RELATIONSHIP BETWEEN CEREBRAL ENERGY FAILURE AND FREE FATTY ACID ACCUMULATION FOLLOWING PROLONGED BRAIN ISCHEMIA

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Abstract—Prolonged ischemia by bilateral carotid artery ligation in rats resulted in cerebral edema with a reduced energy state. Mitochondria isolated from the ischemic brain showed an impairment of oxidative phosphorylation. The ischemic brain was also characterized by a remarkable accumulation of free fatty acids known to have properties as an uncoupling factor. The major components of increased free fatty acids were palmitic, stearic, oleic and arachidonic acids. The analysis of saponified myelin and mitochondrial lipids from the ischemic brain showed a decrease in fatty acid contents. The main components of decreased fatty acids in these subcellular fractions corresponded to those of free fatty acids accumulating in the ischemic brain. These results indicate that cerebral energy failure in the ischemic brain is related to the accumulation of free fatty acids, which are derived from endogenous brain lipids.

Although considerable efforts have been devoted to the study on experimental brain edema, the biochemical alterations associated with the pathogenesis still remain to be elucidated. Current evidence suggests that cerebral swelling is accompanied by a reduction of the cerebral energy state (1–3) and an impairment of mitochondrial function (4–6). A deficiency of available energy is assumed to cause a disturbance of active ionic transport across the cell membrane, which could lead to cerebral swelling or cell damage. In a previous report (7), we showed that unilateral brain edema produced in rats by the combination of ischemia and hypoxic exposure was characterized by a considerable increase of free fatty acids in the brain. Our experiments also demonstrated that oleic and arachidonic acids, which increased markedly in the edematous brain, inhibited oxidative phosphorylation of *in vitro* mitochondrial preparations (7). These observations provided information for further study on the biochemical mechanism of cerebral energy failure in brain edema.

In the present study, we investigated cerebral energy metabolism in another form of brain edema produced in rats by a permanent ligation of bilateral carotid arteries. This paper deals with biochemical events associated with cerebral energy failure and the free fatty acid change in the subcellular lipids of the edematous brain.

MATERIALS AND METHODS

Brain ischemia

Male Wistar rats weighing 80–100 g were anesthetized with sodium hexobarbital (150 mg/

kg, i.p.). A ventral mid-line incision was made through the skin of the neck and the bilateral common carotid arteries were exposed. After ligating the arteries, the skin incision was closed with wound clips. The rats subjected to brain ischemia awoke from anesthesia about 30 min after, and showed behavioral abnormalities, e.g., an impaired gait, an occasional convulsion, a decrease in muscle tone and a loss of righting reflex. On the following day, these rats died.

Analytical methods

Water, sodium and potassium: Rats were decapitated and the forebrain was removed within 1 min. Water and electrolyte contents in the forebrain were determined by the methods as described previously (7).

Metabolic intermediates: Rats were immersed *in situ* in about 5 liters of acetone maintained at −82°C with an excess amount of dry ice, and kept at least for 5 min after vigorous agitation. The frozen rat was decapitated using a saw and the head was kept in dry ice—acetone except for the short time necessary for dissection. The frozen forebrain was removed within 3 min and stored in a freezer (−60°C) until analysis. The tissue extract was prepared by the method of Folbergrova *et al.* (8). ATP, ADP, AMP, phosphocreatine (P-creatine), lactate and glucose were analyzed by the enzymatic methods as described by Lowry *et al.* (9). Energy charge potential (ECP) was calculated from the equation of (ATP+0.5 ADP)/(ATP+ADP+AMP) proposed by Atkinson (10).

Oxidative phosphorylation: Rats were decapitated and the forebrain was removed within 1 min. The mitochondrial fraction was prepared from the fresh forebrain according to the method of Ozawa *et al.* (11). The F_1 fraction was used as mitochondrial preparation for the assay of oxidative phosphorylation. Oxygen uptake was measured manometrically in a Warburg apparatus. The incubation medium had the following composition; 20 mM sodium phosphate buffer (pH 7.4), 15 mM sodium glycylglycine (pH 7.4), 50 mM KCl, 8 mM glucose, 30 mM sodium succinate (pH 7.4), 2.5 mM ADP, 3 mM NaF, 0.2 mg of hexokinase (crystal from yeast, Boehringer Mannheim GmbH) and 0.5 ml of mitochondrial preparation (approx. 0.40 mg of nitrogen). Total volume was 3.3 ml. Oxygen uptake was measured for 25 min at 37°C after equilibrating for 10 min. The reaction was terminated by acidifying with perchloric acid immediately after reading the manometer. ATP formation was calculated from the amount of glucose-6-phosphate formed in the incubation medium (12). Glucose-6-phosphate was determined by the enzymatic method (9) and nitrogen of mitochondrial preparations was analyzed by the micro-Kjeldahl method (13).

Free fatty acids: Rats were frozen in dry ice—acetone (82°C) and the forebrain was removed by dissection in the same manner as mentioned above. The frozen tissue was weighed and homogenized with $CHCl_3$-MeOH (2:1, v/v) (14). Free fatty acids in the lipid extract were isolated by thin-layer chromatography according to the method of Bazan and Joel (15), and then methylated with diazomethane (16). The methyl esters of fatty acids were analyzed by gas-liquid chromatography using benzophenone as an internal standard. The methylated fatty acids were identified by comparing their retention times with those of known reference compounds. The analysis by the combined system of gas-liquid chromato-

graphy and mass spectrometry also served to identify some unknown peaks.

Isolation and analysis of myelin and mitochondrial fractions

Myelin and mitochondria were isolated from the fresh forebrain according to the method of Koeppen *et al.* (17). The resultant fractions, M_1 0.8 (myelin) and M_1p (mitochondria), were used for the analysis of fatty acids. Total lipids of these fractions were extracted with $CHCl_3$-MeOH (2:1, v/v) (14), followed by alkaline saponification (18). The fatty acids were extracted with petroleum ether in the acidic condition (18) and isolated by thin-layer chromatography (15). The analysis of fatty acids was carried out by gas-liquid chromatography following methylation (16). Nitrogen of myelin and mitochondrial fractions was determined by the micro-Kjeldahl method (13).

Statistical analysis

The statistical significance was evaluated by Student's t-test.

RESULTS

Water, electrolyte and metabolic intermediates

Table 1 shows water and electrolyte contents in the brain at various intervals after bilateral carotid artery ligation. Following brain ischemia, water and sodium contents in the brain increased with a simultaneous decrease in potassium content. The results indicate that prolonged brain ischemia led to edema associated with electrolyte disturbance. Cerebral energy metabolism in the ischemic brain showed a remarkable alteration (Table 2). The ATP and P-creatine levels decreased to 5–10% of the normal after 4–6 hr. The ADP level showed a biphasic pattern after ischemia. The transient rise of ADP level at 2 hr is probably attributed to a decrease of ADP utilization due to a suppression of oxidative phosphorylation. The subsequent event of ADP reduction may be caused by a deficiency of available ATP which leads to a decrease of ADP production in the metabolic processes coupling with ATP. The calculated ECP value was lowered due to the reduction of ATP level and the rise of AMP level. The lactate level increased to more than 10 times the normal after 4–6 hr and the glucose level decreased markedly at that time. These biochemical events suggest that the edematous brain was accompanied by a considerable reduction of the cerebral energy state

TABLE 1. Water and electrolyte contents

Time after ischemia		Water (g/100 g wet tissue wt.)	Sodium (μ equiv./g dry tissue wt.)	Potassium (μ equiv./g dry tissue wt.)
Normal	(14)	79.4±0.05	240± 2	511±3
2 hr	(12)	80.6±0.2*	277± 7*	485±7*
4 hr	(12)	81.5±0.1*	334± 9*	442±7*
6 hr	(7)	82.0±0.1*	368±19*	410±8*

Rats were decapitated and the forebrains were removed. Water and electrolyte contents in the brain were determined at various intervals following brain ischemia. Values are means ± S.E.M. Number of rats in each group is given in parentheses. *Differs from the normal group, $P<0.01$.

TABLE 2. Metabolic intermediate levels

Time after ischemia		ATP	ADP	AMP	P-creatine	ECP	Lactate	Glucose
					(μmoles/g wet tissue wt.)			
Normal	(8)	3.20 ±0.04	0.331 ±0.014	0.057 ±0.003	3.09 ±0.06	0.938 ±0.002	2.18 ±0.07	1.47 ±0.06
2 hr	(7)	1.29* ±0.26	0.473* ±0.021	0.449* ±0.052	0.98* ±0.27	0.662* ±0.048	18.3* ±2.3	0.57* ±0.12
4 hr	(5)	0.34* ±0.06	0.276* ±0.039	0.329* ±0.043	0.18* ±0.04	0.500* ±0.024	26.1* ±1.6	0.21* ±0.07
6 hr	(5)	0.22* ±0.02	0.240* ±0.014	0.323* ±0.073	0.17* ±0.04	0.428* ±0.036	27.4* ±2.1	0.09* ±0.02

Rats were immersed *in situ* in dry ice—acetone (−82°C). Metabolic intermediates in the brain were determined by enzymatic methods. Values are means ±S.E.M. Number of rats in each group is given in parentheses. *Differs from the normal group, $P<0.01$.

and a striking increase of cerebral lactate.

Oxidative phosphorylation

As shown in Table 3, oxidative phosphorylation in mitochondria isolated from the ischemic brain was severely damaged. ATP formation was more defective than oxygen uptake, which resulted in a reduction of ATP/O ratio.

TABLE 3. Mitochondrial oxidative phosphorylation

Time after ischemia		ATP formation (nmoles/min/N mg)	Oxygen uptake (natoms/min/N mg)	ATP/O
Normal	(6)	2246 ± 24	1333 ± 30	1.69 ± 0.04
2 hr	(6)	1334 ± 117*	859 ± 68*	1.55 ± 0.04**
4 hr	(5)	792 ± 141*	570 ± 82*	1.37 ± 0.06*

Mitochondria were isolated from both normal and ischemic brains. Oxidative phosphorylation was assayed using succinate as a substrate. Values are means ± S.E.M. Number of rats in each group is given in parentheses. *Differs from the normal group, $P < 0.01$. **Differs from the normal group, $P < 0.05$.

Free fatty acids

The normal brain contained a very small amount of free fatty acids (about 60 μg/g tissue wt.). Following brain ischemia, free fatty acids increased progressively and attained to more than 10 times the normal at 6 hr (Table 4). The major components of increased free fatty acids were palmitic (16:0), stearic (18:0), oleic (18:1) and arachidonic (20:4) acids. An unusual long-chain polyenoic fatty acid of docosahexaenoate (22:6) also increased considerably. The other components, however, showed no or less increase. A notable observation was the accumulation of arachidonic (20:4) and docosahexaenoic (22:6) acids, which were absent in the pre-ischemic brain. Consequently, the free fatty acid composition in the ischemic brain was different from that in the normal brain.

Fatty acids in saponified lipids of subcellular fractions

In this experiment, the rats were subjected to brain ischemia for 6 hr. Myelin and mitochondria were isolated from both the normal and the ischemic brains. Table 5 shows the saponifiable fatty acid contents in brain homogenate, myelin and mitochondria. The contents of palmitic (16:0) and palmitoleic (16:1) acids were combined because these peaks by gas-liquid chromatography were not separated distinctly due to the surplus application of samples. The proportion of palmitoleic acid (16:1) to palmitic acid (16:0) was assumed to be 6–8% by a rough estimate. The analysis of fatty acids in saponified lipids showed that brain homogenate, myelin and mitochondria contained more palmitic (16:0), stearic (18:0), oleic (18:1), arachidonic (20:4) and docosahexaenoic (22:6) acids.

The fatty acid content per nitrogen weight of brain homogenate was unchanged after ischemia. With regard to myelin, there was a low recovery of myelin nitrogen from the ischemic brain. The fatty acid content per nitrogen weight of myelin was also lowered considerably. The significantly decreased fatty acids were palmitic (16:0), stearic (18:0), oleic (18:1), eicosamonoenoic (20:1) and docosahexaenoic (22:6) acids. In contrast to

Table 4. Free fatty acid contents

Time after ischemia		Free fatty acids (μg/g wet tissue wt.) 16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:4	22:4	22:6	Sum
Normal	(5)	24.2 ±2.0	3.6 ±0.4	11.0 ±1.0	9.4 ±1.3	5.9 ±0.6	1.2 ±0.1	4.1 ±0.4	trace	trace	trace	59.5 ±5.4
2 hr	(4)	72.3 ±5.3	5.9 ±0.2	85.0 ±4.6	45.1 ±4.1	6.4 ±1.0	1.3 ±0.1	5.0 ±0.9	49.9 ±5.7	4.4 ±0.4	9.2 ±0.7	284.4 ±20.2
4 hr	(4)	139.0 ±16.6	10.4 ±1.3	161.9 ±24.2	89.8 ±10.1	8.0 −0.8	2.0 ±0.2	5.7 ±0.5	83.9 +12.2	10.5 ±1.3	27.7 ±3.9	538.9 ±66.5
6 hr	(5)	170.1 ±16.7	12.1 ±2.4	211.5 ±19.2	106.8 ±10.7	8.6 ±0.5	1.9 ±0.1	6.0 ±0.3	107.1 ±14.3	13.2 ±1.4	38.1 ±7.4	675.4 ±67.4

Rats were immersed *in situ* in dry ice—acetone (−82°C) and the frozen brains were removed. Total lipids in the brain were extracted with $CHCl_3$-MeOH (2:1, v/v) and free fatty acids were isolated by thin-layer chromatography. After methylating with diazomethane, the methyl esters of fatty acids were analyzed by gas-liquid chromatography. Values are means±S.E.M. Number of rats in each group is given in parentheses. Fatty acids; palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), eicosamonoenoic acid (20:1), arachidonic acid (20:4), docosatetraenoic acid (22:4) and docosahexaenoic acid (22:6).

TABLE 5. Fatty acid contents in saponified lipids of brain homogenate, myelin and mitochondria

		Total nitrogen (mg/g tissue wt.)	Fatty acids (μg/mg nitrogen) 16:0 + 16:1	18:0	18:1	18:2	18:3	20:1	20:4	22:4	22:6	Sum
Brain homogenate												
Normal	(5)	18.9 ±0.8	174 ±5	131 ±5	164 ±4	6 ±0	2 ±0	10 ±0	86 ±3	26 ±1	90 ±3	690 ±20
Ischemic	(4)	17.7 ±0.3	181 ±10	137 ±9	157 ±8	6 ±0	2 ±0	9 ±1	85 ±4	26 ±1	92 ±4	696 ±34
Myelin												
Normal	(3)	0.539 ±0.016	362 ±21	298 ±15	434 ±27	27 ±5	9 ±1	39 ±3	113 ±7	55 ±12	85 ±16	1421 ±73
Ischemic	(3)	0.426** ±0.051	240** ±28	176** ±29	248** ±43	26 ±1	7 ±1	27** ±1	81 ±11	32 ±4	54** ±3	890** ±111
Mitochondria												
Normal	(3)	0.832 ±0.024	231 ±13	158 ±9	214 ±11	29 ±2	3 ±0	10 ±1	139 ±6	18 ±4	89 ±3	891 ±41
Ischemic	(3)	0.773 ±0.076	207 ±11	139 ±7	153** ±11	20 ±2	3 ±0	10 ±1	95* ±6	24 ±2	83 ±2	734** ±39

Rats were subjected to brain ischemia for 6 hr. Myelin and mitochondria were isolated from both normal and ischemic brains by discontinuous sucrose gradient centrifugation. Total lipids were extracted with $CHCl_3$-MeOH (2:1, v/v), followed by alkaline saponification. The liberated fatty acids were isolated by thin-layer chromatography and analyzed by gas-liquid chromatography. Values are means±S.E.M. Number of rats in each group is given in parentheses. Fatty acids; palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), eicosamonoenoic acid (20:1), arachidonic acid (20:4), docosatetraenoic acid (22:4), and docosahexaenoic acid (22:6). *Differs from the normal group, $P<0.01$. **Differs from the normal group, $P<0.05$.

myelin, there was a preferential loss of oleic (18:1) and arachidonic (20:4) acids in mitochondria isolated from the ischemic brain despite no significant change in the recovery of mitochondrial nitrogen. The main components of decreased fatty acids in myelin and mitochondria corresponded to those of free fatty acids accumulating in the ischemic brain.

DISCUSSION

It is well known that cerebral ischemia induces cellular hypoxia and metabolic changes in the brain. An increased rate of glycolytic metabolism is commonly observed in acute cerebral ischemia or hypoxia, but cerebral energy metabolism remains relatively unchanged despite the high oxygen requirement of the brain (19–23). These observations suggest that acute cerebral ischemia or hypoxia does not lead to a crucially decreased cerebral blood flow or critically profound cellular hypoxia compatible with cerebral energy failure. This may be partly due to the fact that cerebral circulation is controlled by an autoregulating mechanism (24–26). In the present study, however, we observed that a considerable reduction of cerebral ATP and P-creatine levels occurred in rats subjected to bilateral carotid artery ligation for 2 hr or longer. These findings indicate that prolonged brain ischemia can produce a derangement in cerebral energy metabolism. As the transport of sodium and potassium ions across the cell membrane is dependent on energy available for metabolic processes (27), it is assumed that the marked reduction of cerebral ATP level results in an electrolyte disturbance, followed by an increase in cerebral water content. Eklöf and Siesjö (28) reported that hypotensive rats subjected to brain ischemia showed a similar reduction in the cerebral energy state and inferred that brain ischemia combined with hypotension caused a grossly inhomogenous reduction in cerebral blood flow. However, it remains uncertain whether the decreased blood flow or subsequent hypoxia in the brain could be a primary cause of cerebral energy failure, because the capillary and tissue oxygen tension sufficient to maintain adequate oxidative phosphorylation is considerably low (29). Duffy *et al.* (20) demonstrated that the cerebral ATP level remained unchanged with a degree of hypoxia sufficient to produce a serious depression in brain function.

As it is generally accepted that ATP is synthesized mainly by mitochondrial oxidative phosphorylation, the estimation of mitochondrial function is indispensable for the investigation of cerebral energy metabolism in the ischemic brain. Our experiments in this study demonstrated that mitochondria isolated from the ischemic brain showed an impairment of ATP formation and oxygen uptake, indicating that the lowering of cerebral ATP level was caused by damage to the mitochondrial function.

The present study provides further evidence that the ischemic brain is characterized by a remarkable accumulation of free fatty acids. As rat brain mitochondria is vulnerable to unsaturated fatty acids such as oleic and arachidonic acids (7), it is likely that the accumulation of these fatty acids in the ischemic brain is responsible for the damage of *in vivo* mitochondrial oxidative phosphorylation.

The observation of free fatty acid accumulation led us to investigate further the fatty acid change in the subcellular fractions. For this purpose, attempts were made to analyze

fatty acids in the myelin and mitochondrial lipids because brain edema was reportedly accompanied by demyelination (30) and structural changes of mitochondria (31). The present data indicated that the major components of saponifiable fatty acids in the brain corresponded to those of free fatty acids accumulating in the ischemic brain. The result suggests that the increase of free fatty acids reflects the change occurring in endogenous brain lipids. In fact, there was a notable alteration with regard to fatty acid contents in myelin and mitochondria from the ischemic brain. The content of fatty acids per nitrogen weight of myelin decreased considerably, but the fatty acid composition was relatively unchanged. This may be explained by a removal of lipid-rich components from myelin or a breakdown of the myelin sheath due to degeneration of morphological integrity, which results in a low recovery of myelin isolated by the procedure devised for the normal myelin. The degenerated myelin fragments are, in our opinion, attacked by lipolytic enzymes, followed by the release of fatty acids. A similar phenomenon of myelin degeneration was reported by Eto *et al.* who observed that brain edema induced by triethyl tin sulfate was characterized by a low yield of myelin (32). In contrast to myelin, mitochondria isolated from the ischemic brain showed a preferential loss of oleic and arachidonic acids despite no significant change in the yield. The result suggests that the greater part of increased oleic and arachidonic acids in the ischemic brain may be derived from mitochondrial lipids. Mellors *et al.* (33) reported that lysosomes from rat liver and heart catalyzed a rapid disruption of mitochondrial structure and function, which was accompanied by a release of free fatty acids. They also noted that mitochondrial dysfunction was not due to lipid digestion by lysosomal enzymes but did closely parallel the liberation of fatty acids.

The biochemical events of cerebral energy metabolism in brain edema produced by prolonged ischemia were similar to those observed previously in unilateral brain edema (7), although there were differences in the latent time before the pathogenetic manifestation and the degree of cerebral energy failure. These data support the concept that the pathogenesis of brain edema involves the production of free fatty acids in the brain and that these acids play an important role in mitochondrial damage and subsequent cerebral energy failure. A notable finding was the decreased fatty acid content in myelin and mitochondria isolated from the ischemic brain. This may account for the accumulation of free fatty acids following brain ischemia.

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