Effect of Benidipine Hydrochloride (KW-3049), on Cerebral Ischemia Induced by Bilateral Occlusion of the Common Carotid Arteries in Rats

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The effect of benidipine on cerebral ischemia was investigated in rats subjected to occlusion of the bilateral common carotid arteries. Benidipine (30 μg/kg, i.p.) improved neurological symptoms such as ataxia, convulsion and loss of righting reflex, and prolonged survival time after occlusion of the bilateral common carotid arteries. In the nicardipine (100 μg/kg, i.p.)-treated group, a similar effect was observed, whereas nifedipine (100, 300 μg/kg, i.p.) and verapamil (300 μg/kg, i.p.) did not show any beneficial effect in this model. Furthermore, pretreatment with benidipine (30 μg/kg, i.p.) suppressed the increase in cerebral water content 3 h after the occlusion. Nicardipine (100 μg/kg, i.p.) showed a tendency to reduce the increase in cerebral water content, though the effect was not statistically significant. Nifedipine (100 μg/kg, i.p.) produced no improvement. After occlusion of the bilateral common carotid arteries, depletion of adenosine triphosphate (ATP) and phosphocreatine (CP) and an accumulation of lactate occurred in a time-dependent manner. Prophylactic administration of benidipine (30 μg/kg, i.p.), 20 min before occlusion, attenuated the depletion of ATP and CP and the accumulation of lactate 3 h after the occlusion. Furthermore, post-treatment with benidipine 30 min after occlusion also suppressed these metabolic disorders. In conclusion, the beneficial effects of benidipine in this severe cerebral ischemia model show that the compound has advantages over nicardipine, nifedipine and verapamil. Thus, these results suggest that benidipine may be useful in the treatment of acute ischemic cerebral damage.

Keywords: benidipine; cerebral ischemia; occlusion; Ca²⁺-antagonist

Introduction

A reduction of cerebral blood flow induces cerebral ischemia, causing a rapid elevation of intracellular calcium (Ca²⁺). Many reports have shown that ischemia disrupts Ca²⁺ haemostasis in the cell and this suggests that Ca²⁺ triggers a following ischemia. Intracellular Ca²⁺ overload impairs mitochondrial function and inhibits adenosine triphosphate (ATP) production. Moreover, a high Ca²⁺ concentration triggers the well-known cascade of lipolytic and proteolytic events which eventually result in cell death. Ca²⁺-antagonists appear to inhibit Ca²⁺ entry into cells via the so-called slow channels in the cell membrane and prevent or reduce metabolic disturbances associated with ischemia. In addition, it is well known that Ca²⁺-antagonists are potent vasodilators and increase cerebral blood flow. Therefore, it is to be expected that Ca²⁺-antagonists will reduce ischemic cerebral damage. In fact, it has already been reported that Ca²⁺-antagonists produce beneficial effects on cerebral damage in various models of acute cerebral ischemia.

Benidipine is a newly-developed 1,4-dihydropyridine Ca²⁺-antagonist that has long-lasting antihypertensive and antianginal activities. Additionally, it has been reported that benidipine increases vertebral blood flow and cerebral cortex blood flow in experimental animal models. In the present study, we have evaluated the possible protective effect of benidipine in cerebral damage after occlusion of the bilateral common carotid arteries in rats, and compared the effects of benidipine with those of nicardipine, nifedipine and verapamil.

Materials and Methods

Animals Male Wistar rats (Japan SLC, Inc.), weighing 230–250 g were used. All animals had free access to standard rat chow and water.

Surgical Procedure and Behavioral Observation Under ether anesthesia, the bilateral common carotid arteries were exposed in the neck and ligated with surgical threads. After ligation, the neck was closed and the rat recovered from anesthesia. The behavior and mortality was monitored at 1 h interval for 8 h. The degree of symptoms was graded 0–5: 0: Normal appearance; 1: slight weakness of fore- and hind-limbs; 2: ataxia; 3: convulsion; 4: loss of righting reflex; 5: death.

Cerebral Water, Sodium (Na⁺) and Potassium (K⁺) Concentrations Three hours after occlusion, the animals were sacrificed. The complete cerebrum was removed from the skull, and the wet weight (W) of the cerebral hemispheres was measured. The cerebral hemispheres were then dried in an oven (Drying sterilizer SH 42, Yamato, Japan) heated at 90 °C for 3 d and then weighed again to obtain the dry weight (D). The cerebral water content was calculated as follows:

\[ \text{cerebral water content (%) = } \left( \frac{W - D}{W} \right) \times 100 \]

The dried tissue was dissolved in 5 ml of 10% (v/v) nitric acid diluted with distilled water as required. The Na⁺ and K⁺ concentrations of each solution were then determined by flame photometry (775-A, Hitachi, Japan) with lithium as the internal standard.

Cerebral ATP, Phosphocreatine (CP) and Lactate Concentration Three hours after occlusion, the animals were subjected to microwave irradiation (5.0 kW, 1.2 s) to prevent further metabolism, and then decapitated. The complete cerebrum was homogenized in 5 ml ice-cold 6% perchloric acid, and the homogenate centrifuged at 18000 g at 0 °C for 15 min. The supernatant was neutralized with 2 M K₂CO₃ and a sample used to determine tissue high-energy phosphates and lactate. ATP concentration was determined by enzymatic assay. In brief, the extract was mixed with 100 mM Tris/HCl, pH 7.5, 5.0 mM MgCl₂, 1 mM glucose, 0.02% BSA, 0.06 mM nicotinamide adenine dinucleotide phosphate, 0.2 mM hexokinase and 0.6 U/ml glucose-6-phosphate dehydrogenase, and then the mixture was incubated for 10 min at 30 °C. Changes in the absorbance at Ex 340 nm and Em 462 nm were monitored. Measurements of CP were performed according to the method of Lowry and Passonneau. The extract was incubated at 30 °C with 0.03 mM ADP and 9.3 U/ml of creatine kinase and CP was converted to ATP. The increased ATP content in the reaction mixture, which is equivalent to the CP content, was determined by the same method as used for determining ATP. Lactate was determined using an assay kit (Kyowa Medex, Japan).

Drugs Benidipine (hydrochloride, KW-3049) and nicardipine (hydrochloride), were synthesized in our laboratories. Nifedipine and verapamil (hydrochloride) were purchased from Sigma Chemical Co. Benidipine, nicardipine and nifedipine were dissolved in saline containing 5% Tween-80 and diluted with saline to an appropriate concentration. Verapamil was dissolved in distilled water. The injection volume was 0.1 ml per 100 g body weight. Nicotinamide adenine dinucleotide phosphate (Sigma),
glucose-6-phosphate dehydrogenase (Sigma), creatine phosphokinase (Sigma), ADP (Boehringer Mannheim), hexokinase (Sigma), BSA (bovine serum albumin, Sigma) were used.

Statistical Analyses Values are expressed as means ± S.E.M. Difference was examined by analysis of variance (ANOVA) followed by Dunnet's test. Survival rate was evaluated by the χ² test. p values of 0.05 or less were considered to indicate statistically significant differences.

Results

Behavioral Effects The rats, after occlusion of their bilateral common carotid arteries, developed various symptoms such as ataxia, convulsions, loss of righting reflex and death. As shown in Fig. 1, pre-treatment with benidipine (30 μg/kg, i.p.) prevented these symptoms. While nicardipine at a dose of 100 μg/kg also prevented these symptoms, 30 μg/kg did not. The protective potency of benidipine was approximately 3 times higher than that of nicardipine. Nifedipine (100 and 300 μg/kg, i.p.) and verapamil (300 μg/kg, i.p.) had no effect.

Fig. 1. Effects of Various Drugs on Stroke Symptoms after Occlusion of the Bilateral Common Carotid Arteries in Rats

(A) Benidipine, (B) nicardipine, (C) nifedipine, (D) verapamil. Score of cumulative occurrence is indicated. Drugs were administered 20 min before occlusion. a) and b) p < 0.05 and p < 0.01 vs. control group, respectively.

Fig. 2. Effects of Various Drugs on the Survival Rate after Occlusion of the Bilateral Common Carotid Arteries in Rats

(A) Benidipine, (B) nicardipine, (C) nifedipine, (D) verapamil. Drugs were administered 20 min before occlusion. a) and b) p < 0.05 and p < 0.01 vs. control group, respectively.
Survival Time The survival rate after occlusion of the bilateral common carotid arteries is shown in Fig. 2. The survival rate for the control group at 5 and 8 h after occlusion was 47% and 18%, respectively. The survival rate for the benidpine (30 μg/kg, i.p.-treated group at 5 and 8 h after occlusion was 92% and 75%, respectively. Thus, benidpine (30 μg/kg, i.p.) produced a significant reduction of mortality. Similarly, nicardipine (100 μg/kg, i.p.) improved the survival rate. On the other hand, nifedpine (100 and 300 μg/kg, i.p.) and verapamil (300 μg/kg, i.p.) did not improve the survival rate.

Cerebral Water, Na⁺ and K⁺ Concentrations The cerebral water, Na⁺ and K⁺ concentrations 3 h after occlusion of the bilateral common carotid arteries are shown in Table I. In the normal group, the water content of the cerebrum was 78.7%. At the end of ischemia, it was 81.2%, the increase being statistically significant, compared with the normals. Benidpine (30 μg/kg, i.p.) significantly prevented the rise in cerebral water 3 h after the occlusion. Nicardipine (100 μg/kg, i.p.) showed a tendency to reduce the cerebral water content, but nifedpine (100 μg/kg, i.p.) did not.

Tissue ATP, CP and Lactate Concentrations The pre-treatment effect of benidpine on cerebral ischemia is shown in Fig. 3. Benidpine (30 μg/kg, i.p.) was administered 20 min before occlusion of the bilateral common carotid arteries and cerebral ATP, CP and lactate content were determined 3 h later. In the control group, a marked reduction in ATP and CP and an increase in lactate were observed; these differences were significant compared with the normals. Benidpine (30 μg/kg, i.p.) significantly prevented the depletion of ATP and CP and inhibited the increase in lactate.

The post-treatment effect of benidpine on cerebral ischemia is shown in Fig. 4. Benidpine (30 μg/kg, i.p.)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dose (μg/kg)</th>
<th>Route</th>
<th>Water content (%)</th>
<th>Na⁺ content (meq/g brain)</th>
<th>K⁺ content (meq/g brain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>i.p.</td>
<td>78.7 ± 0.1</td>
<td>244.9 ± 4.1</td>
<td>475.1 ± 5.2</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>i.p.</td>
<td>81.3 ± 0.3</td>
<td>262.0 ± 7.4</td>
<td>398.5 ± 3.3</td>
</tr>
<tr>
<td>Benidpine</td>
<td>30</td>
<td>i.p.</td>
<td>79.9 ± 0.4</td>
<td>262.0 ± 10.5</td>
<td>441.2 ± 7.3</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>100</td>
<td>i.p.</td>
<td>80.3 ± 0.2</td>
<td>284.2 ± 8.9</td>
<td>414.0 ± 8.0</td>
</tr>
<tr>
<td>Nifedpine</td>
<td>100</td>
<td>i.p.</td>
<td>80.8 ± 0.2</td>
<td>301.6 ± 4.2</td>
<td>403.4 ± 5.3</td>
</tr>
</tbody>
</table>

Drugs were administered 20 min before occlusion of the bilateral common carotid arteries. Values are presented as means ± S.E.M. of 5–6 rats. a) p < 0.01 vs. normal group. b) p < 0.05 vs. control group.

Fig. 3. Changes in Cerebral ATP (A), CP (B) and Lactate (C) after Occlusion of the Bilateral Common Carotid Arteries in Rats

Benidpine (30 μg/kg, i.p.) was administered 20 min before occlusion. Values are presented as means ± S.E.M. of 5 rats. Nor: Normal, Cont: Control, Beni: Benidpine. a) p < 0.01 vs. normal group, b) and c) p < 0.05 and p < 0.01 vs. control group.

Fig. 4. Changes in Cerebral ATP (A), CP (B) and Lactate (C) after Occlusion of the Bilateral Common Carotid Arteries in Rats

Benidpine (30 μg/kg, i.p.) was administered 30 min after occlusion. Values are presented as means ± S.E.M. of 5–6 rats. (●), control (■), benidpine. a) p < 0.05 vs. control group at the point of 3 h.
was administered 30 min after occlusion of the bilateral common carotid arteries and, at 30 min and 3 h after occlusion, cerebral ATP, CP and lactate were measured. In the control group, the concentrations of ATP and CP gradually decreased and the lactate level increased in a time-dependent manner. The reduction in ATP was significantly inhibited by treatment with benidipine (30 µg/kg, i.p.). In addition, the decrease in CP and increase in lactate were also inhibited by treatment with benidipine (30 µg/kg, i.p.), although these changes did not reach statistical significance.

Discussion

In the present study, we have shown that Wistar rats developed various symptoms such as ataxia, convulsions, loss of righting reflex and death after occlusion of the bilateral common carotid arteries. It is well accepted that these changes are closely related to cerebral damage following ischemia. In acute cerebral ischemia, the most remarkable changes are the depletion of tissue stores of ATP and CP. 8,13 The depletion of ATP and CP causes a failure of the enzyme, Na⁺, K⁺-ATPase. The dysfunction of Na⁺, K⁺-ATPase in the cell membrane causes membrane depolarization and accumulation of Na⁺ and water in the cell, finally inducing cytotoxic edema in the ischemic cell. 14 A decline in ATP and an increase in cerebral water content were in fact observed 3 h after the ischemia in the present study.

Ischemic depolarization of the nerve membrane is associated with a rapid influx of Ca²⁺ into the intracellular compartment. 15 Mitochondrial Ca²⁺-overload destroys mitochondrial functions, such as oxidative phosphorylating activity and ATP generating capacity. 16 At the same time, a rise in intracellular Ca²⁺-overload should activate membrane phospholipases, which would result in a release of free fatty acids, particularly arachidonic acid. 17 Polysaturated fatty acids induce edema in slices of rat brain, and free fatty acids inhibit the respiratory activity of brain mitochondria, uncoupling oxidative phosphorylation. 18 Furthermore, metabolites of arachidonic acid, such as leukotrienes and prostaglandins could contribute to cellular disintegration and death. 19 Leukotriene C₄ especially has strong vasoconstrictor activity and promotes vascular permeability, its level being related to the increase in cerebral water content. 20 Thus, excessive entry of Ca²⁺ ions into cells has been assumed to be the final common pathway of cell death. 21

In the present study, pretreatment with benidipine (30 µg/kg, i.p.) or nicardipine (100 µg/kg, i.p.) had a beneficial effect in terms of the occurrence of stroke and the mortality rate whereas nifedipine (100 µg/kg, i.p.) did not. Furthermore, while benidipine (30 µg/kg, i.p.) significantly prevented the increase in cerebral water content after occlusion of the bilateral common carotid arteries, nicardipine (100 µg/kg, i.p.) and nifedipine (100 µg/kg, i.p.) did not. This discrepancy in protective action among these drugs is likely to be due to differences in the potency and duration of action of the Ca²⁺-antagonists studied. In fact, it has been reported that, for benidipine, the potency is higher and the duration of action is longer than in the case of nifedipine and nicardipine. 10 Thus, benidipine seems to be a better candidate drug to protect against ischemic cerebral damage than nifedipine or nicardipine. Additionally, our present results indicate that not only pre-but also post-treatment with benidipine prevents the decline in ATP and CP, which are indices of energy metabolism. These results suggest that benidipine helps restore mitochondrial function following cerebral ischemia.

Regarding these protective effects of benidipine on cerebral ischemia, some possible explanations can be offered. Firstly, benidipine may act to improve the reduced cerebral blood flow resulting from occlusion of the bilateral common carotid arteries, resulting in a reduction in cell damage. Most studies have reported increased cerebral blood flow in ischemic regions of the brain in animals pretreated with Ca²⁺-antagonists. 6,22 Furthermore, Kanasawa et al. have reported that benidipine produced its vasodilating effect on vertebral arteries rather than on other arteries, resulting in a relatively selective increase in the vertebral blood flow of dogs. 10 Additionally, benidipine increased cerebral cortex blood flow in cats for a longer time compared with nicardipine. 10

Secondly, benidipine might have blocked Ca²⁺-entry into ischemic cells and thereby limited Ca²⁺-mediated cell damage. Recently, benidipine has been reported to have beneficial effects on ischemic organ failures of diverse origins, 23 suppressing accumulation of Ca²⁺ in the tissues. In addition, Uematob et al. have reported that the Ca²⁺-antagonist prevents an increase in cytosolic free Ca²⁺ concentration following cerebral ischemia in vivo. 24 Prevention of Ca²⁺ influx into ischemic cells could inhibit the accumulation of free fatty acids and their metabolites, such as prostaglandins, leukotrienes, and endoperoxides. Several investigators have proposed that Ca²⁺-antagonists may prevent this cascade of events. 25 Recently, Akctan et al. showed that nicardipine reduced the levels of leukotriene C₄ and prostaglandin E₂ in rat brain tissue following ischemia. 26 Therefore, benidipine may prevent partly the cerebral damage by directly blocking the entry of Ca²⁺ into the cell. However, in the present study, it was not clear whether benidipine prevented the increase in intracellular Ca²⁺ during ischemia, since we did not measure the Ca²⁺ content. Further investigations are necessary.

In conclusion, benidipine ameliorated the stroke symptoms, improved the mortality rate and prevented the depletion of high energy phosphates, such as ATP and CP, in the early phases of the cerebral ischemia. These results suggest that benidipine helps restore mitochondrial function during ischemia. The protective mechanisms involved seem to be the improvement of cerebral blood flow and/or direct reduction of Ca²⁺ entry into the ischemic cell. Benidipine could be a useful drug for the treatment of acute cerebrovascular diseases.

References


17) P. H. Chann and R. A. Fishmann, J. Neurochem., 35, 1004 (1980);


24) A. Karasawa and K. Kubo, J. Pharmacobiol.-Dyn., 11, 722 (1988);

