**Effects of Monochlorobimane on Cerebral Ischemia-Induced Damage to Mitochondria**

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A possible involvement of inhibitory effects of monochlorobimane (MCB) on the opening of mitochondrial permeability transition (MPT) pore in the cerebroprotection against the ischemic brain injury was examined. MCB (1 mM) inhibited the opening of MPT pore in vitro. Sustained cerebral ischemia was induced by injecting 900 microspheres (48 μm in diameter) into the right hemisphere of rats. At 12 to 72 h after microsphere embolism (ME), the mitochondrial activity was determined histochemically by staining cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) of the brain sections. The COX and SDH stainings in the hippocampus were observed intensively in the pyramidal neurons in the CA2-3 and dentate gyrus rather than those in the CA-1 region. The staining was decreased with time after the embolism. Thus, we have hypothesized that MCB may play an important role in the cerebroprotection against cerebral ischemic injury.

**Key words** cerebral ischemia; mitochondria; monochlorobimane; cytochrome c oxidase; succinate dehydrogenase

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**MATERIALS AND METHODS**

**Animals** Male Wistar rats, weighing 180—220 g (Charles River Japan, Atsugi, Japan) were used in the present study. The rats were housed in cages, maintained in a room having a 12-h light/12-h darkness cycle, a temperature of 23 ± 1 °C, and a humidity of 55 ± 5% throughout the experiment. The animals had free access to food and water according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available. The study protocol was approved by the Committee of Animal Care and Welfare of Tokyo University of Pharmacy and Life Sciences.

**Effects of MCB on the Opening of MPT Pore Isolation of Mitochondria** In the first set of experiments, the effect of MCB (Molecular Probes, Inc., Eugene, OR, U.S.A.) on the MPT in vitro was determined. Male Wistar rats, weighing 180—220 g (Charles River Japan, Atsugi, Japan) were used in the present study. The mitochondria were isolated by the method of Sims et al. Animals were anesthetized with diethyl ether and decapitated. The cerebral cortex was quickly isolated and homogenized in 10 volumes of ice-cold isolation buffer containing 0.32 M sucrose, 10 mM Tris/HCl, 1 mM EDTA, 0.25% fatty acid-free bovine serum albumin, pH 7.4. The homogenate was mixed with an equal volume of 12% Percoll. The mixture was layered on Percoll gradients, 40 and 26%, and centrifuged at 30700 × g for 10 min. This procedure yielded a dense fraction 3, followed by two washing steps of centrifugation at 167000 × g and 7300 × g, respectively, for 10 min. The final pellets were suspended in an adequate buffer and used for measurement of MPT.

The opening of MPT pore was performed according to the method of Friberg et al. The isolated mitochondria was incubated for 5 min with buffer containing 150 mM KCl, 20 mM MOPS, 10 mM Tris/HCl, pH 7.0, 2 mM nitrotriacetic acid
(NTA), 0.5 mM rotenone, 0.5 μM antimycin, 2 μM A23187, 30 μM phenylarsine oxide, and 250 μM calcium chloride in the presence or absence of different concentrations of MCB. Changes in the absorbance of the reaction mixture at 540 nm were monitored by a spectrophotometer (Ubest-30, JASCO, Tokyo). Protein concentrations were determined by the Brad- ford method.

**Treatment with MCB and Microsphere Embolism**

Rats were anesthetized i.p. with 40 mg/kg pentobarbital and fixed to the stereotactic apparatus (SR-6, Narishige, Japan). The head skin was dissected from the rat skull. The pore was made in the skull at the point of 0.8 mm posterior from the bregma and 1.5 mm lateral to the right from the midline. Then, the syringe was positioned at the 4.0 mm depth from the surface and 10 μg/5 μl MCB was injected through this syringe at a speed of 5 μl/min. Thirty minutes after the injection of MCB, the microsphere embolism was conducted. The dose of MCB used in vivo study was determined on the basis of data in a preliminary dose–response study.

Microsphere-induced cerebral embolism was performed by the method described previously. The right carotid and right pterygo-palatine arteries were temporarily ligated with strings. Nine hundred microspheres (47.5 μm in diameter, NEN-0.05, New England Nuclear, Boston, MA, U.S.A.), suspended in 20% dextran solution, were injected into the right internal carotid artery through the needle cannula. The needle was removed and then the needle bore wound was closed with surgical glue. Thereafter, the blood was recirculated. It took approximately 2 min to recirculate in the occluded region. Sham-operated rats were injected with the same volume of vehicle without microspheres. Fifteen hours after the operation, the neurological deficits of the operated animals were scored on the basis of paucity of movement, truncal curvature and force circling during locomotion, which was considered to be typical symptoms of stroke in rodents.

The score of each item was rated from 3 to 0 (3, very severe; 2, severe; 1, moderate; 0, faint or none). The rats with a total score of 7—9 points were used in the present study.

**Mitochondrial Activity: COX and SDH Staining**

The rats were anesthetized i.p. with 40 mg/kg pentobarbital and their brains were intraventricularly perfused with a cold glyc-erin solution (500 ml) at 4 °C at the injection speed of 10 ml/min. The blood and perfusate were drained through the right atrium. The isolated brain was coronally sectioned with their brains were intraventricularly perfused with cold glyc-erin solution (500 ml) at 4 °C at the injection speed of 10 ml/min. The blood and perfusate were drained through the right atrium. The isolated brain was coronally sectioned with 10 ml/min. The blood and perfusate were drained through the right atrium. The isolated brain was coronally sectioned with their brains were intraventricularly perfused with cold glyc-erin solution (500 ml) at 4 °C at the injection speed of 10 ml/min. The blood and perfusate were drained through the right atrium. The isolated brain was coronally sectioned with 10 ml/min. The blood and perfusate were drained through the right atrium. The isolated brain was coronally sectioned with

The tissue sections with a 20-OCT compound (Miles-Sankyo, Tokyo) and frozen with liquid nitrogen in 5—10 min. The tissue sections were embedded with the right atrium. The isolated brain was coronally sectioned with

**RESULTS**

**Effect of Monochlorobimane on the Opening of MPT Pore**

The effects of MCB on the opening of MPT pore were examined in vitro (Table 1, n=6 each). The opening of MPT pore was depressed with increased concentrations of MCB. MCB at the concentration of 1 mM significantly attenuated the opening of MPT pore.

**Operation**

In the present study, we injected microspheres into 15 rats. Among them, one rat died by the 3rd day after ME. Twelve rats with severe stroke-like symptoms (A type; 80%) were used in the following study. The symptoms of two rats were less than those of the A type rats. Six rats were sham-operated without microspheres.

**Changes in Neurological Deficits**

Typical stroke-like symptoms in rodents (scores more than 7) were observed in 13 out of 16 microsphere-embolized rats. Table 2 shows changes in neurological deficits of the ME and MCB-treated ME rats. The neurological deficits were gradually decreased with time after ME. There were no significant differences in the score between two groups. No stroke-like symptoms were seen in any sham-operated rat.

**Histochemical Examination of the Mitochondrial Enzymes**

Histochemical examination of the COX and SDH staining of the cerebral cortex and hippocampus was con

### Table 1. Changes in Neurological Deficits of ME Rats with and without Monochlorobimane Treatment

<table>
<thead>
<tr>
<th>Day</th>
<th>ME</th>
<th>ME+MCB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.0±0.9</td>
<td>8.1±0.9</td>
</tr>
<tr>
<td>2</td>
<td>6.8±0.8</td>
<td>6.7±0.7</td>
</tr>
<tr>
<td>3</td>
<td>5.7±0.6</td>
<td>5.5±0.6</td>
</tr>
</tbody>
</table>

There was no significant difference in values between the microsphere-embolized and monochlorobimane-treated microsphere-embolized groups.

### Table 1. Effects of Monochlorobimane on the Mitochondrial Permeability Transition in Vitro

<table>
<thead>
<tr>
<th>Monochlorobimane (mM)</th>
<th>0</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>100±4.7</td>
<td>94.8±5.9</td>
<td>82.8±4.9</td>
<td>58.8±4.7*</td>
<td></td>
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</tbody>
</table>

Control value (a decrease in absorbance/mg protein/5 min, determined in the absence of MCB) was 2.9±0.14 (n=6). Expressed values (% of control) represent the mean±S.E.M. of 6 experiments. * Significantly different from control value (p<0.05).
ducted \((n=4 \text{ each})\). There were no significant differences in the degree of the COX and SDH staining between control (naïve) and sham-operated rats in the histochemical examination. Figure 1 shows the time course of changes in COX (upper panels) and SDH (lower panels) staining of the hippocampus of the control (0 h) and ME rats at 12, 24, and 72 h after ME. The COX and SDH stainings in the hippocampus were seen strongly in the stratum oriens rather than the stratum radiatum and lacunosum. Furthermore, the pyramidal neurons in the CA2-3 and dentate gyrus were intensively stained, rather than those in the CA-1 region. In microsphere-embolized rats, the staining was gradually decreased, particularly in the upper layers of CA2-3 and the molecular layer of the dentate gyrus of the hippocampus, along with time after the operation. At 24 h after the operation, the staining of COX and SDH was globally decreased. A similar trend in the time course of changes of the staining profile was seen in the cerebral cortex, particularly, strong stainings of the COX and SDH were observed in the 4th layer of the cerebral cortex.

Figure 2 shows time course of changes in COX and SDH activities in the hippocampus obtained from semi-quantitative analysis. The COX activity markedly decreased at 12 h after the operation and the decreased activity was continued for 72 h after the operation. The SDH activity decreased from 24 h after the operation.

Figure 3 shows the COX and SDH staining of the parietal cortex and hippocampus of the right hemisphere of ME and MCB-treated ME rats at 24 h after the operation. Reduction (arrowheads) in the staining of the COX and SDH after the operation was attenuated by treatment with MCB.

Figure 4 shows changes in COX and SDH activities in the cerebral cortex, particularly, strong stainings of the COX and SDH were observed in the 4th layer of the cerebral cortex.
parietal cortex and hippocampus obtained from semi-quantitative analysis. Treatment with MCB significantly attenuated the decreased-activities of COX and SDH in the hippocampus, whereas there were no changes in the activities of COX and SDH in the parietal cortex between animals-treated with and without MCB at 24 h after ME.

DISCUSSION

In the present study, we at first determined whether MCB is capable of inhibiting the opening of MPT pore in vitro. The agent significantly inhibited the opening of MPT pore, suggesting the possibility of the inhibitory effects of MCB on the MPT.

Then, we performed histological examination to explore alterations in the mitochondria of the cerebral cortex and hippocampus after ME. COX and SDH are localized to the inner membrane in the mitochondria. Both enzymes are pivotal components of the respiratory chain in the oxidative phosphorylation process, and thus play a critical role in the energy production in mitochondria. Staining of enzymatic activity in tissues is considered to represent not only the location of markers but also the mitochondrial activity in the targeting region. The time course of changes in the staining in the right hippocampus showed that the staining of COX and SDH was decreased with time after ME, suggesting that the ischemic injury to mitochondria was aggravated with time after microsphere embolism. In contrast, the cerebral cortex showed a mild alteration in the stainings of COX and SDH at 24 h, but appreciably damaged at 72 h after ME. The difference of the alterations in brain regions may be due to that in the sensitivity to ischemic insult or that in the activity of aerobic glucose metabolism. COX and SDH activities in the CA2-3 regions were high, whereas the activities were low in the CA1 region. Although the exact mechanism for the differences in the activities of the hippocampal subfields, such difference appears to be linked to vulnerability of the CA1 and resistance of CA2-3 regions to ischemia.

In a previous study, we reported that high-energy phosphate levels of the right hemisphere of this model were markedly decreased on the first day and the mitochondrial SDH activity was greatly reduced on the 3rd and 5th days after the operation. This was consistent with the present finding. The shortage of energy may lead to inhibition of COX and SDH protein synthesis and/or enhancement of the degradation of these proteins mediated by ischemia-induced proteolysis.

Pretreatment with MCB attenuated the ischemia-induced reduction in the stainings of COX and SDH at 24 h after ME. Opening of the MPT pore is suggested to induce a decline in the mitochondrial membrane potential and a release of cytochrome c, thereby leading to mitochondrial dysfunction and eventually to cerebral cell death. Prevention of the opening of MPT pore by MCB may play an important role in the cerebroprotection against cerebral ischemic injury.

REFERENCES