Effects of Clentiazem on Cerebral Ischemia Induced by Carotid Artery Occlusion in Stroke-Prone Spontaneously Hypertensive Rats

Kohei Kikkawa, MS; Rikako Yamauchi, MS; Toshikazu Suzuki, PhD; Kiyoshi Banno, PhD; Sakae Murata, BS; Tsunao Tetsuka, MD; Taku Nagao, PhD

Background and Purpose We examined metabolic and functional changes when forebrain ischemia was induced in stroke-prone spontaneously hypertensive rats by bilateral carotid artery occlusion. In addition, the protective effect of clentiazem was evaluated in this model.

Methods Rats were anesthetized with urethane. Cerebral blood flow was measured with a laser Doppler flowmeter. Cerebral high-energy phosphates and intracellular pH were measured by phosphorus magnetic resonance spectroscopy. Electroencephalographic activity was evaluated as the summation of its amplitude. These parameters were monitored during a 30-minute period of ischemia and recirculation. Clentiazem was given orally as pretreatment (10 mg/kg twice a day for 3.5 days).

Results Bilateral carotid occlusion caused a decrease in cerebral blood flow to approximately 5% of the preischemic level and the disappearance of electroencephalographic activity. Occlusion also caused a decrease in ATP and phosphocreatine (to 48.7±4.3% and 23.7±2.2% of preischemic levels, respectively) as well as intracellular pH (from 7.3±0.1 to 6.0±0.1). During recirculation the reversal of these changes was variable: high-energy phosphates were partially restored, but electroencephalographic activity and intracellular pH showed little improvement. Hypoperfusion (55.7±11.5% of the preischemic flow) developed after reactive hyperemia. Pretreatment with clentiazem lessened the decrease in cerebral blood flow (control, 4.8±1.4%; clentiazem, 14.1±4.1% of the preischemic level; P<.05) and prevented the disappearance of electroencephalographic activity in some rats during ischemia. Clentiazem also prevented postischemic hypoperfusion and accelerated the restoration of high-energy phosphates, intracellular pH, and electroencephalographic activity during recirculation.

Conclusions Carotid artery occlusion induced stable forebrain ischemia in stroke-prone spontaneously hypertensive rats. Clentiazem improved the metabolic and functional disturbances that occurred in this ischemic model, and its beneficial effect appeared to be due mainly to the relative preservation of cerebral blood flow during carotid occlusion.

Key Words • calcium antagonists • carotid arteries • cerebral ischemia • occlusion • rats

Hypertension is one of the important factors contributing to the progression of cerebral ischemia. Many studies using spontaneously hypertensive rats (SHR) have demonstrated that bilateral carotid artery occlusion (BCAO) causes forebrain ischemia because of an upward shift in cerebral blood flow autoregulation. However, there have been few studies on cerebral ischemia in stroke-prone spontaneously hypertensive rats (SHRSP), which develop more severe hypertension than SHR. 4-5 In the present study we evaluated whether clentiazem could improve cerebral ischemia in this rat model.

Materials and Methods

Animal Preparation

Stroke-prone spontaneously hypertensive rats derived from the Okamoto-Aoki strain of SHR (obtained from Dr K. Okamoto of Kinki University Medical School, Osaka, Japan) were bred by Marugo Research Service Co, Ltd, Saitama, Japan. All experiments were performed on male SHRSP (22 to 33 weeks old, with a systolic blood pressure of 220 to 260 mm Hg by the tail-cuff method, F1 to F2 generations). The rats were given normal laboratory chow (CE-2, Nihon Clea, Tokyo, Japan) and water ad libitum. Anesthesia was induced with an intraperitoneal injection of urethane (1.0 g/kg). After tracheotomy the animals were artificially ventilated (70 breaths per minute, 1 mL/100 g). Both common carotid arteries were carefully dissected from the vagus nerves via a ventral midline cervical incision. A catheter was placed in the femoral artery for blood gas measurements. Cerebral ischemia was produced by clamping the bilateral carotid arteries. After ischemia had been maintained for 30 minutes, recirculation...
The cerebral blood flow response to carbon dioxide inhalation was checked, and the flow data were recorded continuously and was evaluated by summation of the constant of 1.5 seconds. The cerebral blood flow response to clentiazem was initiated by removing the clamps. Arterial blood gases and pH were measured with a blood gas analyzer (Radiometer ABL30, Copenhagen, Denmark). The body temperature was controlled with a heating pad, and the rectal temperature was adjusted to 36.2°C to 37.0°C during the experiment. In the nuclear magnetic resonance (NMR) spectroscopy experiment, respiration was spontaneous because of technical limitations. Clentiazem was administered at a dose of 10 mg/kg twice a day for 3.5 days. The final dose was given 90 minutes before BCAO. In control rats distilled water was administered, and a sham operation was carried out.

### Local Cerebral Blood Flow

Eighteen rats were used for the measurement of cerebral blood flow. After the head was fixed in a head holder (SR-6, Narishige, Tokyo, Japan), the skull was exposed by retraction of the soft tissues. A hole was drilled in the calvarium at a site 3 mm frontal and 3 mm lateral to the bregma. The cerebral cortical blood flow was measured with a laser Doppler flow-meter (Laser Flo, BPM 403A, TSI, St Paul, Minn). The probe (diameter, 0.5 mm) was placed vertical to the cortical surface and was dissolved in deionized water for use in these experiments. Clentiazem maleate was synthesized by the Organic Research Laboratory of Tanabe Seiyaku Co, Ltd, Saitama, Japan, and was dissolved in deionized water for use in these experiments.

### Drug Concentrations in Plasma and Brain Tissue

Thirty rats were used for the measurement of drug levels. The concentration of clentiazem in plasma and in whole brain tissue was measured by high-performance liquid chromatography, as described previously.

### Statistical Analysis

Quantitative data are expressed as mean±SEM. Repeated-measures ANOVA was performed for comparisons between the control and clentiazem-treated groups during recirculation. Because a significant interaction between group and time was not found in each experiment, we did not perform any post hoc tests to determine the significance on each time point. Student's unpaired t-test was also performed for comparisons of the two groups during ischemia. The results were considered statistically significant at P<0.05.

### Chemicals

Clentiazem maleate was synthesized by the Organic Research Laboratory of Tanabe Seiyaku Co, Ltd, Saitama, Japan, and was dissolved in deionized water for use in these experiments.

### Results

#### Blood Gas Parameters and Drug Concentration

Table 1 shows physiological parameters in blood. BCAO caused a decrease in $P_{CO_2}$ and an increase in $P_{O_2}$ under both spontaneous and artificial respiration, but the changes were smaller in ventilated animals. There was no difference in blood pH under both types of

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-BCAO</th>
<th>BCAO (30 min)</th>
<th>Reflow (1 h)</th>
<th>Reflow (2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous respiration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P_{CO_2}$, mm Hg</td>
<td>Control</td>
<td>44.9±1.3</td>
<td>33.9±0.9</td>
<td>51.8±1.2</td>
</tr>
<tr>
<td>Clentiazem</td>
<td>42.5±1.1</td>
<td>27.6±2.3</td>
<td>46.3±2.5</td>
<td>46.6±2.5</td>
</tr>
<tr>
<td>$P_{O_2}$, mm Hg</td>
<td>Control</td>
<td>89.5±4.8</td>
<td>111.7±4.3</td>
<td>95.5±5.5</td>
</tr>
<tr>
<td>Clentiazem</td>
<td>88.6±0.64</td>
<td>118.6±3.5</td>
<td>90.4±1.2</td>
<td>96.9±3.9</td>
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<td>pH</td>
<td>Control</td>
<td>7.40±0.01</td>
<td>7.46±0.01</td>
<td>7.34±0.01</td>
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<tr>
<td>Clentiazem</td>
<td>7.41±0.01</td>
<td>7.36±0.02</td>
<td>7.38±0.02</td>
<td>7.37±0.03</td>
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<tr>
<td>Artificial respiration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P_{CO_2}$, mm Hg</td>
<td>Control</td>
<td>31.6±1.4</td>
<td>27.7±2.0</td>
<td>31.5±2.3</td>
</tr>
<tr>
<td>Clentiazem</td>
<td>28.0±1.1</td>
<td>23.9±0.9</td>
<td>24.9±1.1</td>
<td>24.5±0.7</td>
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<tr>
<td>$P_{O_2}$, mm Hg</td>
<td>Control</td>
<td>109.3±2.3</td>
<td>120.8±5.5</td>
<td>114.7±11.2</td>
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<tr>
<td>Clentiazem</td>
<td>109.2±1.6</td>
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<td>133.0±5.2</td>
<td>134.8±8.0</td>
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<tr>
<td>pH</td>
<td>Control</td>
<td>7.50±0.01</td>
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<td>7.47±0.02</td>
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<tr>
<td>Clentiazem</td>
<td>7.51±0.01</td>
<td>7.54±0.01</td>
<td>7.51±0.02</td>
<td>7.51±0.02</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=5). BCAO indicates bilateral carotid artery occlusion. Clentiazem was administered at a dose of 10 mg/kg twice a day for 3.5 days. The final dose was carried out 90 minutes before BCAO.

mm diameter), and the probe was inserted into an NMR spectrometer (JNM-GX270W, JEOL, Tokyo, Japan) with a vertical-bore superconducting magnet (field strength, 6.34 T). A free induction decay pulse sequence was used with a pulse repetition time of 0.25 seconds and a flip angle of 45 degrees in the center of the surface coil. The flip angle at 5 mm before the coil was 9 degrees. A short pulse-repetition rate was used to achieve a sufficiently good signal-to-noise ratio in an examination time of 2.5 minutes. The pH was determined from the chemical shift difference between the phosphocreatine and inorganic phosphate peaks. 11

Electroencephalographic Activity

Twelve rats were used for measurement of EEG activity. After the same operative procedure as for the measurement of cerebral blood flow, EEG leads were implanted into the surface of the cerebral cortex and connected to a biophysical amplifier (BR-5, Nihon Koden, Tokyo, Japan). The reference leads were placed on another part of the body. The EEG was recorded continuously and was evaluated by summation of the amplitudes (low-cut filter, 0.1 Hz; high-cut filter, 30 Hz).

### Nuclear Magnetic Resonance Spectroscopy

Twelve rats were used for $^{31}$P NMR spectroscopy. Each rat was placed in an 8.9-cm-diameter probe with a surface coil (8
TABLE 2. Concentrations of Clentiazem and Its Main Basic Metabolites in Plasma and Whole Brain (Before, 90, and 240 Minutes After Final Dose)

<table>
<thead>
<tr>
<th></th>
<th>Before Final Dose</th>
<th>90 Min After Final Dose</th>
<th>240 Min After Final Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma, ng/mL</td>
<td>Brain, ng/g</td>
<td>Plasma, ng/mL</td>
</tr>
<tr>
<td>Clentiazem</td>
<td>ND</td>
<td>3.6±1.4</td>
<td>6.6±2.2</td>
</tr>
<tr>
<td>MB-1</td>
<td>ND</td>
<td>ND</td>
<td>6.7±1.1</td>
</tr>
<tr>
<td>MB-2</td>
<td>ND</td>
<td>ND</td>
<td>14.1±2.1</td>
</tr>
<tr>
<td>MB-3</td>
<td>ND</td>
<td>ND</td>
<td>12.6±1.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=10). MB-1 indicates 3-deacetyl clentiazem; MB-2, N-demethyl clentiazem; MB-3, 3-deacetyl N-demethyl clentiazem; and ND, not detected. Clentiazem was administered at a dose of 10 mg/kg twice a day for 3.5 days. The final dose was carried out 90 minutes before bilateral carotid artery occlusion.

Local Cerebral Blood Flow

Fig 1 shows the changes in cortical blood flow during ischemia and recirculation in control and clentiazem-treated rats. BCAO caused a rapid decrease in cerebral blood flow to approximately 5% of the preischemic level in control rats. During recirculation the cerebral blood flow increased to 172.1±54.6% of the preischemic level (reactive hyperemia). Subsequently, the blood flow showed a paradoxical decrease at 90 minutes after recirculation (delayed hypoperfusion). Cerebral blood flow decreased to 55.7±11.5% of the preischemic level at 190 minutes after recirculation. Pretreatment with clentiazem significantly lessened the decrease in cerebral blood flow during ischemia. Two minutes after the start of ischemia, the cerebral blood flow in control and clentiazem-treated rats was 4.8±1.4% and 14.1±4.1%, respectively, of the preischemic level (P<.05) (Fig 1, top right panel). Clentiazem also reduced the severity of delayed hypoperfusion.

Electroencephalography, Blood Pressure, and Heart Rate

Fig 2 shows typical EEG tracings obtained during ischemia and recirculation in control and clentiazem-treated rats. BCAO caused the disappearance of EEG activity in all control rats. During recirculation, EEG activity reappeared in three of six control rats, but the restoration was very slight (Fig 2B). In contrast, treatment with clentiazem prevented the disappearance of EEG activity during ischemia in two of six rats (Fig 2D).

Fig 3 shows the changes in mean arterial blood pressure, heart rate, and EEG activity during ischemia and recirculation in control and clentiazem-treated rats. BCAO caused a transient increase in blood pressure in control rats (before ischemia, 104.2±6.0 mm Hg; after 2 minutes of ischemia, 188.1±9.0 mm Hg). The blood pressure then gradually returned to the preischemic level with the prolongation of ischemia. Thereafter, the blood pressure decreased transiently after recirculation (64.4±4.4 mm Hg after 2 minutes of recirculation). BCAO caused an increase in heart rate (before ischemia, 288.3±6.8 beats per minute; after 15 minutes of ischemia, 390.8±3.5 beats per minute). The heart rate increased to 300 beats per minute after ischemia, and it returned to normal after recirculation.
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non-treated

preischemic 30 min after
A
B
clentiazem-treated
C
D

rapidly returned to normal during recirculation. BCAO caused a decrease in EEG activity, and EEG activity disappeared after 30 minutes of ischemia. EEG activity showed little restoration during recirculation in control rats. Treatment with clentiazem had no significant influence on blood pressure and heart rate during ischemia and recirculation. However, it delayed the disappearance of EEG activity during ischemia (Fig 3, right panel). In addition, clentiazem significantly accelerated the restoration of EEG activity during recirculation.

Nuclear Magnetic Resonance Spectrometry

Fig 4 shows a typical $^{31}$P NMR spectrum of the cerebral cortex obtained in a control rat. $\beta$-ATP, $\alpha$-ATP (including a small amount of $\alpha$-ADP), $\gamma$-ATP (including a small amount of $\beta$-ADP), and phosphocreatine were detected by their chemical shift. BCAO caused a decrease in these high-energy phosphates that was accompanied by an increase in inorganic phosphate, and these changes were partially corrected during recirculation.

Fig 5 shows the changes in $\beta$-ATP and phosphocreatine in the cerebral cortex during ischemia and recirculation in control and clentiazem-treated rats. BCAO caused a gradual decrease in ATP, followed by a decrease in phosphocreatine. After 30 minutes of ischemia, the phosphocreatine and ATP concentrations reached 23.7±2.2% and 48.7±4.3% of the preischemic levels, respectively. These high-energy phosphates showed a partial recovery during recirculation. Pretreatment with clentiazem accelerated the restoration of phosphocreatine (control, 65.0±7.9%; clentiazem, 86.9±5.0% versus preischemic level; $P<.05$). Clentiazem treatment also tended to accelerate the restoration of ATP.

Fig 6 shows the changes in inorganic phosphate during ischemia and recirculation in control and clentiazem-treated rats. BCAO caused an increase in inorganic phosphate, and this parameter did not return to

Fig 2. Typical cortical electroencephalographic tracings obtained during ischemia and recirculation in stroke-prone spontaneously hypertensive rats. A and B, Two representative patterns from control rats. C and D, Two representative patterns from clentiazem-treated rats. Ischemia was induced by bilateral carotid artery occlusion in rats under urethane anesthesia.
the preischemic level during recirculation in control rats. Although pretreatment with clentiazem did not have a significant influence on the increase in inorganic phosphate during ischemia, there was a complete return to the preischemic level during recirculation.

Fig 7 shows the changes in cerebral intracellular pH during ischemia and recirculation in control and clentiazem-treated rats. BCAO caused a decrease in intracellular pH (before ischemia, 7.3±0.1; after 30 minutes of ischemia, 6.0±0.1) in control rats, and the acidosis persisted during recirculation. Pretreatment with clentiazem had no influence on the severity of intracellular acidosis during ischemia, but it significantly ameliorated acidosis during recirculation.

Discussion

Carotid artery occlusion can induce forebrain ischemia in SHR because of an upward shift in the autoregulation of cerebral blood flow.13 However, the severity of ischemia is quite variable among rats. BCAO appeared to induce more stable forebrain ischemia in SHRSP than in SHR, perhaps because the hypertension was more severe in the former rats and the autoregulation threshold for cerebral blood flow was shifted to a higher pressure region compared with SHR. As shown in this study, BCAO in SHRSP decreased cerebral blood flow to approximately 5% of the preischemic level and caused a disappearance of EEG activity in all control rats. These findings indicate that the residual blood flow after BCAO was below the threshold for the loss of neuronal electric activity. BCAO also caused a gradual decrease in high-energy phosphates and progressive intracellular acidosis during ischemia in all control rats. Because our preliminary study using microdialysis showed that BCAO caused an increase in extracellular K+ in the SHRSP cortex, the residual blood flow was considered to be below the threshold for the loss of ionic homeostasis. During recirculation the normalization of these parameters was variable: ATP and phosphocreatine showed partial and rapid restoration toward normal, while intracellular acidosis showed little improvement and EEG activity was not improved. A similar dissociation of these parameters has been reported in several studies of global cerebral ischemia.13-15 Thus, we confirmed that BCAO could induce stable forebrain ischemia in SHRSP. In cerebral isch-
vasodilation during the period of ischemia. It seems likely that clentiazem would cause cerebral vasodilation. Therefore, it is possible that even a slight improvement of cerebral blood flow could prevent the disturbance of ionic homeostasis and thus protect the brain against more severe damage. This hypothesis is supported by the observation that the extent of cerebral blood flow preservation by clentiazem (Fig 1, top right panel) was in proportion to its improvement of EEG activity (Fig 3, right panel). We measured the concentrations of clentiazem and its main metabolites in plasma and whole brain tissue in this study. The plasma concentrations of clentiazem at 90 minutes (immediate before ischemia) and 240 minutes after the final dose were 6.6 ng/mL (1.2×10^-8 mol/L) and 2.6 ng/mL, respectively. These concentrations are high enough to cause dilation of the cerebral arteries.

In this ischemic model we evaluated the cerebral protective action of clentiazem, which has a selective cerebral vasodilatory action and a protective effect on neurons subjected to in vitro ischemia. Pretreatment with clentiazem had a clear protective effect against the metabolic and functional disturbances caused by ischemia. This drug slightly but significantly ameliorated the decrease in cerebral blood flow during ischemia, and the preservation of cerebral blood flow was considered to be related to the protective action of clentiazem. Many investigations into the relation between cerebral blood flow and neuronal function have indicated that the threshold for the loss of neuronal electric activity is slightly higher than that for the loss of cellular ionic homeostasis. Therefore, it is possible that even a slight improvement of cerebral blood flow could prevent the disturbance of ionic homeostasis and thus protect the brain against more severe damage. This hypothesis is supported by the observation that the extent of cerebral blood flow preservation by clentiazem (Fig 1, top right panel) was in proportion to its improvement of EEG activity (Fig 3, right panel). We measured the concentrations of clentiazem and its main metabolites in plasma and whole brain tissue in this study. The plasma concentrations of clentiazem at 90 minutes (immediately before ischemia) and 240 minutes after the final dose were 6.6 ng/mL (1.2×10^-8 mol/L) and 2.6 ng/mL, respectively. These concentrations are high enough to cause dilation of the cerebral arteries. Therefore, it appears likely that clentiazem would cause cerebral vasodilation during the period of ischemia.

BCAO caused an increase in arterial blood pressure and heart rate through stimulation of the carotid sinus baroreceptors. This increase in perfusion pressure combined with the selective cerebral vasodilatory action of clentiazem would probably have caused an increase in vertebral blood flow and thus an increase in the blood supply to the ischemic region via collateral arteries.

Clentiazem also prevented postischemic hyperperfusion and accelerated the restoration of high-energy phosphates, intracellular pH, and EEG activity during recirculation. These effects of clentiazem may have been related to the relative preservation of cerebral blood flow during ischemia. However, its calcium overload blocking action on neurons may also be involved. Clentiazem readily crosses the blood-brain barrier because of its lipophilicity. In fact, a high concentration of clentiazem was detected in the brains of the rats in this study. In addition, it has been reported that a specific binding site for clentiazem exists in the brain as well as in peripheral tissues. Interestingly, Bleakman et al have reported that clentiazem could inhibit calcium influx (as barium current) induced by depolarization and stimulation of the N-methyl-D-aspartate receptor in cultured rat neuronal cells. In addition, Kobayashi et al have reported that clentiazem and its main metabolite MB-1, which showed a persistent brain level, both inhibit high potassium-induced calcium influx in cultured rat neuronal cells. Therefore, the inhibition of calcium influx into the neurons by clentiazem may be related to its neuroprotective effect in our ischemia model. In addition, clentiazem prevents neuronal death induced by anoxia (potassium cyanide and nitrogen) at a concentration of 3×10^-4 mol/L in cultured rat hippocampal cells. Taken together, these findings suggested that clentiazem may have a direct effect on neurons and may ameliorate metabolic and functional dysfunction in cerebral ischemia.

In summary, BCAO induced stable forebrain ischemia in urethane-anesthetized SHRSP. This rat model pretreatment with clentiazem improved various metabolic and functional disturbances due to ischemia. These beneficial effects of clentiazem may have been related mainly to its relative preservation of cerebral blood flow during carotid occlusion.

Acknowledgments

The authors would like to thank Dr H. Narita for helpful discussion and Mr C. Ishikawa for statistical analysis of the data.

References


Editorial Comment

The accompanying article by Kikkawa et al provides evidence that clentiazem (8-chloro diltiazem, TA-3090), a new calcium antagonist, may have important neuroprotective effects in an animal model of cerebral ischemia. This article carefully establishes the effect of forebrain ischemia induced in spontaneously hypertensive rats by 8-chloro diltiazem (TA-3090), a new calcium antagonist with potential cerebral-protective compounds. Further research with this compound and some of its derivatives may provide important clinical therapeutic strategies for stroke and brain injury.

Restoration of high-energy phosphates, intracellular pH, and EEG activity during recirculation. Clentiazem is a calcium channel antagonist comparable to the dihydropyridines. However, this compound not only has cerebrovascular effects but has also been shown to protect cultured neuronal cells against ischemia. This compound also crosses the blood-brain barrier and more readily reaches therapeutic levels in the brain than other diltiazem-type compounds. The specific mechanisms of cerebral protection of this compound need to be further studied. However, this article provides an important advance in the development of potential cerebral-protective compounds. Further research with this compound and some of its derivatives may provide important clinical therapeutic strategies for stroke and brain injury.

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