmethylsulfonyl fluoride, an inhibitor of phospholipase C, inhibits the accumulation of FFAs in rat neocortex during the first 2 min of ischemia, while minimally affecting the later phospholipase A<sub>2</sub>-mediated release of fatty acids from phosphatidylethanolamine and phosphatidylcholine (Uemura et al., 1992).

In the present study, we aim to define mechanisms of FFA accumulation during seizures in the posts ischemic phase, correlating the data to changes in calcium metabolism and changes in levels of cyclic nucleotides.

## MATERIALS AND METHODS

### Operative and sampling techniques

The animal experiments were performed in the Laboratory for Experimental Brain Research, Experimental Research Center, Lund University Hospital, Lund, Sweden. Male Wistar rats (weighing 300–350 g) allowed free access to tap water, were fasted overnight. The animals were anesthetized with 3% halothane in 70% N<sub>2</sub>O and 30% O<sub>2</sub>, intubated, and artificially ventilated. Halothane was maintained at 1.5% during the operative procedure and, after the operation, at 0.5% throughout the whole experimental procedure. All procedures followed the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Lund University Animal Ethical Committee. A tail artery and tail vein were cannulated for blood sampling, blood pressure recording, and injection of a muscle relaxant (vecuronium bromide; Norcuron; 2 mg h<sup>-1</sup>), and the right jugular vein was cannulated for withdrawal of blood during ischemia or during seizure. The common carotid arteries were isolated via a neck incision. For EEG recording, a pair of needle electrodes was inserted into the muscles lateral to the skull bone. Skull temperature was monitored by insertion of a temperature probe under the skin of the scalp and maintained at 37°C during ischemia.

### Induction of ischemia and of continuous seizures

The experimental protocol was similar to that described by Katsura et al. (1994). In summary, incomplete ischemia was induced by lowering the blood pressure to 50 mm Hg by exsanguination of blood via the jugular vein, followed by clamping both common carotid arteries with neurosurgical clips for 15 min (Smith et al., 1984). After 6 h of recovery, the animals were reanesthetized and artificially ventilated. The skin overlapping the scalp was incised, and a plastic funnel was placed over the skull bone for freezing the brain in situ at 30 s

or 5 min after the induction of seizures. Bicuculline hydrochloride (1.2 mg kg<sup>-1</sup>) was injected intravenously to induce seizures for 5 min (Chapman et al., 1977). Body temperature was maintained close to 37°C, and the mean blood pressure was maintained at ~120 mm Hg during the seizure activity. Bicuculline seizures were induced in both nonischemic rats and in those subjected to ischemia 6 h previously.

### Extracellular calcium measurements

Separate groups of animals were used for electrode measurements of extracellular calcium concentration (Ca<sup>2+</sup>) and direct current potential. Ca<sup>2+</sup>-sensitive microelectrodes were prepared with a calcium ion exchanger (Cocktail A, 21048; Fluka) (see Kristián et al., 1994). The tip of the double-barrel electrode (diameter of ~5 μm) was positioned in the frontoparietal cortex at a depth of 750 μm. An Ag/AgCl<sub>2</sub> reference electrode in a physiological saline-agar bridge was inserted subcutaneously in the neck.

### Analytical techniques

After the brains were frozen in situ (Pontén et al., 1973), they were chiselled out and stored at -80°C. The superficial 1 mm layer of the neocortex was dissected at -25°C. Samples of 20 mg were used for cyclic nucleotides, and ~150-mg samples were used for quantification of FFAs and DAGs, as described previously (Katsura et al., 1993). In brief, frozen tissue was homogenized with a Polytron in 2 ml of chloroform/methanol (2:1, vol/vol), and lipids were extracted and purified (Folch et al., 1957). FFAs and DAGs were isolated by monodimensional TLC on silica gel GHL plates (Analtech, Newark, DE, U.S.A.) using hexane/ethyl ether/acetic acid (45:55:1.3, by volume) as a chromatographic system. Lipid bands were identified by spraying the plates with 0.005% 2',7'-dichlorofluorescein in methanol and visualized under UV light. FFAs and DAGs were derivatized to fatty acid methyl esters and quantified by GLC using 17:0 and 21:0 methyl esters as internal standards (Marcheselli and Bazan, 1990). Duplicate aliquots from each lipid extract were taken for determination of total lipid phosphorus content (Rouser et al., 1970).

For measurements of cAMP levels, the cortical samples were extracted with HCl-methanol followed by perchloric acid, and the extracts were neutralized with KOH-imidazole base-KCl mixture (Folbergrová et al., 1969). cAMP was quantified with a protein binding technique (cyclic AMP assay kit; Amersham, U.K.). For the measurements of cGMP levels, an acidic extract was used (Lowry and Passonneau, 1972). KHCO<sub>3</sub> and acetate buffers (pH 4.6) were added to maintain pH at 4.6–5.0. For measuring cGMP levels, we used radioimmunoassay, using a cyclic GMP <sup>125</sup>I-labeled radioimmunoassay kit (New England Nuclear, Boston, MA, U.S.A.) (Steiner et al., 1972).

### Statistics

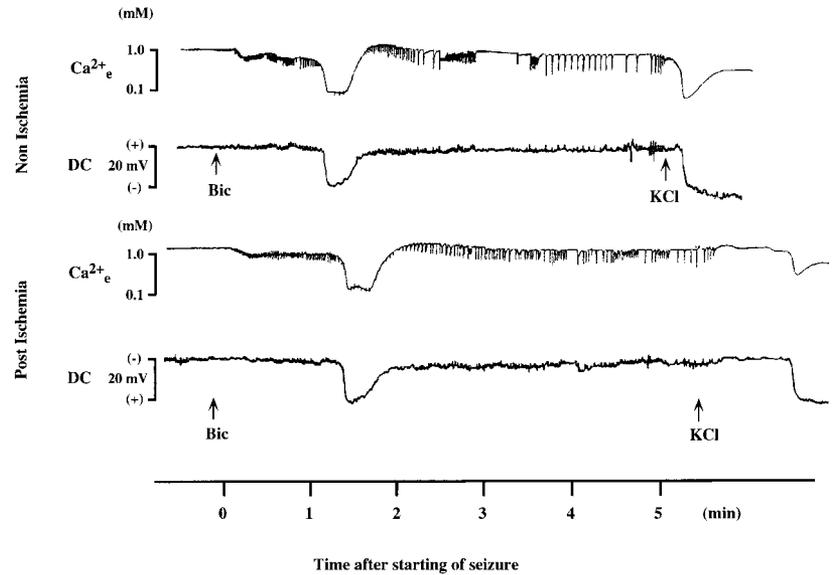
All data are expressed as mean ± SD values. Statistical differences were calculated using one-factor ANOVA followed by Scheffé's test and two-factor ANOVA followed by Tukey's test, as well as Student's *t* test.

## RESULTS

### Physiological parameters and changes in Ca<sup>2+</sup> levels

Physiological parameters in all experimental groups were within the ranges generally considered normal. There were no significant differences in temperature,

**FIG. 1.** Changes in  $\text{Ca}^{2+}_e$  and direct current (DC) potential in the rat cortex during bicuculline (Bic)-induced seizure discharge. The upper two traces show the response in a nonischemic animal, and the lower two traces show the response in a postischemic animal. In both nonischemic and postischemic animals, a spreading depression-like wave occurred at  $\sim 1$  min after Bic injection.



mean arterial blood pressure (MABP),  $P_{aO_2}$ ,  $P_{aCO_2}$ , pH, or plasma glucose levels between groups.

Figure 1 shows a typical recording with the  $\text{Ca}^{2+}$ -sensitive double-barrel microelectrode. Approximately 15 s after bicuculline injection, seizure activity started, and at 30 s,  $\text{Ca}^{2+}_e$  slightly decreased. A spreading depression-like depolarization wave followed, and  $\text{Ca}^{2+}_e$  decreased to values of 0.1–0.2 mM. Recovery from the depolarization was relatively slow, and, at 5 min after seizure onset,  $\text{Ca}^{2+}_e$  levels remained at or slightly below baseline. The changes were similar during nonischemic and postischemic seizures. Thus, no significant differences were found between the two groups in time to seizure onset after injection of bicuculline, in time to depolarization from the onset of seizure activity, in duration of depolarization, in baseline  $\text{Ca}^{2+}_e$  levels, in  $\Delta\text{Ca}^{2+}$  decrease at 30 s or 5 min after the onset of seizure, or in maximal  $\Delta\text{Ca}^{2+}$  during the initial depolarization (Table 1). We conclude therefore that although postischemic tissue was metabolically depressed, the calcium transient caused by seizures or spreading depression was not altered.

#### Accumulation of FFAs and DAGs

Previous studies have shown that bicuculline-induced seizures in nonischemic animals give rise to the accumulation of FFAs and DAGs, particularly free 20:4, free 18:0, and 20:4-DAGs (Siesjö et al., 1982; Rodriguez de Turco et al., 1983; Bazan et al., 1993). The present study gave similar results (Table 2). During early seizures (0.5 min after bicuculline injection), the FFA pool was increased 2.2-fold above basal, with free 20:4 displaying the greatest relative change (18-fold), followed by 18:0 (2.5-fold), 18:1 (1.9-fold), and 22:6 (1.8-fold). After 5 min of seizure activity, FFAs continued to accumulate (3.4-fold above basal), with 20:4 and 18:0 levels reaching 24- and 4.8-fold increases, respectively. At this later time, free 16:0 content was also increased by 1.5-fold.

After a 6-h recovery from the ischemic insult, the basal FFA pool was the same as that of control animals. The accumulation of FFAs during early seizures was also similar to that of nonischemic animals, in which 20:4 and 18:0 levels reached 24- and threefold increases, respectively (Table 2). A difference in the response to bicuculline with respect to nonischemic animals was observed after 5 min of seizures. Free 20:4 values did not increase after 0.5 min of seizures, whereas other FFAs continued to accumulate.

In nonischemic animals, DAG accumulation during early seizures was only significant for 20:4 (1.4-fold increase), whereas after 5 min of seizures, a significant accumulation of 20:4-DAG (1.7-fold) as well as 22:6-DAG (1.6-fold) and 18:1-DAG (1.4-fold) was observed

**TABLE 1.**  $\text{Ca}^{2+}_e$  changes during 5 min of seizure

	Seizure	
	Nonischemic	Postischemic
No.	6	5
Time(s) to		
Seizure after injection of bicuculline	14.2 ± 4.2	17.0 ± 8.4
Depolarization from onset of seizure	44.6 ± 22	70.8 ± 28
Duration of initial depolarization (s)	35.2 ± 17	39.6 ± 9.4
Baseline $\text{Ca}^{2+}_e$ levels (mM)	1.10 ± 0.12	1.04 ± 0.13
$\Delta\text{Ca}^{2+}_e$ decrease (mM) after onset of seizure		
At 30 s	0.15 ± 0.03	0.12 ± 0.10
At 5 min	0.20 ± 0.05	0.19 ± 0.13
Maximal $\Delta\text{Ca}^{2+}_e$ during initial depolarization (mM)	0.99 ± 0.14	0.92 ± 0.10

No significant differences were found between nonischemic seizure and postischemic seizure.

**TABLE 2.** Changes in cAMP, cGMP, and FFAs (F) and fatty acid composition of DAGs (D) during seizure activity

	Basal	After seizure	
		0.5 min	5 min
<b>Nonischemic</b>			
cAMP	1.28 ± 0.18		
cGMP	0.12 ± 0.02 <sup>a</sup>		
F16:0	13.10 ± 3.99	16.37 ± 4.36	20.26 ± 2.06 <sup>a</sup>
F18:0	11.35 ± 1.80	28.37 ± 5.56 <sup>a</sup>	54.05 ± 7.01 <sup>a,b</sup>
F18:1	5.15 ± 0.84	9.65 ± 1.77 <sup>a</sup>	11.65 ± 2.03 <sup>a</sup>
F18:2	2.37 ± 0.71	2.47 ± 0.33	2.89 ± 1.16
F20:4	0.96 ± 0.15	17.64 ± 2.58 <sup>a</sup>	23.14 ± 5.75 <sup>a,b</sup>
F22:6	0.78 ± 0.26	1.38 ± 0.28 <sup>a</sup>	1.66 ± 0.66 <sup>a</sup>
F <sub>Total</sub>	35.2 ± 5.8	77.0 ± 11.0 <sup>a</sup>	118.8 ± 16.6 <sup>a,b</sup>
D16:0	17.55 ± 3.38	17.97 ± 2.89	21.28 ± 3.05
D18:0	22.46 ± 2.44	23.62 ± 1.51	24.22 ± 1.75
D18:1	9.41 ± 2.07	11.74 ± 1.07	13.25 ± 1.64 <sup>a</sup>
D18:2	1.09 ± 0.53	1.13 ± 0.17	1.13 ± 0.20
D20:4	11.72 ± 2.45	16.20 ± 0.94 <sup>a</sup>	19.96 ± 2.05 <sup>a,b</sup>
D22:6	2.46 ± 0.44	3.30 ± 0.46	4.01 ± 0.91 <sup>a</sup>
D <sub>Total</sub>	64.7 ± 7.6	74.0 ± 3.2	83.9 ± 6.5 <sup>a</sup>
<b>At 6 h of recovery after ischemia</b>			
cAMP	1.27 ± 0.15	4.96 ± 0.48 <sup>a</sup>	2.41 ± 0.19 <sup>a,b</sup>
cGMP	0.067 ± 0.01 <sup>c</sup>	0.14 ± 0.02 <sup>a</sup>	0.17 ± 0.03 <sup>a</sup>
F16:0	14.55 ± 1.75	16.95 ± 4.17	20.00 ± 2.68 <sup>a</sup>
F18:0	11.60 ± 0.65	34.49 ± 7.22 <sup>a</sup>	51.94 ± 5.83 <sup>a,b</sup>
F18:1	5.64 ± 1.19	9.39 ± 1.77 <sup>a</sup>	11.01 ± 1.99 <sup>a</sup>
F18:2	2.07 ± 0.25	2.87 ± 0.64	2.55 ± 0.91
F20:4	0.88 ± 0.19	21.12 ± 4.99 <sup>a</sup>	19.02 ± 2.10 <sup>a</sup>
F22:6	0.88 ± 0.21	1.53 ± 0.24	1.89 ± 0.67 <sup>a</sup>
F <sub>Total</sub>	35.6 ± 3.0	86.3 ± 15.9 <sup>a</sup>	106.4 ± 10.4 <sup>a,b</sup>
D16:0	16.80 ± 2.38	19.85 ± 2.54	17.77 ± 4.39
D18:0	21.34 ± 3.47	25.54 ± 4.56	26.20 ± 4.70
D18:1	8.79 ± 0.30	10.63 ± 1.18	10.95 ± 1.81
D18:2	0.73 ± 0.09	1.33 ± 0.59	1.06 ± 0.18
D20:4	8.04 ± 2.26 <sup>d</sup>	15.36 ± 3.40 <sup>a</sup>	14.79 ± 2.60 <sup>a,d</sup>
D22:6	2.49 ± 0.76	3.64 ± 1.00	3.83 ± 0.66
D <sub>Total</sub>	58.2 ± 4.2	76.4 ± 10.8 <sup>a</sup>	74.6 ± 7.9 <sup>a</sup>

Data are mean ± SD values, expressed in nmol/mg of lipid phosphorus in fatty acids and  $\mu\text{M kg}^{-1}$  in cyclic nucleotides.

<sup>a</sup>  $p < 0.05$  against basal value, <sup>b</sup>  $p < 0.05$  against 0.5 min value by one-factor ANOVA, followed by Scheffé's test.

<sup>c</sup>  $p < 0.05$  against nonischemic basal value by Student's *t* test.

<sup>d</sup>  $p < 0.01$  against corresponding nonischemic value by two-factor ANOVA, followed by Tukey's post hoc test.

(Table 2). In postischemic animals, two differences were observed: First, the basal level of 20:4-DAGs was 30% lower than in nonischemic animals ( $p < 0.01$  by Student's *t* test). Second, after 5 min of seizure discharge, accumulation of 20:4-DAGs had not increased above the value of 0.5 min. Furthermore, the level of 20:4-DAGs at 5 min was significantly lower than the nonischemic seizure value ( $p < 0.05$  by two-factor ANOVA followed by Tukey's post hoc test).

Table 3 shows the net amounts of FFAs and DAG-acyl group accumulated during the first 0.5 min, between 0.5 and 5 min, and after 5 min of seizure activity. In both groups (nonischemic and postischemic) the nanomolar amount of FFA accumulated during the first 0.5 min of seizure was very similar, with 18:0 and 20:4 levels increasing in a 1:1 molar relationship. The difference

between nonischemic and postischemic seizure then lies in the accumulation of free 20:4 and 20:4-DAG between 0.5 and 5 min, which was observed only in nonischemic seizures, and in the significantly higher accumulation of total DAGs after 0.5 min of seizure activity in postischemic animals. Despite these differences, the total free 20:4 and 20:4-DAG accumulation during the 0–5-min period of seizure activity was similar in both groups, with the only difference being a significant accumulation of 18:1- and 22:6-DAGs in the nonischemic animals.

### Cyclic nucleotides

Because changes in content of cyclic nucleotides during bicuculline-induced seizures in nonischemic animals have already been established (Siesjö et al., 1983), only postischemic values were measured. The cAMP values recovered to basal control levels at 6 h after 15 min of ischemia. After 30 s of seizure activity, cAMP content increased approximately fourfold and then decreased to ~200% of control at 5 min. These changes were similar to those recorded in nonischemic animals (Siesjö et al., 1983). After 6 h of postischemic recovery, cGMP content was significantly lower than control, but seizures resulted in an expected rise in cGMP concentration. The relative increase in postischemic animals during the initial phase of seizure activity was not markedly different (two- to threefold increase) from that in nonischemic animals [approximately fourfold increase (Folbergrová et al., 1981)]; however, the absolute value at 30 s or 5 min of seizure activity seemed somewhat lower in nonischemic animals, which had values of  $\sim 0.4 \mu\text{M kg}^{-1}$  after 1–20 min of seizure activity (Folbergrová et al., 1981; Siesjö et al., 1983).

### DISCUSSION

In the present study we have established the following: (a) When animals are subjected to 15 min of transient ischemia and seizures are induced by bicuculline after 6 h of recovery, the seizure-induced decrease in  $\text{Ca}^{2+}_e$  or the subsequent decrease of  $\text{Ca}^{2+}_e$  during the spreading depression-like depolarization is not altered from that seen in control groups, i.e., nonischemic animals, subjected to seizures, nor is the rate of calcium extrusion altered after the spreading depression-like transient. (b) In both control and postischemic animals, seizures are similarly accompanied by a marked increase in FFA concentrations, particularly 18:0 and 20:4. (c) The baseline content of 20:4 in the DAG fraction decreased after 15 min of ischemia and 6 h of recovery. The content notably increased during seizures in both control and postischemic animals; however, the level attained by 20:4-DAGs in postischemic animals after 5 min of seizures was lower than in controls. (d) In postischemic animals, accumulation of free 20:4 and 20:4-DAGs was observed only during the first 30 s of seizure activity. In control animals, although the bulk of 20:4 mobilization also occurred during the first 30 s of seizures, it continued throughout the remainder of the 5 min of seizure

**TABLE 3.** Accumulated FFAs and DAGs during 0.5 or 5 min of seizure activity

	FFAs			DAGs		
	0–0.5 min	0–5 min	0.5–5 min	0–0.5 min	0–5 min	0.5–5 min
Nonischemia						
16:0	—	7.2	—	—	—	—
18:0	17.0	42.7	25.7	—	3.8 <sup>a</sup>	—
18:1	4.5	6.5	—	—	—	—
20:4	16.7	22.2	5.5 <sup>a</sup>	4.5	8.2	3.8 <sup>a</sup>
22:6	0.6	0.9	—	—	1.6 <sup>a</sup>	—
Total	41.8	83.6	41.8	—	19.2	—
Postischemia						
16:0	—	5.5	—	—	—	—
18:0	22.9	40.3	17.5	—	—	—
18:1	3.8	5.4	—	—	—	—
20:4	20.2	18.1	—	7.3	6.8	—
22:6	0.7	1.0	—	—	—	—
Total	50.7	70.8	20.1	18.2 <sup>a</sup>	16.4	—

Data, expressed as nmol/mg of lipid phosphorus, are the accumulated values during the first 30 s, 5 min, and from 30 s to 5 min of seizure activity. A dash indicates not significantly accumulated. The "total" values were calculated as the difference between individual total values and the basal values or 0–0.5 min values shown in Table 2. For the 0–0.5 and 0–5 min groups, only experimental values that were significantly different from basal values in Table 2 were used. For the 0.5–5 min groups, calculations were performed if the difference between the 0–0.5 min value and 0.5–5 min value became significant in Table 2.

<sup>a</sup> Significant difference between nonischemic and postischemic seizure.

activity. The net accumulation of free 20:4 and 20:4-DAGs during 0–5 min of seizures, however, was similar for both groups. (e) After 6 h of recovery, following 15 min of ischemia, cAMP content returned to normal, but cGMP content was clearly reduced. Although both cyclic nucleotides showed the expected relative increases during the seizure discharge, the absolute increase in cGMP content in postischemic animals seemed lower than that in nonischemic animals.

The background information outlined in the introductory section showed that transient ischemia leads to a sustained reduction of metabolic rate and blood flow. Furthermore, other studies demonstrate a disturbance of the stimulus–response–metabolism coupling (Dietrich et al., 1986; Ueki et al., 1988). However, this effect represents a functional block because both spreading depression and seizure discharge trigger normal increases in metabolic rate and blood flow (Kocher, 1990; Katsura et al., 1994), indicating that the metabolic and circulatory capacity of the tissue is preserved.

The present study was undertaken to explore whether ischemia alters the activation of phospholipases or the accumulation of cyclic nucleotides during seizures in postischemic animals. As these events depend on agonist stimulation of surface receptors and on influx of calcium into cells, the results should provide information on membrane function. Additional information was obtained by measurements of  $\text{Ca}^{2+}_e$  during seizure discharge. The analysis of calcium fluxes was facilitated by the fact that seizure discharge results in spreading de-

pression-like depolarization and a reduction of  $\text{Ca}^{2+}_e$  to 0.1–0.2 mM.

### Preseizure metabolic state

Previous results in rats have demonstrated that mitochondrial metabolism and cerebral energy states are normalized after brief to moderately long periods of ischemia (Ljunggren et al., 1974; Pulsinelli and Duffy, 1983; Hillered et al., 1985; Katsura et al., 1994). Levels of FFAs are increased during ischemia, and the increase persists during the initial recirculation period; however, a gradual normalization occurred, and the ischemic changes were essentially reversed at 30 min after restoration of circulation (Rehncrona et al., 1982). Our results provide additional information about FFAs, DAGs, and cyclic nucleotides, as well as on spreading depression-induced calcium fluxes in the later phase of recirculation. The results reveal a paucity of changes induced by the preceding ischemia. Thus, calcium levels were similar to those observed in nonischemic animals, and levels of FFAs and DAGs had largely normalized. The only lasting changes were a reduction in cGMP concentration, as well as a reduction in level of 20:4-DAGs. Thus, despite the massive perturbation of lipid metabolism by the preceding ischemia, we found that 6 h of recirculation is sufficient to achieve almost complete normalization of cerebral metabolic state. However, such normalization may be transient because both cerebral energy state and FFA content are altered after longer recovery periods, probably paralleling the delayed cell death (Pulsinelli and Duffy, 1983; Nakano et al., 1990; Abe et al., 1992).

### Response to bicuculline-induced seizures

Six hours after 15 min of ischemia, values for metabolic rate and blood flow are reduced to ~50% of control; yet, seizures raise these values to levels similar to those observed in nonischemic animals. Furthermore, changes in content of labile metabolites are similar between control and postischemic animals (Katsura et al., 1994). Thus, the section of the signal transduction pathway that involves  $\text{Ca}^{2+}$ , cyclic nucleotides, and DAGs/FFAs does not seem to be measurably altered by the preceding ischemia. We conclude therefore that the reduction of metabolic rate and blood flow, as well as the perturbed signal transduction, are caused by more subtle changes than those recorded in the present experiments.

The present results give indirect evidence for the involvement of phospholipase C in the accumulation of FFAs (Table 3). During the first 30 s of seizure discharge, increases in levels of free 18:0 and 20:4 were identical in both controls and postischemic animals. In other experimental conditions such as ischemia (Avelaño and Bazan, 1975) and electroconvulsive seizures (Reddy and Bazan, 1987), hydrolysis of inositol lipids gives rise to 18:0- and 20:4-DAGs, which are further degraded by lipases, contributing to the enlargement of the pools of free 18:0 and 20:4 (Bazan et al., 1993). During early seizures induced by bicuculline, only levels of 20:4-DAGs were significantly increased. Therefore, it is possible that phospholipase C-mediated release of 18:0- and 20:4-DAGs is efficiently coupled to their subsequent degradation by DAG-lipase and monoacylglyceride-lipase. During the later phase, i.e., in the period 30 s to 5 min, more 18:0 accumulated than 20:4, suggesting the involvement of phospholipases other than phospholipase C. Even if phospholipases  $A_2/A_1$ , together with phospholipase C, were involved in early and late accumulation of FFAs, the present results would suggest that inositol lipids, highly enriched in 18:0 and 20:4 species, are the primary targets during early seizures induced by bicuculline. As shown in Table 3, the increase in total DAG content at 30 s after seizure onset was significant, showing a larger change in postischemic than in nonischemic animals. This response may reflect a higher phospholipase C activity due to down-regulation of the feedback inhibition by protein kinase C. It has been established that protein kinase C is translocated and down-regulated after ischemia (Wieloch et al., 1991) and that its feedback regulation is hampered (Seren et al., 1989; Kirino et al., 1992).

Figure 1 shows that the later phase, i.e., the period 30 s to 5 min, is dominated by ongoing seizure discharge with a superimposed spreading depression-like wave. Because the spreading depression-like discharge leads to massive cellular influx of calcium, the rise in intracellular  $\text{Ca}^{2+}$  concentration may lead to activation of phospholipase  $A_2/A_1$ . This response is consistent with the different composition of FFAs during this phase ( $\Delta 18:0$  was not equal to  $\Delta 20:4$ ). However, there was no additional accumulation of arachidonic acid in either the free or the DAG fraction in this period of postischemic animals. It

remains to be seen whether the lack of changes in 20:4 content during the later phase can be explained by depletion of phospholipid substrates by the preceding ischemia. Because in postischemic animals the lack of 20:4 mobilization between 0.5 and 5 min followed an early apparently more active phase of accumulation of free 20:4 and 20:4-DAGs, it is also possible that there is a limited pool of 20:4-phospholipids that is susceptible to hydrolysis and thus depleted more rapidly in postischemic than in control animals.

**Acknowledgment:** This study was supported by the Swedish Medical Research Council, by U.S. Public Health Service grant NS23002 from the National Institutes of Health, and by Grant-in Aid for Scientific Research (C), 10670610, 1999, from the Ministry of Education of Japan.

### REFERENCES

- Abe K., Kogure K., Yamamoto H., Imazawa M., and Miyamoto K. (1987) Mechanism of arachidonic acid liberation during ischemia in gerbil cerebral cortex. *J. Neurochem.* **48**, 503–509.
- Abe K., Araki T., Kawagoe J., Aoki M., and Kogure K. (1992) Phospholipid metabolism and second messenger system after brain ischemia. *Adv. Exp. Med. Biol.* **318**, 183–195.
- Avelaño M. and Bazan N. (1975) Rapid production of diacylglycerols enriched in arachidonate and stearate during early brain ischemia. *J. Neurochem.* **25**, 919–920.
- Bazan N. G., Geoffrey A., and Rodriguez de Turco E. B. (1993) Role of phospholipase  $A_2$  and membrane-derived lipid second messengers in excitable membrane function and transcriptional activation of genes: implications in cerebral ischemia. *Prog. Brain Res.* **96**, 247–257.
- Cardell M., Koide T., and Wieloch T. (1989) Pyruvate dehydrogenase activity in the rat cerebral cortex following cerebral ischemia. *J. Cereb. Blood Flow Metab.* **9**, 350–357.
- Chapman A., Meldrum B., and Siesjö B. (1977) Cerebral metabolic changes during prolonged epileptic seizures in rats. *J. Neurochem.* **28**, 1025–1035.
- Dietrich W. D., Ginsberg M. D., and Busto R. (1986) Effect of transient cerebral ischemia on metabolic activation of a somatosensory circuit. *J. Cereb. Blood Flow Metab.* **6**, 405–413.
- Folbergrová J., Passonneau J. V., Lowry O. H., and Schulz D. W. (1969) Glycogen, ammonia and related metabolites in the brain during seizures evoked by methionine sulphoximine. *J. Neurochem.* **16**, 191–203.
- Folbergrová J., Ingvar M., and Siesjö B. K. (1981) Metabolic changes in cerebral cortex, hippocampus, and cerebellum during sustained bicuculline-induced seizures. *J. Neurochem.* **37**, 1228–1238.
- Folch J., Lees M., and Sloane-Stanley G. H. (1957) A simple method for the purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497–509.
- Hillered L., Smith M.-L., and Siesjö B. K. (1985) Lactic acidosis and recovery of mitochondrial function following forebrain ischemia in the rat. *J. Cereb. Blood Flow Metab.* **5**, 259–266.
- Ikeda M., Yoshida S., Busto R., Santiso M., and Ginsberg M. D. (1986) Polyphosphoinositides as a probable source of brain free fatty acids accumulated at the onset of ischemia. *J. Neurochem.* **47**, 123–132.
- Katsura K., Rodriguez de Turco E. B., Folbergrová J., Bazan N. G., and Siesjö B. K. (1993) Coupling among energy failure, loss of ion homeostasis, and phospholipase  $A_2$  and C activation during ischemia. *J. Neurochem.* **61**, 1677–1684.
- Katsura K., Folbergrová J., Gidö G., and Siesjö B. K. (1994) Functional, metabolic, and circulatory changes associated with seizure activity in the postischemic brain. *J. Neurochem.* **62**, 1511–1515.
- Kirino T. (1982) Delayed neuronal death in the gerbil hippocampus following transient ischemia. *Brain Res.* **239**, 57–69.

- Kirino T., Robinson H. P., Miwa A., Tamura A., and Kawai N. (1992) Disturbance of membrane function preceding ischemic delayed neuronal death in the gerbil hippocampus. *J. Cereb. Blood Flow Metab.* **12**, 408–417.
- Kocher M. (1990) Metabolic and hemodynamic activation of postischemic rat brain by cortical spreading depression. *J. Cereb. Blood Flow Metab.* **10**, 564–571.
- Kozuka M., Smith M.-L., and Siesjö B. K. (1989) Preischemic hyperglycemia enhances postischemic depression of cerebral metabolic rate. *J. Cereb. Blood Flow Metab.* **9**, 478–490.
- Kristián T., Katsura K., Gidö G., and Siesjö B. K. (1994) The influence of pH on cellular calcium influx during ischemia. *Brain Res.* **641**, 295–302.
- Ljunggren B., Ratcheson R. A., and Siesjö B. K. (1974) Cerebral metabolic state following complete compression ischemia. *Brain Res.* **73**, 292–307.
- Lowry O. H. and Passonau J. V. (1972) *A Flexible System of Enzymatic Analysis*. Academic Press, New York.
- Marcheselli V. L. and Bazan N. G. (1990) Quantitative analysis of fatty acids in phospholipids, and diacylglycerols, free fatty acids, and other lipids. *J. Nutr. Biochem.* **1**, 382–388.
- Nakano S., Kogure K., and Fujikura H. (1990) Ischemia-induced slowly progressive neuronal damage in the rat brain. *Neuroscience* **38**, 115–124.
- Pontén U., Ratcheson R. A., Salford L. G., and Siesjö B. K. (1973) Optimal freezing conditions for cerebral metabolites in rats. *J. Neurochem.* **21**, 1127–1138.
- Pulsinelli W. A. and Duffy T. E. (1983) Regional energy balance in rat brain after transient forebrain ischemia. *J. Neurochem.* **40**, 1500–1503.
- Pulsinelli W. A., Brierley J. B., and Plum F. (1982a) Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann. Neurol.* **11**, 491–498.
- Pulsinelli W. A., Levy D. E., and Duffy T. E. (1982b) Regional cerebral blood flow and glucose metabolism following transient forebrain ischemia. *Ann. Neurol.* **11**, 499–509.
- Reddy T. S. and Bazan N. G. (1987) Arachidonic acid, stearic acid, and diacylglycerol accumulation correlates with the loss of phosphatidylinositol 4,5-bisphosphate in cerebrum 2 seconds after electroconvulsive shock: complete reversion of changes 5 minutes after stimulation. *J. Neurosci. Res.* **18**, 449–455.
- Rehncrona S., Westerberg E., Åkesson B., and Siesjö B. K. (1982) Brain cortical fatty acids and phospholipids during and following complete and severe incomplete ischemia. *J. Neurochem.* **38**, 84–93.
- Rodriguez de Turco E. B., Morelli de Liberti S., and Bazan N. G. (1983) Stimulation of free fatty acid and diacylglycerol accumulation in cerebrum and cerebellum during bicuculline-induced status epilepticus. Effect of pretreatment with  $\alpha$ -methyl-*p*-tyrosine and *p*-chlorophenylalanine. *J. Neurochem.* **40**, 252–259.
- Rouser G., Fleischer S., and Yamamoto A. (1970) Two-dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5**, 494–496.
- Seren M. S., Aldinio C., Zanoni R., Leon A., and Nicoletti F. (1989) Stimulation of inositol phospholipid hydrolysis by excitatory amino acids is enhanced in brain slices from vulnerable regions after transient global ischemia. *J. Neurochem.* **53**, 1700–1705.
- Siesjö B. K., Ingvar M., and Westerberg E. (1983) The influence of bicuculline-induced seizures on free fatty acid concentrations in cerebral cortex, hippocampus, and cerebellum. *J. Neurochem.* **39**, 796–802.
- Siesjö B. K., Ingvar M., Folbergrová J., and Chapman A. G. (1983) Local cerebral circulation and metabolism in bicuculline-induced status epilepticus: relevance for development of cell damage. *Adv. Neurol.* **34**, 217–230.
- Smith M.-L., Bendek G., Dahlgren N., Rosén I., Wieloch T., and Siesjö B. K. (1984) Models for studying long-term recovery following forebrain ischemia in the rat. 2. A 2-vessel occlusion model. *Acta Neurol. Scand.* **69**, 385–401.
- Steiner A. L., Ferrendelli J. A., and Kipnis D. M. (1972) Radioimmunoassay for cyclic nucleotides III. Effects of ischemia, changes during development and regional distribution of adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in mouse brain. *J. Biol. Chem.* **247**, 1121–1124.
- Sun G. Y., Yoa F.-G., and Lin T.-N. (1990) Degradation of polyphosphoinositides in brain subcellular membranes in response to decapitation insult. *Neurochem. Int.* **174**, 529–535.
- Ueki M., Linn F., and Hossmann K.-A. (1988) Functional activation of cerebral blood flow and metabolism before and after global ischemia of rat brain. *J. Cereb. Blood Flow Metab.* **8**, 486–494.
- Uemura A., Mabe H., and Nagai H. (1992) A phospholipase C inhibitor ameliorates postischemic neuronal damage in rats. *Stroke* **23**, 1163–1166.
- Wieloch T., Cardell M., Bingren H., Zivin J., and Saitoh T. (1991) Changes in the activity of protein kinase C and the differential subcellular redistribution of its isozymes in the rat striatum during and following transient forebrain ischemia. *J. Neurochem.* **56**, 1227–1235.
- Yoshida S., Ikeda M., Busto R., Santiso M., Martinez E., and Ginsberg M. D. (1986) Cerebral phosphoinositide, triacylglycerol, and energy metabolism in reversible ischemia: origin and fate of free fatty acids. *J. Neurochem.* **47**, 744–757.