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Effects of naftidrofuryl oxalate on microsphere-induced changes in acetylcholine and amino acid content of rat brain regions

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Abstract Effects of naftidrofuryl oxalate (naftidrofuryl) on neurotransmitter, acetylcholine, and amino acid content of brain regions following microsphere-induced cerebral embolism were examined to elucidate its possible therapeutic effects on ischemic brain. Rats received 900 microspheres (48 μm in diameter) via the right internal carotid artery, followed by ligation of the right common carotid artery; and histological and biochemical alterations were examined on the 3rd, 5th, and 28th days after embolism. The embolism induced increases in triphenyltetrazolium chloride- (TTC)-unstained areas and decreases in acetylcholine, glutamate, aspartate, and γ-aminobutyric acid (GABA) contents in the cerebral cortex, striatum, and hippocampus of the right hemisphere, suggesting that microsphere embolism causes severe damage to these brain regions. Hematoxylin-eosin staining of the right cortical sections after embolism showed degeneration and necrosis of nerve cells with chromatolytic nuclei and eosinophilic cytoplasm. Changes in neurotransmitters of the left hemisphere were relatively small. Treatment with naftidrofuryl of the embolized rats with stroke-like symptoms took place from postoperative day 1 to 28. Treatment resulted in a reduction in TTC-unstained areas, less morphological damage to cerebral cortex on the 3rd and 5th days, and an appreciable restoration of acetylcholine content of three brain regions of the right hemisphere throughout the experiment, but restoration of neurotransmitter amino acids was observed to a smaller degree. The results suggest that naftidrofuryl is capable of preventing the development of ischemia-induced, sustained damage to brain regions vulnerable to oxygen deficiency, particularly by improving impaired acetylcholine metabolism.

Key words Acetylcholine · Asparte · Brain ischemia
Glutamate · Microsphere embolism
Naftidrofuryl oxalate · Rat

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Introduction

Naftidrofuryl oxalate [2-(diethylamino)ethyltetrahydro-α-(1-naphthylmethyl)-2-furanpropionate ester oxalate] is a cerebral and peripheral vasodilator that is commercially available in several European countries. The agent has been shown to increase cerebral blood flow in anesthetized animals (Young et al. 1983; Hiramatsu et al. 1988). In previous studies, we induced cerebral ischemia and infarction by administration of microspheres into the right internal carotid artery in rats, which was associated with various functional and biochemical alterations of the ipsilateral hemisphere. These include a sustained decrease in cerebral blood flow (Miyake et al. 1993), a pronounced disturbance in brain glucose metabolism (Takeo et al. 1991), high-energy phosphate production (Takeo et al. 1992), and mitochondrial activity relating to energy production (Miyake et al. 1989; Takeo et al. 1989) of microsphere-embolized rats. It was also found in these studies that naftidrofuryl diminished or reduced the microsphere-induced damage of these pathophysiological alterations during 3–5 days of microsphere embolism to an appreciable degree. Since cerebral ischemia or infarction has been demonstrated to induce alterations in cerebral neurotransmitter acetylcholine and amino acids (Saito et al. 1985; Yasumatsu et al. 1987; Scremin and Jenden 1989a), the microsphere embolism may induce a disturbance in cerebral neurotransmitters. In the present study, we attempted to elucidate possible effects of naftidrofuryl on neurotransmitters in ischemic brain regions. For this purpose, we examined acetylcholine, choline, and neurotransmitter amino acid contents of

the following brain regions: cerebral cortex, striatum, and hippocampus. These regions are believed to be vulnerable to ischemic or hypoxic insult (Pulsinelli et al. 1982; Smith et al. 1984; Kirino 1982).

Materials and methods

Surgical procedure

Male Wistar rats weighing 180–220g (Charles River Japan, Atsugi, Japan) were used. The animals were maintained under artificial conditions at 23±1° C, with a constant humidity of 55±5%, a cycle of 12 h light and 12 h dark, and free access to food and tap water, according to the Guidelines of Experimental Animal Care issued by the Prime Minister's Office of Japan. The operation to induce microsphere cerebral embolism was performed by a method previously described (Takeo et al. 1992). One hundred and seventy-six rats were anesthetized with 30 mg/kg pentobarbital sodium i.p. and fixed in the supine position on an operation plate. After cervical incision, the right common carotid artery was isolated; the right external carotid and the right pterygopalatine arteries were ligated with threads. A polyethylene catheter (3 Fr, 1.0 mm in diameter; Atom, Tokyo) was inserted into the right common carotid artery. Nine hundred microspheres (47.5±0.5 μm in diameter; NEN-005; New England Nuclear, Boston, Mass., USA), suspended in 20% dextran solution, were injected into the right internal carotid artery through this catheter. After injection, the right common carotid artery was ligated and the wound was sutured. Seventy-six rats that underwent sham operation were injected with the same volume of vehicle, without microspheres. Nineteen nonoperated rats served as control.

Observation of animal behavior

Fifteen hours after the operation, the behavior of the operated rats was scored on the basis of paucity of movement, trunk curvature, and forced circling during locomotion, which are considered to be typical symptoms of stroke in animals (Furlow and Bass 1976; McGraw 1977). The score of each item was ranked from 3 to 0 (3, very severe; 2, severe; 1, moderate). the rats that scored 7 or more points were considered to be type A, 6–4, type B, and less than 4, type C. In the present study, we used only type A animals.

Treatment with naftidrofuryl

After ensuring stroke-like symptoms of the microsphere-injected rats, the animals were treated at both 0900 hours and 1800 hours with i.p. injection of 15 mg/kg naftidrofuryl for 3–28 consecutive days. The rats were killed on postoperative days 3, 5, and 28, 1 h or more after administration of the agent, and their metabolic changes were determined. The number of doses administered was thus 5 for the 3-day treatment, 9 for the 5-day treatment, and 55 for the 28-day treatment, respectively. Numbers of animals used in the present study are listed in Table 1. The treatment with naftidrofuryl employed in the present study was the same as that which exerted beneficial effects on cerebral glucose metabolism of the microsphere-embolized rat (Takeo et al. 1991).

Histological study

Determination of triphenyltetrazolium chloride-stained area

The microsphere-injected and sham-operated rats were lightly anesthetized with ether and decapitated at different time intervals after embolism. The brains were rapidly isolated and cooled in a stainless steel container, precooled in an icebox. The brain was positioned on a brain holder and coronally sectioned 3, 5, and 7 mm from the frontal pole with razor blades. These sections optimally faced striatal, cortical, and hippocampal regions of the rat brain, respectively. The sectioned brain tissue was incubated at 37° C for 30 min with 2% 2,3,5,-triphenyltetrazolium chloride (TTC) in physiological saline, according to a modified method of Bederson et al. (1986). The tissue slices were transiently immersed in a 10% formalin solution and then photographed. The sum of TTC-stained, and TTC-unstained (including weakly stained) areas of three brain slices was estimated by a planimetric method.

Hematoxylin-eosin staining of brain sections

For microscopic examination, microsphere-injected and sham-operated rats, with and without naftidrofuryl-treatment, and control rats were decapitated on postoperative days 3 and 5. The brain was quickly isolated. The specimens were fixed for 1 week in 10% formalin solution buffered with phosphates, pH 7.4. Thereafter, serial 2-mm sections were cut from the frontal area of each brain and embedded in paraffin. The paraffin sections were stained with hematoxylin and eosin, and observed by light microscopy. The microscopic observation of the sample was performed by a person unaware of the study group.

Determination of brain metabolites

At an appropriate point in the experimental sequences, the rats with and without naftidrofuryl treatment, and the nonoperated rats (control) were instantly killed by microwave irradiation to the head, using a strength of 5.0 kW for 0.85 s from a microwave applicator (TMW-6402c; Muromachi Kikai, Tokyo, Japan). After decapitation, the head of the animal was immersed in liquid nitrogen and left for 10 s (near freezing). The cerebral hemispheres were isolated. Three brain regions, cerebral cortex, striatum, and hippocampus, were separated under ice cooling. After their wet weights were determined, each region was homogenized in 0.2 M $HClO_4$ and 0.01% disodium ethylenediaminetetraacetate with a Polytron homogenizer (PT-10, Kinematica, Switzerland) for 15 s at the maximal speed. The extracting solution contained 1 μM ethylhomocholine as internal standard for detection of choline

Table 1 Numbers of control, sham-operated, and microsphere-embolized animals used for histological examination and determination of tissue metabolite contents of the brain

	Control	Sham-operated						Microsphere-embolized					
		−Naf			+Naf			−Naf			+Naf		
		3	5	28	3	5	28	3	5	28	3	5	28 day
TTC study (*n*)	5	6	4	6	6	4	5	5	5	7	6	7	8
Microscopic study (*n*)	2	2	2	–	2	2	–	4	4	–	4	4	–
Metabolite study (*n*)	14	8	8	6	8	8	7	8	8	8	8	8	8

−Naf, groups without naftidrofuryl treatment; +Naf, groups with naftidrofuryl treatment; TTC, triphenyltetrazolium chloride

and acetylcholine. After being left for 10 min at 0° C, the homogenate was centrifuged at 10000 *g* for 15 min at 4° C. A part of the supernatant solution was taken and kept as a sample for determination of tissue amino acid content. Alternatively, the resultant supernatant solution was taken and neutralized with 2.5 M K_2CO_3. Then, the resultant solution was centrifuged at 10000 *g* for 15 min at 4° C. The supernatant solution was filtered through a membrane filter (0.45 μm), and the filtrate was sampled for determination of acetylcholine and choline contents.

Tissue acetylcholine and choline contents

Tissue acetylcholine and choline contents were determined by high-performance liquid chromatographic analysis. For acetylcholine determination, the tissue extract in the chromatography column (Eicompak AC-gel + AC-enzymepak; Eicom, Kyoto, Japan) was eluted at 33° C with a solution of 0.1 M Na_2HPO_4 containing 65 mg 1-decanesulfonic acid sodium salt, pH 8.5, at a flow rate of 1.0 ml/min (EC-10; Eicom, Kyoto, Japan). The eluate was detected at 450 mV by an electrochemical detector (ECD-100; Eicom, Kyoto, Japan).

Tissue neurotransmitter amino acid content

The supernatant solution which had been kept as a sample for determination of neurotransmitter amino acids, glutamic acid, aspartic acid, and γ-aminobutyric acid (GABA), was filtered through a membrane filter with a pore size of 0.45 μm (Advantec Toyo, Tokyo, Japan) and then applied to an amino acid analyzer (Hitachi-835; Hitachi, Tokyo, Japan). Amino acids were eluted with a solvent of triketohydrindenehydrate (Ninhydrin reagent 835-set; Wako Pure Chemical Industries, Tokyo, Japan).

Expression of specific amount of metabolites

In the present study tissue metabolite contents are expressed as nanomoles or micromoles per frozen tissue weight. In a previous study we examined water content of wet and frozen tissues of the cerebral cortex, striatum, and hippocampus of microsphere-injected and sham-operated rats (Miyake et al. 1992). The frozen weights of brain regions were almost the same as the wet ones. There were slight increases in water content of the brain regions of the right hemisphere on the 3rd day as compared with control. However, no significant increase in water content was seen in any tissue and at any experimental time period examined, when estimated by analysis of variance followed by Scheffe test.

Statistics

The results are expressed as the mean ± SEM. Statistical significance for comparison of the metabolite content with and without microsphere injection or that with and without naftidrofuryl treatment was evaluated using the *F*-test, followed by either Student's or Welch's *t*-test. A confidence level of more than 95% was considered significant ($P < 0.05$).

Results

Microsphere embolism

In the present study, 53 rats (26%) out of 204 rats that were injected with microspheres died within 24 h of surgery. Animal behavior was observed on postoperative day 1. It was found that 127 rats showed type A symptoms (62%), among which 25 rats died within 3 days of the operation; 16 rats showed B-type symptoms (8%), and 8 rats, type C symptoms (4%). The sham-operated rats showed no stroke-like symptoms and survived throughout the experimental period.

Changes in TTC-stained area

The TTC-stained and -unstained areas of the brain slices of microsphere-injected rats were determined. The TTC unstained areas contained white and pink regions, which are considered to represent necrotic and border zone areas, respectively. Gross observation of the colored photographs of the TTC-stained slices could not apparently differentiate these two areas. Figure 1 shows changes in the TTC-stained area of the right hemisphere of microsphere-injected animals with and without naftidrofuryl treatment. Approximately 21, 32, and 20% of the TTC-stained areas of the right hemisphere were seen in the microsphere-injected rat brain slices on the 3rd, 5th, and 28th day, respectively, indicating that the embolism-induced infarction was extensively developed by the 3rd day under the present experimental conditions. Treatment with naftidrofuryl significantly attenuated the development of the TTC-unstained area of the right hemisphere on the 3rd and 5th days, but not on the 28th day. In the left hemisphere, no appreciable development of the TTC-unstained area was detected in any brain slice at any period during the experiments. There was no TTC-unstained area of either hemisphere of the sham-operated rat brain slices, at any period after em-

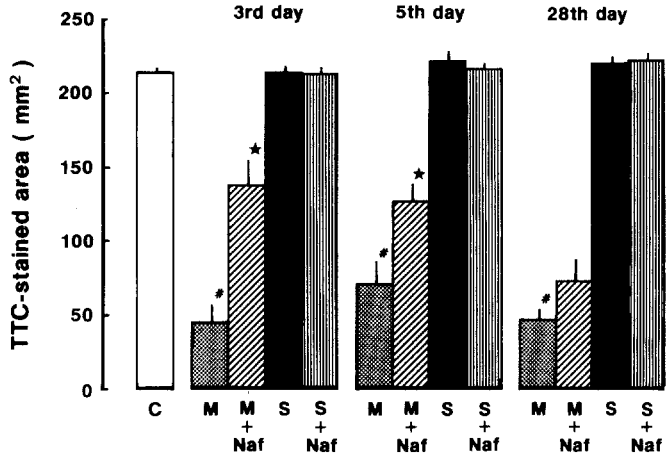

Fig. 1 The triphenyltetrazolium chloride- (TTC)-stained area of the right hemisphere of microsphere-injected rat brain with (*M* + Naf, ▨) and without naftidrofuryl treatment (*M*, ▩) and those of sham-operated rat brain with (*S* + Naf, ▥) and without naftidrofuryl treatment (*S*, ■) on the 3rd, 5th, and 28th days after embolism. Each value represents the mean ± SEM of 4–9 experiments. The TTC-stained area of the right hemisphere of control animals (*C*, □) was 212.7 ± 1.87 mm² ($n = 5$). There was no significant change in the TTC-stained area of the left hemisphere of either microsphere-injected or sham-operated rats, regardless of treatment with or without naftidrofuryl. # Significant difference from the sham-operated rats; ★ significant difference from the microsphere-injected rats ($P < 0.05$)

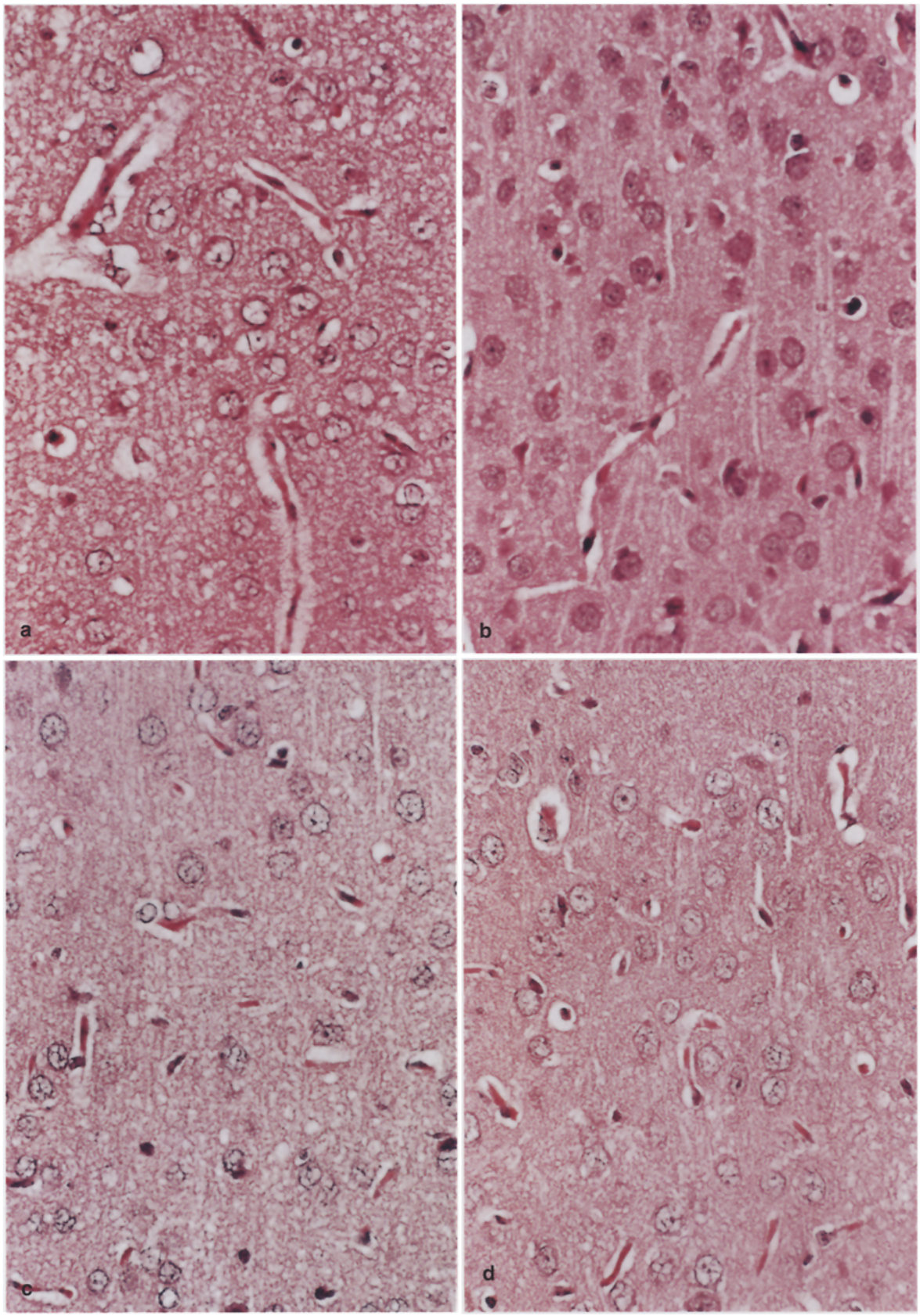

Fig. 2a–d Photomicrographs of right cerebral cortex of rats on the 3rd and 5th days after microsphere-injection without and with naftidrofuryl treatment. **a** Brain slice on the 3rd day without treatment. Marked edema is seen in the intercellular space, which has a spongy appearance. Nerve cells show degeneration, with decrease in cytoplasmic basophilia and with pale, vacuolated nuclei. **b** Brain slice on the 3rd day of naftidrofuryl treatment. Although the intercellular space is edematous, nerve cells have basophilic cytoplasm and round nuclei with fine granular chromatin. **c** Brain slice on the 5th day without treatment. Although intercellular edema is still found, the nerve cells show regenerative changes. The cytoplasmic basophilia of these cells is slightly increased. In a large number of the nerve cells, the nuclear chromatin has became finely granular. **d** Brain slice on the 5th day of naftidrofuryl treatment. The nerve cells have basophilic cytoplasm and nuclei with finely granular chromatin. Intercellular edema still remains. H & E , $\times 400$

bolism, regardless of treatment with or without naftidrofuryl.

Histological study

Histological study was performed using brains isolated from rats on the 3rd and 5th days after embolism. Histological examination was not performed in rats on the 28th day after embolism because no appreciable improvement of biochemical alteration and development of TTC-stained areas was seen on this day.

Brain sections on the 3rd day

The right brain hemisphere or rats without naftidrofuryl treatment were swollen and edematous. On the cut surfaces, areas supplied by the right medial cerebral arteries, such as the thalamus, lenticular nucleus, inner capsule, and hippocampus, were pale and swollen and showed typical early anemic infarction. Small hemorrhagic foci were frequently observed in these necrotic areas. Histologically, the necrotic areas were usually shrunken and necrotic with chromatolytic nuclei and eosinophilic cytoplasm. Areas spared from anemic infarction were usually found in the brain cortices. Nerve cells of these areas had degenerated to various degrees (Fig. 2a). Intercellular spaces were markedly edematous, with a spongy appearance.

The right hemispheres of the rats with naftidrofuryl treatment were macroscopically similar to those of the rats without naftidrofuryl treatment. Under histological examination, however, edema was less marked in the cortical areas without necrosis. The nerve cells of these areas were preserved better than those of the rats without naftidrofuryl treatment. The cytoplasm of these cells was basophilic, nuclear chromatin was finely granular, and there was a distinct nucleolus (Fig. 2b).

Brain sections on the 5th day

Necrotic areas of the right hemispheres of rats without naftidrofuryl treatment showed some softening. Histologically, microglial lipophagocytes proliferated mainly in the marginal zones of the necrotic areas. Cortical areas spared from necrosis were still edematous, but the nerve cells of these areas became slightly regenerative. Cytoplasmic basophilia of the cortical cells increased. Although many nerve cells contained a pale and vacuolated nucleus with margination of chromatin, nerve cells with a nucleus showing finely granular chromatin were found in the cortical areas (Fig. 2c).

Macroscopic findings in the right hemispheres of the rats with naftidrofuryl treatment were almost identical to those of the rats without naftidrofuryl treatment. In the histological examination, numerous lipophagocytes were found in the necrotic areas, as seen in the rats without naftidrofuryl treatment. Although intercellular edema still remained, the nerve cells of the cortical areas saved from necrosis were well preserved (Fig. 2d). The cytoplasm of these cells was basophilic and nuclear chromatin was finely granular.

Changes in acetylcholine content

In microsphere-injected rats, a significant reduction in acetylcholine content of three brain regions of the right hemisphere was seen on postoperative days 3, 5, and 28 when the values were compared with those of sham-operated animals (Fig. 3). Treatment of microsphere-embolized rats with naftidrofuryl resulted in a significant

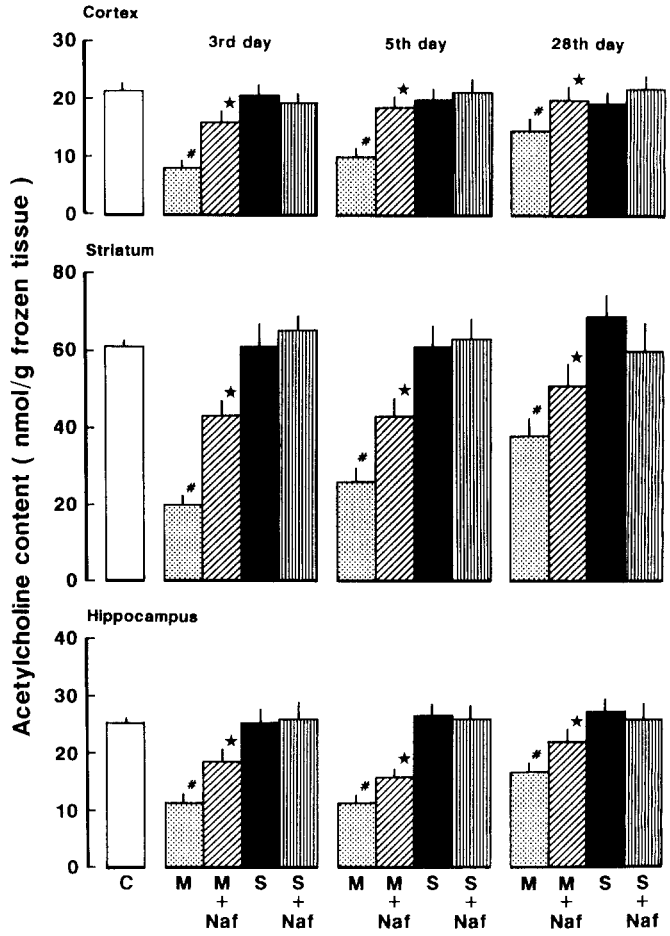

Fig. 3 Acetylcholine content of the cerebral cortex, striatum, and hippocampus of the right hemisphere on the 3rd, 5th, and 28th days after microsphere embolism. Values represent the mean ± SEM of 6–14 experiments. *M* + Naf (▨) and *M* (▩) indicate acetylcholine content of each brain region of the right hemisphere of the microsphere-injected group with and without naftidrofuryl treatment; and *S* + Naf (▥) and *S* (■), those of the sham-operated group with and without naftidrofuryl treatment, respectively. # Significant difference between the sham-operated and microsphere-injected groups; * significant difference between the microsphere-injected groups with and without naftidrofuryl treatment ($P < 0.05$). Acetylcholine content of the cortex, striatum, and hippocampus of the right hemisphere of control animals (*C*, □) were 21.43 ± 1.92, 61.48 ± 3.22, and 25.00 ± 0.98 nmol/g frozen tissue, respectively

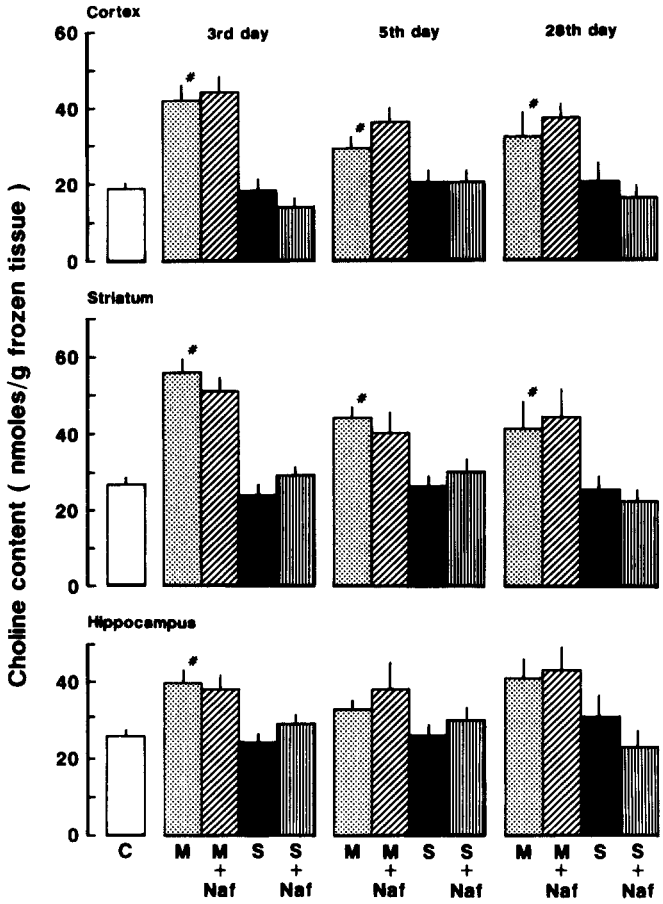

Fig. 4 Choline content of the cerebral cortex. striatum, and hippocampus of the right hemisphere on the 3rd, 5th, and 28th days after microsphere embolism. Values represents the mean ± SEM. Symbols, numbers of experiments, and statistics are the same as those in Fig. 3. # Significant difference between the sham-operated and microsphere-injected groups; * significant difference between the microsphere-injected groups with and without naftidrofuryl treatment ($P < 0.05$). Choline content of the cortex, striatum, and hippocampus of the right hemisphere of control animals was 18.61 ± 0.81, 27.09 ± 2.00 and 26.35 ± 1.58 nmol/g frozen tissue, respectively

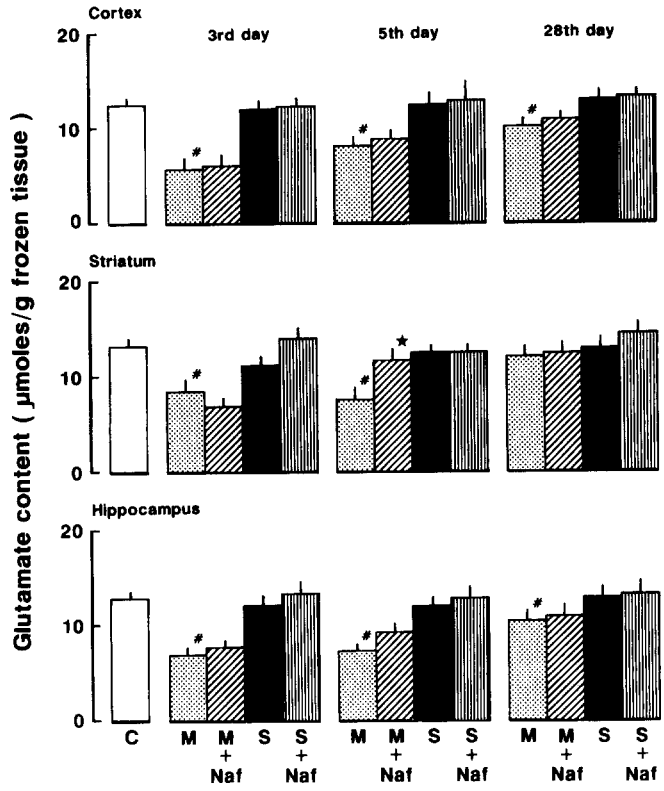

Fig. 5 Glutamic acid content of the cerebral cortex, striatum, and hippocampus of the right hemisphere on the 3rd, 5th, and 28th days after microsphere embolism. Values represents the mean ± SEM. Symbols, numbers of experiments, and statistics are the same as those in Fig. 3. # Significant difference between the sham-operated and microsphere-injected groups; * significant difference between the microsphere-injected groups with and without naftidrofuryl treatment ($P < 0.05$). Glutamic acid content of the cortex, striatum, and hippocampus of the right hemisphere of control animals was 12.46 ± 0.35, 13.08 ± 0.40, and 13.55 ± 1.42 µmol/g frozen tissue, respectively

restoration of acetylcholine content in the right hemisphere on the 3rd, 5th, and 28th days. In the brain regions of the left hemisphere of microsphere-embolized rats, slight but significant decreases in acetylcholine content were observed in some areas and at some periods during the experiments. That is, the cortical acetylcholine content on the 3rd day was 15.41 ± 0.79 nmol/g frozen tissue. The cortical, striatal, and hippocampal acetylcholine contents on the 5th day were 14.90 ± 0.97, 50.23 ± 3.89, and 19.53 ± 2.14 nmol/g frozen tissue, and the striatal acetylcholine content on the 28th day was 58.40 ± 1.56 nmol/g frozen tissue. The reduction in acetylcholine content of brain regions in the left hemisphere was reversed toward the values of the sham-operated group by treatment with naftidrofuryl. In sham-operated rats, tissue acetylcholine content of either hemisphere was not altered at any time after vehicle treatment, regardless of treatment with or without naftidrofuryl.

Changes in choline content

Choline contents in the right hemisphere of cerebral cortex, striatum, and hippocampus of control rats were 18.61 ± 0.81, 27.09 ± 2.00, and 26.35 ± 1.58 nmol/g frozen tissue, respectively. The choline contents of the three brain regions of the right hemisphere in microsphere-injected rats markedly increased on postoperative day 3 (Fig. 4). The increase in choline content of the cortex and striatum, but not hippocampus, lasted up to day 28. In the left hemisphere of microsphere-embolized rats, the changes were observed to a lesser degree; the cortical choline content on the 3rd day (20.91 ± 1.36 nmol/g frozen tissue) was only significantly higher than that of the corresponding sham-operated group (16.79 ± 1.11 nmol/g frozen tissue). No significant restoration of the choline content of either hemisphere was seen throughout the experiment as a result of treatment with naftidrofuryl at any period of the embolism or in any brain region measured. In sham-operated rats, there were no significant differences in the choline con-

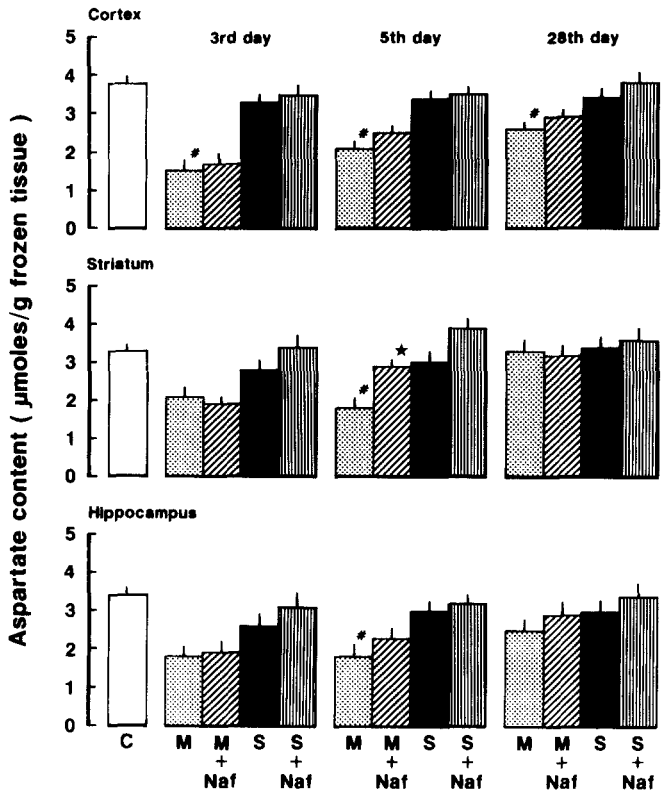

Fig. 6 Aspartic acid content of the cerebral cortex, striatum, and hippocampus of the right hemisphere on the 3rd, 5th, and 28th days after microsphere embolism. Values represents the mean ± SEM. Symbols, numbers of experiments, and statistics are the same as those in Fig. 3. # Significant difference between the sham-operated and microsphere-injected groups; * significant difference between the microsphere-injected groups with and without naftidrofuryl treatment ($P < 0.05$). Aspartic acid content of the cortex, striatum, and hippocampus of the right hemisphere of control animals was 3.78 ± 0.14, 3.31 ± 0.12, and 3.40 ± 0.21 μmol/g frozen tissue, respectively

tent of either hemisphere from control throughout the experiment.

Changes in glutamic acid, aspartic acid and GABA contents

In the right hemisphere

In sham-operated animals, there were no significant changes in glutamic acid, aspartic acid, and GABA contents in the cerebral cortex, striatum, and hippocampus throughout the experiment, regardless of treatment with or without naftidrofuryl.

In microsphere-injected rats, significant decreases were detected in glutamic acid content of the three brain regions on the 3rd, 5th, and 28th days after the embolism, except or that of the striatum on the 28th day (Fig. 5). Aspartic acid content also decreased in the cortex on the 3rd day, the cortex, striatum and hippocampus on the 5th day, and the cortex on the 28th day (Fig. 6). There was a decrease in GABA content of the three brain regions on the 3rd day, and the striatum and hippocampus on the 5th day after the operation (Table 2). Treatment with naftidrofuryl attenuated the decrease in the striatal glutamic acid on the 5th day, the striatal aspartic acid on the 5th day, and the hippocampal GABA content on the 3rd day. These three amino acid contents in the specified brain regions and at any other time after embolism were not restored by treatment with naftidrofuryl.

In the left hemisphere

The amino acid contents of these brain regions of the left hemisphere, as described above, were similar to those of the right hemisphere of control rats. In the left

Table 2 Cortical, striatal, and hippocampal GABA content of the right hemisphere on the 3rd, 5th, and 28th days after microsphere embolism

Values are expressed as micromoles per milligram of frozen tissue; each value represents the mean ± SEM. GABA content of the cerebral cortex, striatum, and hippocampus of the right hemisphere of the control rats was 1.67 ± 0.14, 2.68 ± 0.20, and 2.09 ± 0.14 μmol/g frozen tissue, respectively
* Significantly different from the sham-operated group (Sham);
** Significantly different from the microsphere-injected group (Microsphere; $P < 0.05$)

	Experimental group	Time after operation		
		Day 3	Day 5	Day 8
Cortex	Sham	1.69 ± 0.11	1.30 ± 0.08	1.50 ± 0.15
	Sham + Naf	1.34 ± 0.03	1.48 ± 0.12	1.49 ± 0.11
	Microsphere	0.74 ± 0.12*	1.01 ± 0.13	1.40 ± 0.09
	Microsphere + Naf	0.75 ± 0.14	1.02 ± 0.10	1.24 ± 0.04
Striatum	Sham	2.63 ± 0.31	2.45 ± 0.22	2.43 ± 0.12
	Sham + Naf	2.22 ± 0.20	2.75 ± 0.38	2.63 ± 0.12
	Microsphere	1.36 ± 0.22*	1.44 ± 0.24*	2.51 ± 0.18
	Microsphere + Naf	1.29 ± 0.12	1.85 ± 0.19	2.74 ± 0.25
Hippocampus	Sham	2.00 ± 0.15	1.98 ± 0.14	1.79 ± 0.78
	Sham + Naf	1.71 ± 0.12	1.94 ± 0.18	1.80 ± 0.11
	Microsphere	1.09 ± 0.10*	1.06 ± 0.09*	1.84 ± 0.13
	Microsphere + Naf	1.61 ± 0.17**	1.42 ± 0.18	1.85 ± 0.21

hemisphere of the microsphere-injected rats, significant decreases (vs sham-operated group) were detected only in striatal aspartic acid on the 5th (2.004 ± 0.273 vs 3.055 ± 0.341 μmol/g frozen tissue) and 28th days (2.642 ± 0.160 vs 3.333 ± 0.190 μmol/g frozen tissue), and striatal GABA on the 5th day (1.828 ± 0.207 vs 2.476 ± 0.116 μmol/g frozen tissue). Only the decreases in these amino acids on the 5th day were significantly reserved by treatment with naftidrofuryl. There were no changes in amino acid content of the sham-operated rats, regardless of treatment with or without naftidrofuryl.

Discussion

In the present study, we observed a sustained decrease in acetylcholine content of three brain regions (cerebral cortex, striatum, and hippocampus) throughout the experiment following microsphere embolism. Short periods of hypoxic insults have been shown to impair acetylcholine synthesis in vivo (Gibson and Blass 1976; Gibson and Duffy 1981). Furthermore, various ischemic insults induced by four-vessel ligation in rats (Yasumatsu et al. 1987) and bilateral carotid artery ligation in Mongolian gerbils (Saito et al. 1985) also resulted in a decrease in acetylcholine and an increase in choline of brain regions in the early stage of cerebral ischemia. A difference in the pathogenesis of ischemia in rats with middle cerebral artery occlusion has been reported; that is, a minor or no significant change in acetylcholine content of brain regions was detected 2.5 and 24 h after induction of ischemia (Scremin and Jenden 1989b). On this issue, microsphere embolism may be characteristic in inducing sustained disturbance of neurotransmitter metabolism in these brain regions. The results in the current study also provided evidence that treatment with naftidrofuryl restored acetylcholine content of microsphere-injected animals to the control level, which may be interpreted, at least in part, as a sign of recovery from impairment of cerebral acetylcholine metabolism.

The present study has demonstrated that choline content of brain regions, particularly in the right hemisphere, increased after microsphere embolism and remained elevated for 28 days. Choline content has been shown to increase after cerebral ischemia, or hypoxia, in vivo (Scremin and Jenden 1989a, b) and in vitro (Kosh et al. 1980; Zeisel 1985). Our findings of an embolism-induced increase in choline content are consistent with these observations. Acetylcholine is synthesized from choline and acetyl-CoA, but de novo synthesis of choline is limited (Tuček 1984). Furthermore, it has been demonstrated that there is no correlation between choline formation and the degree and distribution of cholinergic innervation (Freeman and Jenden 1976; Kosh et al. 1980). Thus, it is unlikely that an increase in choline content of the brain upon ischemia is attributable to breakdown of acetylcholine, but it would more likely be the result of hydrolysis of phospholipids (Zeisel 1985; Scremin and Jenden 1989a). If this were the case, virtually complete absence of effects of naftidrofuryl treatment on choline content of the microsphere-embolized rats suggests that naftidrofuryl neither prevents ischemia-induced phospholipid breakdown nor acetylcholine breakdown itself.

There is increasing evidence that amino acids, such as glutamic acid and aspartic acid, which are released in large quantities during cerebral ischemia, may exert neurotoxic effects (Choi et al. 1988; Mattson and Kater 1989; Meldrum 1985; Rothman and Olney 1986) or may be involved in delayed neuronal death in the ischemic brain (Jørgensen and Diemer 1982; Mitani et al. 1990). Obviously, it would be premature to conclude that either release, synthesis, or catabolism of acetylcholine is deranged by microsphere embolism, since no attempt was made in the present study to elucidate a possible mechanism involving the decrease in acetylcholine content. It should be noted that, in rats with middle cerebral artery occlusion, a sustained elevation in interstitial glutamic acid and aspartic acid concentrations was observed in the striatal region shortly (30–180 min) after induction of ischemia (Hillered et al. 1989). Thus, the sustained low levels of glutamic acid and aspartic acid in the embolized rat brain regions studied here appear to result from a massive release of the amino acids in these regions. We did not detect consistently that the microsphere embolism-induced decrease in neurotransmitter amino acids was reversed by treatment with naftidrofuryl; therefore, the effect of naftidrofuryl treatment on the decrease in neurotransmitter amino acids might be less than its effect on acetylcholine content.

In the present study, we observed a diverse time course of changes in development of TTC-unstained areas and acetylcholine and neurotransmitter amino acid contents of the right hemisphere of untreated, microsphere-injected rats over a period of 28 days after embolism. That is, stabilization of TTC-unstained areas as early as on day 3 and severe reduction in acetylcholine and neurotransmitter amino acid contents shortly after the embolism were followed by a gradual restoration with time. The cerebral infarction would be elicited even by a period of short, but severe, ischemia; whereas metabolic disturbances may be induced by consequences of several factors, such as a loss of linkage of metabolic reactions and a loss of substrates, which apparently resulted from reduction of cerebral blood flow. Several reports have shown dissociated abnormalities in the brain of the experimental animal with ischemic or hypoxic insult. These include dissociation between electrical and ion pump failure and histological damage (Astrup et al. 1982), between membrane failure and morphological damage (Heiss 1983), and between metabolic recovery and histologic determination (Pulsinelli et al. 1982; Smith et al. 1984; Kirino 1982). Thus, it is likely that dissociation in histological observations and metabolic sequences may be the result of a pathophysiological profile frequently seen in ischemic

brain. Presumably, diverse time courses of changes in various parameters as described above may be due to differences in their vulnerability to, resistance to or compensation for, cerebral ischemia or infarction. For example, in spite of pronounced development of the TTC-unstained area, an appreciable recovery acetylcholine and amino acid contents was detected on the day 28. This might be attributable to high vulnerability of these brain regions to ischemia (Pulsinelli et al. 1982; Smith et al. 1984; Kirino 1982) and improvement of regional blood flow toward control levels with time after embolism (Miyake et al. 1993), which may possibly result from a compensatory blood flow from the contralateral hemisphere through Willis's circle. We also observed diverse effects of naftidrofuryl on various parameters and periods examined; possibly, different impairments of the parameters examined may partly be related to effectiveness of naftidrofuryl.

As described in the Introduction, naftidrofuryl has been shown to increase cerebral blood flow under normal conditions in animals. In a recent study in our laboratory, we observed that treatment of the microsphere-embolized rats with naftidrofuryl resulted in a partial restoration of the striatal and hippocampal blood flow on the 3rd day after the embolism (Miyake and Takeo 1992). The findings suggest that naftidrofuryl is capable of improving cerebral circulation of the embolized brain to some extent. Such delayed improvement of cerebral circulation in the ischemic region has also been demonstrated in rats with middle cerebral artery ligation (Hakim et al. 1992). Furthermore, we have observed in previous studies, in which the same treatment was employed, that the aggravated brain glucose metabolism in microsphere-injected rats was improved by treatment with naftidrofuryl. This was associated with recovery of cerebral high-energy phosphates (Takeo et al. 1991; Miyake et al. 1992). The agent also improved the in vitro oxidative phosphorylation activity of mitochondria isolated from microsphere-embolized rats (Takeo et al. 1989), suggesting direct effects on the mitochondrial activity. Both restoration of cerebral blood flow and improvement of brain energy metabolism may result in recovery of acetylcholine content and prevention of ischemic damage in the cerebral cortex, striatum, and hippocampus.

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