

Protective Effect of Minaprine against the Abnormal Changes of 2-Deoxyglucose Uptake by Rat Hippocampal Slices Induced by Hypoxia/Hypoglycemia

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ABSTRACT—Effect of minaprine on hypoxia- or hypoxia/hypoglycemia (ischemia)-induced impairment of 2-deoxyglucose (2DG) uptake by rat hippocampal slices was evaluated. Since minaprine was found to possess both a stimulating effect on acetylcholine release and a blocking effect on 5-HT₂ receptors, the improving effect of minaprine on impaired 2DG uptake was compared to the findings obtained with oxotremorine, ketanserin and pentobarbital. Hippocampal slices were exposed to 20-min ischemia, and then these slices were returned to oxygenated and glucose-containing buffer for 6 hr. Ischemia reduced 30 mM KCl-induced 2DG uptake by the hippocampus. Pretreatment with minaprine, oxotremorine, pentobarbital and ketanserin attenuated the ischemia-induced decline of 2DG uptake. In addition, minaprine, oxotremorine and pentobarbital relatively recovered the increase of 2DG uptake in the hippocampal slices under hypoxia for 45 min. The present results suggest that minaprine exerts a neuroprotective action against ischemia-induced deficit of energy metabolism *in vitro*.

Keywords: Ischemia, 2-Deoxyglucose uptake, Hippocampus, Serotonin, Acetylcholine

Minaprine, 3-(2-morpholino-ethylamino)-4-methyl-6-phenylpyridazine dihydrochloride, is an atypical antidepressant drug (1, 2). In addition to this property, minaprine is effective for the treatment of multi-infarct and Alzheimer-type senile dementia (3). In addition, minaprine improves the memory impairment in Mongolian gerbils (4) and in rats (5) caused by cerebral ischemia. Minaprine prevents both the reduction of hippocampal theta waves and a delayed neuronal death in the hippocampal CA1 subregion induced by the bilateral occlusion of the carotid arteries of Mongolian gerbils (6). In biochemical experiments, Muramatsu et al. demonstrated that minaprine increases the release of acetylcholine (ACh) from the hippocampus by a blocking action on the 5-HT₂ receptors located on cholinergic nerve terminals (7).

Because glucose is the major energy substrate for nervous tissue, 2-deoxyglucose (2DG) uptake is an index of regional energy consumption, which is linked in turn to neuronal activity (8, 9). Recently we reported that hypoxia or ischemia changed the 2DG uptake by

hippocampal slices, and this change was attenuated by treatments with *N*-methyl-D-aspartate receptor antagonists and pentobarbital, suggesting that the impairment of 2DG uptake can serve as a marker of ischemia-induced functional deficits *in vitro* (10, 11).

The present study was therefore undertaken to investigate the protective effect of minaprine against the abnormal change of 2DG uptake by hippocampal slices induced by hypoxia or ischemia. This result was compared with other drugs, pentobarbital, the 5-HT₂ receptor antagonist ketanserin, and the cholinergic receptor agonist oxotremorine.

MATERIALS AND METHODS

Materials and procedure

Male Wistar rats weighing 250–300 g were used. Animals were decapitated and the cerebrum was removed quickly from the skull and cooled in ice-cold Krebs-Ringer solution for about 1 min. The coronal hippocampal slices (450- μ m-thick) was prepared using a tissue chopper. Slices were isolated within 5 min after decapitation. The composition of the control Krebs

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solution, equilibrated with 95% O₂–5% CO₂, was: 129 mM NaCl, 1.3 mM MgSO₄, 22.4 mM NaHCO₃, 1.2 mM KH₂PO₄, 4.2 mM KCl, 10.0 mM glucose and 1.5 mM CaCl₂. The slices were held between two sheets of nylon mesh.

The chamber design, slice transfer methods, and incubation procedures were described in our previous reports (10, 12). The buffer had a pH of 7.3–7.4 throughout the experiment. The temperature was 37°C in all phases of the experiment. For induction of hypoglycemia, the glucose in the incubation medium was replaced by 10 mM sucrose. Hypoxia solution was equilibrated with a 95% N₂–5% CO₂ gas mixture for at least 1 hr.

Treatment with drugs

Preparations were preincubated with standard Krebs solution for 1 hr. Slices were exposed to normal Krebs-Ringer solution containing the drug for 20 min before the induction of hypoxia/hypoglycemia (ischemia) and then removed. These were then placed in ischemic solution containing the drug for 10 or 20 min. Following this procedure, the slices were removed from the ischemic solution. After 10-min ischemia, the slices were washed with normal Krebs-Ringer solution for 2 min, and then the slices were incubated with Krebs-Ringer solution containing [¹⁴C]2DG. In the 20-min ischemia group, the slices were placed in normal Krebs-Ringer solution for 6 hr and then incubated with Krebs-Ringer solution containing [¹⁴C]2DG. Some slices were incubated with normal Krebs-Ringer solution containing [¹⁴C]2DG and the drug for 45 min.

2DG uptake

Slices were incubated with Krebs-Ringer solution containing 0.2 μ Ci/ml of [¹⁴C]2DG [Spec. act. 50 mCi/mmol; Amersham] and 30 mM KCl for 45 min. The incubation chamber was arranged to recirculate 14 ml of buffer at 4.4 ml/min with continuous bubbling of humidified 95% O₂–5% CO₂ through the buffer as it entered the chamber. To initiate an incubation, the slices held between the two mesh sheets were removed from the preincubation chamber and drained briefly; 1 ml of the isotope buffer was pipetted over the slices, which were then placed in the incubation chamber within 10 sec of removal from the first chamber. In this manner, a square-wave pulse of radioactivity was delivered to the slices. Incubations were terminated by removing the disk preparations from the incubation chamber, rinsing it with 20 ml of warm buffer and placing it in a chamber identical to the preincubation chamber for 30 min. At the end of the washout period, slices were placed on dry ice to stop the metabolism and were

then frozen at –80°C. The time from the final buffer drainage to freezing was within 10 sec.

For biochemical assay, the frozen tissue was homogenized in 1 ml phosphate solution containing 0.5% perchloric acid, and a 450- μ l aliquot of homogenate was used to determine the total amount of protein. The radioactivity in another 450 μ l of the homogenate was measured in a liquid scintillation counter after the homogenate was solubilized.

Drugs

The drugs used in this study were minaprine (Taisho Pharmaceu. Inc., Japan), oxotremorine (Funakoshi Inc., Japan), pentobarbital (Tanabe Inc., Japan), and ketanserin tartrate (Funakoshi Inc., Japan). All drugs were dissolved in distilled water and then diluted with Krebs-Ringer solution. The Krebs-Ringer solution containing drugs (100 μ M) had a pH between 7.2–7.4, and these values were not different from those of the non-treated solution (pH 7.3–7.4).

Statistical analyses

Data are expressed as means \pm S.E. Significant differences between groups were determined with an analysis of variance followed by the Duncan test.

RESULTS

2DG and hypoxia

The 2DG uptake was observed under the hypoxic condition for 45 min. Under the hypoxic condition, 2DG uptake in the hippocampal slices increased to $296 \pm 35\%$ ($n = 12$) that of normoxic control slices. The hypoxia-induced increase in 2DG uptake was significantly attenuated by minaprine, pentobarbital and oxotremorine (Fig. 1). Under the normoxic condition, 2DG uptake by the hippocampus, which was compared to the control vehicle-treated slices, was not significantly affected by minaprine ($105 \pm 6.4\%$, $n = 6$ by 10 μ M and $107 \pm 4.4\%$, $n = 5$ by 100 μ M), pentobarbital ($114 \pm 8.1\%$, $n = 5$ by 10 μ M and $89 \pm 3.87\%$, $n = 5$ by 100 μ M), and oxotremorine ($103 \pm 3.3\%$, $n = 12$ by 1 μ M and $90 \pm 2.7\%$, $n = 12$ by 10 μ M).

2DG uptake and ischemia

When normal slices were incubated with 2DG following a 1-hr preincubation, 30 mM KCl-induced uptake of 2DG in the hippocampus reached 33.2 ± 1.5 , ($n = 3$) dpm/mg protein/45 min. The time course of the recovery rate of 2DG uptake in slices following 10-min and 20-min ischemia was compared to that of normal slices (Fig. 2). Slices were exposed to 10-min ischemia, and immediately after 2-min washout with normal Krebs-

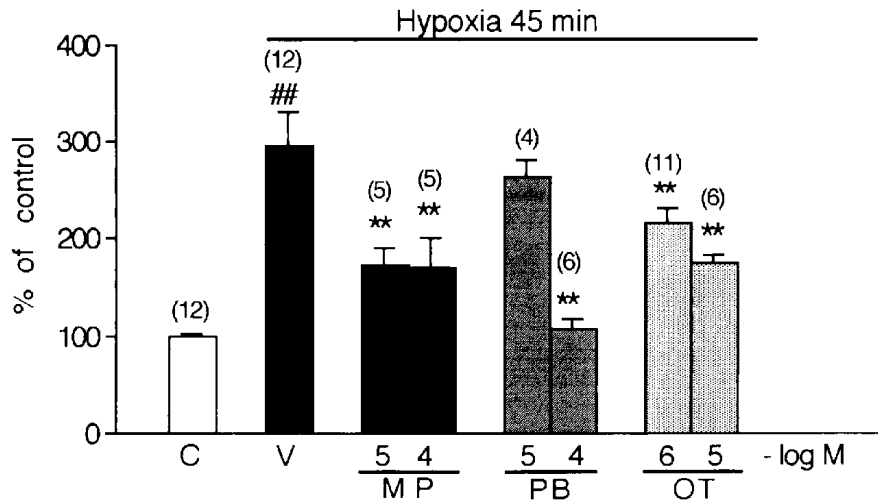


Fig. 1. Effects of minaprine (MP), pentobarbital (PB) and oxotremorine (OT) on the 2-deoxyglucose (2DG) uptake increase in hippocampal slices induced by 45-min hypoxia. The 2DG uptake by normal hippocampal slices was designated as 100%. C: non-hypoxia-treated slices, V: vehicle (distilled water)-treated slices. Values in the figure are means \pm S.E. Numbers in the parentheses are the number of slices. ** $P < 0.01$ vs. vehicle-treated group. ## $P < 0.01$ vs. normal non-hypoxia group.

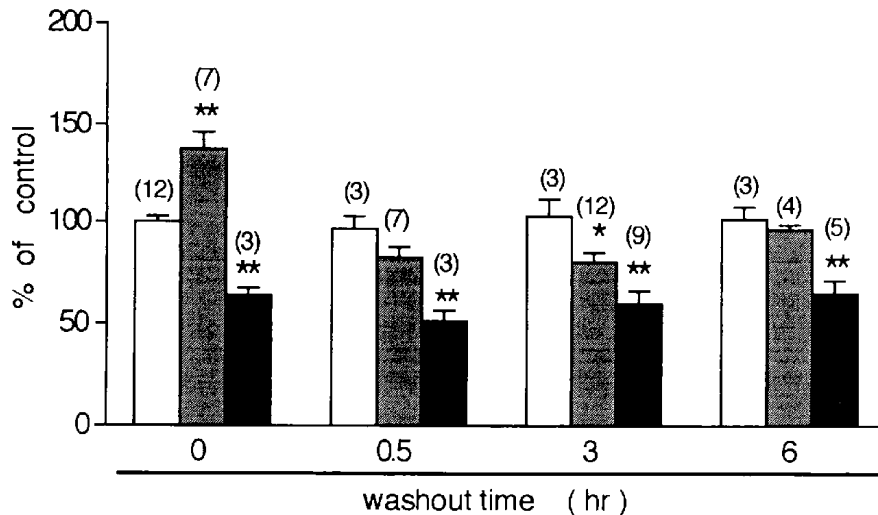


Fig. 2. Effect of 10- or 20-min hypoxia/hypoglycemia (ischemia) on 30 mM KCl-induced elevation of 2-deoxyglucose (2DG) uptake by hippocampal slices. The 2DG uptake by normal hippocampal slices after 0-min washout was designated as 100%. □: non-ischemia-treated slices, ■: 10-min-ischemia-treated slices, ■: 20-min-ischemia-treated slices. Values in the figure are means \pm S.E. Numbers in the parentheses are the number of slices. * $P < 0.05$, ** $P < 0.01$ vs. normal non-ischemia group.

Ringer solution, the slices were incubated with [14 C]2DG for 45 min. The 2DG uptake by hippocampal slices was significantly increased. However after the 30-min or 6-hr washout, 2DG uptake was recovered to the control levels. The 20-min ischemia dramatically decreased 2DG uptake after the 0.5-hr washout, but there was little recovery of 2DG uptake after the 6-hr washout. The 2DG uptake of slices exposed to 20-min ische-

mia was, however, still declined by about 40% after the 6-hr washout.

When 2DG uptake was examined immediately after 10-min ischemia, it was significantly increased in hippocampal slices (Fig. 3). The ischemia-induced increase in 2DG uptake was significantly attenuated by treatment with minaprine (100 μ M) or pentobarbital (100 μ M).

The 2DG uptake of the control slices was 33.8 ± 2.0

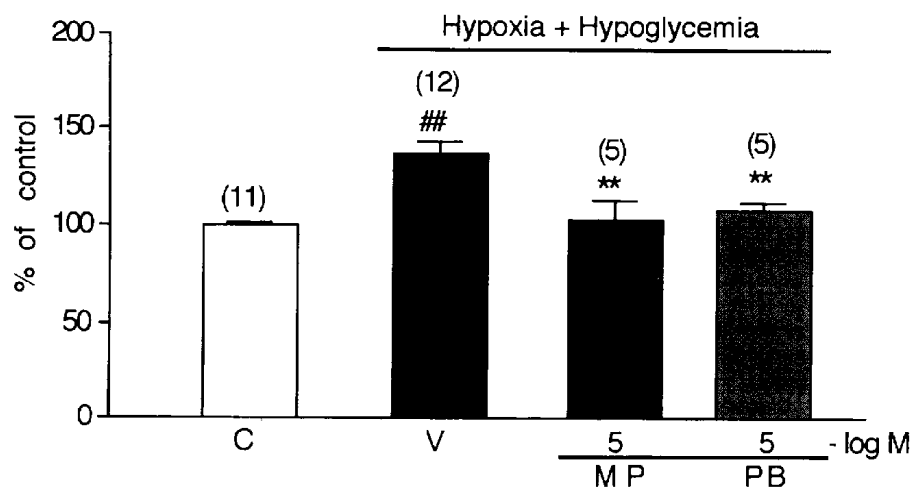


Fig. 3. Effects of minaprine (MP) and pentobarbital (PB) on the increase in 2-deoxyglucose (2DG) uptake in hippocampal slices induced by 10-min hypoxia/hypoglycemia (ischemia). The 2DG uptake was measured at 0-min washout after 10-min ischemia. The 30 mM KCl-induced 2DG uptake by normal hippocampal slices was designated as 100%. C: non-ischemia-treated slices, V: vehicle (distilled water)-treated slices. Values in the figure are means \pm S.E. Numbers in the parentheses are the number of slices. ** $P < 0.01$ vs. vehicle-treated group. ## $P < 0.01$ vs. normal non-ischemia group.

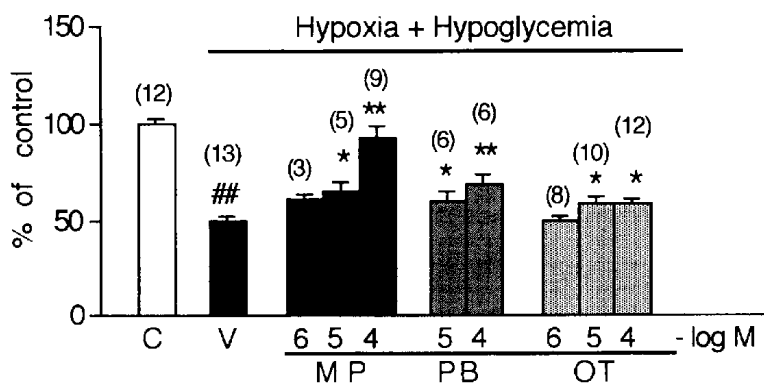


Fig. 4. Effects of minaprine (MP), pentobarbital (PB) and oxotremorine (OT) on the decrease in 2-deoxyglucose (2DG) uptake in hippocampal slices induced by 20-min hypoxia/hypoglycemia (ischemia). The 2DG uptake was measured at 6-hr washout after 20-min ischemia. The 30 mM KCl-induced 2DG uptake by normal hippocampal slices was designated as 100%. C: non-ischemia-treated slices, V: vehicle (distilled water)-treated slices. Values in the figure are means \pm S.E. Numbers in the parentheses are the number of slices. * $P < 0.05$, ** $P < 0.01$ vs. vehicle treated group. ## $P < 0.01$ vs. normal non-ischemia group.

($n = 12$) dpm/ μ g protein/45 min, and this was designated as 100% uptake. The 2DG uptake of slices exposed to 20-min ischemia was still decreased to 50% that of normal control slices after the 6-hr washout. Treatment with minaprine, pentobarbital and oxotremorine significantly attenuated the decline of 2DG uptake induced by ischemia in a dose-related manner (Fig. 4). In addition, treatment with the 5-HT₂ receptor antagonist ketanserin also had an attenuative effect against the impairment of 2DG uptake. The 2DG uptake by hippocampal slices exposed to 20-min ischemia

is $49 \pm 2.3\%$ ($n = 12$), and the decrease of 2DG uptake is attenuated by ketanserin at doses of 1 μ M ($55 \pm 8.3\%$, $n = 4$, $P > 0.05$), 10 μ M ($62 \pm 4.9\%$, $n = 8$, $P < 0.05$) and 100 μ M ($64 \pm 2.9\%$, $n = 9$, $P < 0.05$).

DISCUSSION

In the present experiment, 45-min hypoxia and 10-min ischemia increased 2DG uptake in hippocampal slices, but 20-min ischemia decreased it. At present, the reason for these differences is uncertain, but a possible

explanation is that hypoxia or mild ischemia such as 10-min ischemia may increase anaerobic metabolism and result in an increase in the rate of 2DG phosphorylation, whereas 20-min ischemia may decline energy demands due to metabolic injury. Newman et al. (12) already demonstrated similar changes using thick slices as an *in vitro* ischemia model. A transient increase in release of neurotransmitters including glutamate occurs during ischemia (13, 14). Therefore, glutamate may depolarize and stimulate 2DG uptake of hippocampal neurons in the early stage of ischemia. Treatment with minaprine (10 and 100 μ M) attenuated the increase of 2DG uptake under the hypoxic condition, but the same concentration of this drug did not change 2DG uptake under normal conditions. When the 2DG uptake was examined immediately after 10-min ischemia, it was significantly increased in the hippocampus. The treatment with minaprine (10 μ M) attenuated this increase. In addition, 2DG uptake of slices exposed to 20-min ischemia was impaired, and this impairment of 2DG uptake was also attenuated by the treatment with minaprine (10 and 100 μ M). In the previous *in vivo* experiment, it was demonstrated that minaprine prevented both ischemia-induced hippocampal damage and the passive avoidance deficit (4, 6). In addition, minaprine shows a protective effect against cerebral hypoxia and anoxia in a variety of animal models (15). The present results demonstrate that hypoxia- or ischemia-induced abnormal change of 2DG uptake, an index of regional energy consumption, is significantly attenuated by the treatment with minaprine. This *in vitro* result is well-consistent with previous *in vivo* results. These findings suggest that the attenuating effect of minaprine on energy metabolism may play an important role in the amelioration of behavioral deficits in a variety of ischemic models (4, 5).

At present, the mechanism of protection by minaprine is uncertain, and the precise mechanism should be clarified in the future. Biochemical experiments suggest that minaprine acts as a cholinergic drug, because minaprine increases the release of ACh from the hippocampus by its blocking action on 5-HT₂ receptors (7). Therefore in the present experiment, we examined the effects of oxotremorine and a 5-HT₂ receptor antagonist on the abnormal change of 2DG uptake induced by hypoxia or ischemia. This abnormal change of 2DG uptake was significantly attenuated by treatment with oxotremorine. At present, the precise mechanism of muscarinic receptor agonist-induced neuroprotection is uncertain. Most studies have emphasized the detrimental role of excessive release of glutamate in producing ischemic injury (13, 14). The stimulation of muscarinic receptor by oxotremorine or ACh inhibits an en-

dogeneous release of glutamate and aspartate from the hippocampus (16, 17). Thus inhibition of glutamate and aspartate release by oxotremorine might have important pharmacological implications in the protection against a loss of 2DG uptake during ischemia.

The stimulation of 5-HT₂ receptor modulates the increase of inositol phospholipid turnover and release of arachidonic acid (18, 19), and these may also stimulate the release of intracellular calcium. The increase of cellular calcium ions is thought to be an important trigger in ischemic cell death (20, 21). Although autoradiographic studies indicate the existence of 5-HT₂ binding sites in the hippocampus (22–24), their density is low in this region. However Muramatsu et al. (7) has observed binding sites for [³H]-ketanserin in hippocampal homogenates. In addition, release of arachidonic acid and inositol phosphate mediated by 5-HT₂ receptors are observed in cultures of hippocampal cells (19). Taking these facts into consideration, we propose that ketanserin may reduce ischemia-induced impairment of 2DG uptake in hippocampal slices, through its blocking action on 5-HT₂ receptors. 5-HT₂ receptor antagonists, naftidrofuryl and emopamil, exhibited a protective effect against ischemic neuronal damage in gerbils and rats (25, 26). In addition, behavioral experiments demonstrated that 5-HT₂ receptor antagonists can ameliorate a hypoxia-induced performance deficits in an animal model of learning and memory (27). These lines of evidence along with the present results have suggested possible interactions between cholinergic and serotonergic receptor mechanisms in the neuroprotective action of minaprine.

Pentobarbital prevented hypoxia- or ischemia-induced abnormal change of 2DG uptake by hippocampal slices. In *in vivo* experiments, it was reported that pentobarbital prevented the ischemia-induced neuronal death of CA1 pyramidal cells. Thus pentobarbital may ameliorate neuronal damage to hippocampal cells by either inhibiting protein kinase C activity or enhancing the GABA-ergic inhibitory effect (28, 29).

In summary, the present results suggest that minaprine possesses a neuroprotective action against ischemia-induced impairment of energy metabolism *in vitro*. This neuroprotective effect of minaprine may be related to the amelioration of behavioral deficits by minaprine in the *in vivo* model of ischemia.

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