Pretreatment of Transient Focal Cerebral Ischemia in Rats With the Calcium Antagonist AT877

Masafumi Ohtaki, MD; Bruce Tranmer, MD, FRCSC

Background and Purpose The efficacy of pretreatment with the recently developed intracellular calcium antagonist AT877 against transient focal cerebral ischemia was investigated in rats subjected to middle cerebral artery occlusion and reperfusion using the endovascular suture method.

Methods Halothane-induced moderate hypotension (60 mm Hg) was used during 100 minutes of temporary middle cerebral artery occlusion. In the treated animals (n=10), an intravenous infusion of AT877 (0.03 mg/kg per minute) was initiated 30 minutes before the ischemic event and continued during the ischemic period. The control rats (n=10) received physiological saline in a similar fashion. Local cerebral blood flow was measured by the hydrogen clearance technique. Neurological examinations were performed daily during the 48-hour observation period, and infarct size was assessed by triphenyltetrazolium chloride staining.

It is well known that calcium plays a major role in the excitation-contraction mechanism of vascular smooth muscle, and tension of cerebral vascular muscle is largely dependent on extracellular calcium.1,2 During cerebral ischemia the massive influx of calcium into the arteriole smooth muscle cells is considered the cause of early vasoconstriction of cortical conducting vessels in the core areas of brain ischemia.3-5 Intracellular calcium homeostasis also plays a very crucial role in maintaining neuronal integrity. In certain disease states such as brain ischemia, hypoglycemia, and epileptic seizure, however, this critical homeostasis is disrupted, extracellular calcium enters into the neuron through a damaged cell membrane, and cell death may result as a consequence of overstimulation of phospholipases and proteases.6-8

Calcium channel antagonists have attracted considerable attention because of their direct vasodilating effects on cerebral pial vessels and their potential protective effects against ischemic damage of brain tissue through the blockade of excessive calcium influx into neurons. Among the various types of calcium channel antagonists, the dihydropyridine derivatives, such as nimbodipine, nifedipine, and nicardipine, have been expected to be the most promising agents because of their direct vasodilating effects against ischemic damage of brain tissue through the blood-brain barrier.9,10 In recent experimental studies, however, the neuroprotective effects of conventional calcium channel antagonists have yielded conflicting results.11-17

The reason for these results may be the fact that only the L-type calcium channel, which is one of three voltage-dependent calcium channels in neurons, is blocked by these calcium channel antagonists.18 It is also speculated that at least part of the rise in intracellular calcium may be secondary to the internal release of calcium from both mitochondria and endoplasmic reticulum.8,19 Additionally, hypotension induced by these calcium channel antagonists may offset the augmentation in cerebral blood flow (CBF) in the ischemic brain.

AT877 ([hexahydro-1-5-isoquinoline sulfonyl]-1H,4-diazepine hydrochloride, also named HA1077) is a novel type of calcium antagonist different from the currently available calcium channel antagonists. It is believed that this agent does not inhibit the influx of extracellular calcium into the cell but rather acts on the excess intracellular calcium at internal sequestering sites and induces the vascular relaxation process in smooth muscle cells. In 1989 Asano and colleagues demonstrated that HA1077 potently inhibited protein kinases such as cyclic nucleotide–dependent protein kinases and calcium/calmodulin–dependent myosin light chain kinase. Recent experimental studies have shown that AT877 dilates the spastic canine basilar artery,20 augments local CBF,21 and reduces delayed neuronal degeneration in the CA1 subfield of the gerbil hippocampus after temporary ischemia.22 AT877 has a...
Materials and Methods

Adult male rats of the Sprague-Dawley strain that had been fasted (weight, 280 to 360 g) were used in this experiment. After intramuscular administration of atropine sulfate (0.05 mg), anesthesia was induced with 4% halothane in a 1:2 mixture of O₂/N₂O. The rats were then intubated and mechanically respired with a mixture of 50% O₂, 50% N₂O, and 0.5% to 1.0% halothane using a Harvard rodent ventilator (Harvard Apparatus). Muscle relaxation was achieved with intravenous pancuronium bromide (0.1 mg) as needed. Ventilation was adjusted to maintain PaCO₂ constant at 35 to 40 mm Hg and PaO₂ at greater than 100 mm Hg.

The left femoral artery and vein were cannulated for blood sampling (arterial blood gases, pH, blood glucose, and hematocrit), continuous arterial blood pressure monitoring, and administration of drugs and fluids. Arterial blood gases and pH (1304 pH/Blood Gas Analyzer, Allied Instrumentation Laboratory) were determined serially before each local CBF measurement. Blood glucose was determined using Glucostix reagent strips and a reflectometer system (Ames Co). Body temperature was maintained at 37.0°C to 37.5°C with a warmed water blanket throughout the experiment. After completion of the last CBF measurement, anesthesia was discontinued. The rats were returned to their cages with free access to food and water after full recovery from anesthesia.

The left middle cerebral artery (MCA) was occluded with the endovascular suture technique initially described by Longa et al.²⁴ as modified by Kawamura et al. In brief, after the left cervical carotid bifurcation was exposed, the internal carotid artery (ICA) was carefully dissected distally free from the adjacent vagus, hypoglossal, and glossopharyngeal nerves under the operating microscope. The origin of the pterygopalatine artery (the first branch of the ICA) was easily identified beneath the digastric muscle, and a microvascular clip was placed on this artery. The external carotid artery (ECA) was electrocauterized and cut distal to the origin of the ECA, so that the proximal segment of the anterior cerebral artery and the origin of the ECA were occluded by its thickness.

A 3-0 monofilament nylon suture was introduced through the ICA lumen, and both the ECA stump and the nylon suture were sealed with a 6-0 silk suture. After removal of the ECA clip, this nylon suture was gently advanced into the ICA. After intramuscular administration of atropine sulfate (0.05 mg/kg per minute 30 minutes before the ischemic event and subsequently maintained until reperfusion was achieved (ie, >130 minutes), AT877 was dissolved in saline solution (0.9% NaCl) and infused at 2 mL/h. Control rats (n=10) received only physiological saline at 2 mL/h.

Local CBF was also measured to determine whether the hydrogen concentration of the inhaled gas was approximately 5%. Local CBF values were calculated from their clearance curves using the initial slope index.²⁶

In treated rats (n=10), an intravenous infusion of AT877 (Asahi Chemical Industry) was begun at a dosage of 0.03 mg/kg per minute 30 minutes before the ischemic event and subsequently maintained until reperfusion was achieved (ie, >130 minutes). AT877 was dissolved in saline solution (0.9% NaCl) and infused at 2 mL/h. Control rats (n=10) received only physiological saline at 2 mL/h.

The scores of the neurological status in individual rats were carefully evaluated 3 hours after surgery and daily afterward according to the grading scale of Bederson et al.²⁷ grade 0, neurologically normal observations; grade 1, flaccid contralateral forelimb or hindlimb; grade 2, forelimb flexion and decreased resistance to lateral push toward the paretic side; and grade 3, spontaneous contralateral circling. For evaluation, rats were held by the tail and lifted slowly to check for forelimb flexion; next, gentle lateral pressure was inflicted on rats on a soft plastic-coated paper over the smooth stainless plate. The rats were allowed to move freely in all directions to observe for circling behavior. The cannulation of the femoral vessels was performed on the left side after careful separation of the femoral nerve in both treated and control rats. The inferior abdominal wall artery and vein were preserved so that the blood flow through the collateral pathway to the hind limb was maintained. Therefore, this procedure did not affect resistance to lateral push in experimental or sham-operated rats.

Local CBF values were calculated from their clearance curves using the initial slope index.²⁶

AT877 possesses a predominant action as a potent vasodilator on intracranial vessels as opposed to extracranial vessels; however, with increasing doses, AT877-mediated systemic hypotension can reduce cerebral perfusion pressure further in the ischemic brain. In the preliminary experiments, the threshold dosage for significant reduction in MABP under light halothane anesthesia (inspired concentration of halothane, 0.5%)
Physiological Parameters in a Rat Model of Transient Focal Cerebral Ischemia

<table>
<thead>
<tr>
<th>Group</th>
<th>MABP, mm Hg</th>
<th>pH</th>
<th>Paco₂, mm Hg</th>
<th>PaO₂, mm Hg</th>
<th>Blood Glucose, mmol/L</th>
<th>Hematocrit, %</th>
<th>Rectal Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before occlusion</td>
<td>85±3</td>
<td>7.34±0.01</td>
<td>35.6±0.8</td>
<td>106±5</td>
<td>5.3±0.3</td>
<td>47±1</td>
<td>37.2±0</td>
</tr>
<tr>
<td>During MCA-O</td>
<td>61±1*</td>
<td>7.33±0.01</td>
<td>36.6±0.5</td>
<td>102±5</td>
<td>5.0±0.3</td>
<td>...</td>
<td>37.3±0</td>
</tr>
<tr>
<td>30 min after reperfusion</td>
<td>83±3</td>
<td>7.32±0.01</td>
<td>36.9±0.8</td>
<td>104±3</td>
<td>5.4±0.3</td>
<td>46±1</td>
<td>37.2±0.1</td>
</tr>
<tr>
<td>AT877 (n=10)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Before occlusion</td>
<td>83±3</td>
<td>7.35±0.01</td>
<td>36.7±0.6</td>
<td>111±3</td>
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<td>46±1</td>
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<td>During MCA-O</td>
<td>60±0*</td>
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<td>85±3</td>
<td>7.32±0.01</td>
<td>39.0±0.5</td>
<td>108±4</td>
<td>5.5±0.2</td>
<td>45±1</td>
<td>37.2±0</td>
</tr>
</tbody>
</table>

MABP indicates mean arterial blood pressure; MCA-O, middle cerebral artery occlusion. Values are mean±SEM.

*Significantly different from preocclusion value (P<.01).

was determined. At a dose of 0.03 mg/kg per minute (n=5), no significant alteration in MABP from 84±3 mm Hg to 82±3 mm Hg was observed; however, doses of 0.1 mg/kg per minute (n=4) and 0.05 mg/kg per minute (n=4) produced substantial reductions in MABP, from 89±2 to 77±4 mm Hg and from 85±3 to 78±4 mm Hg, respectively. Therefore, we decided to use a continuous infusion of AT877 at a dose of 0.03 mg/kg per minute in the treated group.

No intergroup differences in the baseline values of physiological parameters were noted, as shown in the Table. These physiological data in control and treated animals also did not significantly alter during the experimental period, except for planned systemic hypotension during ischemia from 85±3 to 61±1 mm Hg (P<.01) and from 83±3 to 60±0 mm Hg (P<.01) observed in control and treated animals, respectively. There was no significant difference in the average inspired concentration of halothane during this ischemic period between the control group and the AT877-treated group (1.34±0.05% and 1.24±0.04%, respectively).

Immediately after MCA occlusion, local CBF within the ipsilateral MCA territory markedly decreased to less than 40% of baseline values in both experimental groups (Fig 1). A continuous infusion of AT877 initiated before ischemia, however, brought about an improvement in local CBF during transient ischemia as follows. Local CBF in the AT877-treated group was significantly greater than that of the control group not only in ischemic brain region A shortly after MCA occlusion (41±5 versus 26±3 mL/100 g per minute, P<.05) but also in both ischemic brain regions A and B before reperfusion (53±6 versus 31±3 mL/100 g per minute, P<.01, and 52±5 versus 36±3 mL/100 g per minute, P<.05, respectively). Interestingly, in the treated animals local CBF tended to increase during MCA occlusion in both ischemic brain regions (both P<.05 by paired t test with Bonferroni correction).

Local CBF values in the AT877-treated group 30 minutes after reperfusion were also significantly higher in both ischemic brain regions compared with those in the control group (both P<.01).

Among both control and treated animals, no rat died during the 48-hour observation period under postoperative care. The treated animals had an obvious tendency to show a better neurological status throughout this observation period compared with the control animals (Fig 2). Although the difference in neurological outcome observed both 3 hours and 48 hours after surgery had not reached statistical significance, significant neurological improvement was found in the AT877-treated group 24 hours after surgery (P<.05 specifically different in grade 0 by Fisher's exact test).

The hemispheric volume of ischemic damage in the AT877-treated group at 87±20 mm³ was significantly reduced compared with the control group at 160±30 mm³ (P<.05 by unpaired t test with Bonferroni correction).
Neurological Grading Scale

- Grade 0
- Grade 1
- Grade 2

Fig 2. Bar graph shows neurological outcome in control (C) and AT877-treated (AT) rats. No animal showed contralateral circling behavior (grade 3) in either group at individual observation times. AT877 significantly ameliorated the neurological status 24 hours after surgical intervention (P<.05).

Fig 3. Bar graph shows hemispheric, neocortical, and striatal infarct volumes, as assessed by triphenyltetrazolium chloride staining, in control and AT877-treated rats. Columns indicate average volumes of infarct; vertical bars represent SEM. AT877 significantly reduced ischemic brain damage in both the striatum and the neocortex (*P<.05, **P<.01, respectively).

Fig 4. Scatterplot shows mean values of all recorded ischemic local cerebral blood flow (CBF) measurements plotted against neocortical infarct volumes of corresponding rats. Neocortical infarct volume was reduced with increasing mean ischemic local CBF, and their relation was found to be significant (y=595.05-20.28x+0.17x²; r=.923, P=.0001). The symbols • and ○ indicate individual animals in the control and AT877-treated groups, respectively.

Discussion

The results of the present study demonstrate that continuous intravenous infusion of AT877 started 30 minutes before the induction of ischemia significantly improves local CBF in the ischemic MCA territory and improves neurological outcome. The drug also dramatically reduced ischemic brain damage in both the neocortex and striatum in this rat model of transient focal cerebral ischemia.

AT877 has been shown to be a potent inhibitor of intracellular free calcium and preferentially exerts a vasodilating effect on cerebral vasculature. Asano et al reported that AT877 significantly antagonized phenylephrine-induced contraction of spiral strips of rabbit aorta in calcium-free solution, which is attributed almost entirely to release of intracellular calcium, whereas the usual calcium channel antagonists failed. In conscious rats this agent, at doses of 1 to 3 mg/kg, increased CBF with only minimal changes in systemic arterial blood pressure. Moreover, in the canine model of subarachnoid hemorrhage intravenous administration of AT877 was reported to dilate the basilar artery and improve cerebral circulation. A recent prospective randomized trial using AT877 in patients with aneurysmal subarachnoid hemorrhage also has provided evidence of its significant reduction of angiographically verified vasospasm. Although the mechanism of action of AT877 remains to be clarified, based on the available data...
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Mies et al\textsuperscript{32} showed that the ischemic threshold for the deple- tion of ATP, ie, energy failure, was 18 mL/100 g per minute in the early ischemic period after MCA occlusion in rats. Early ischemic injury can be found to occur at CBF values of approximately 25 to 34 mL/100 g per minute in similar focal ischemic rat models.\textsuperscript{33,34} Based on the above data, therefore, it seems that in our model ischemic brain region A for CBF measurements was located almost inside the infarct border, whereas ischemic brain region B involved the ischemic penumbra surrounding the ischemic core.\textsuperscript{35} In the present study we found that CBF values during the transient ischemic period were significantly higher in the AT877-treated animals than in the control animals and that a negative correlation between mean CBF values during transient ischemia and size of the neocortical infarct existed among experimental animals. These results indicate that the brain tissue within the ischemic zone was able to be salvaged from an irreversible process leading to cell death by the intravenous infusion of AT877 through its primary action of increasing CBF.

There may be several possible mechanisms to explain how AT877 affects cerebral vasculature to improve perfusion to areas of brain ischemia. It has been shown that severe focal ischemia causes an immediate vasoconstriction of both pial arteries and penetrating arterioles in the ischemic cortex and that certain calcium antagonists can attenuate this ischemic vasoconstriction.\textsuperscript{35} This reversal effect on ischemia-induced vasospasm should result in increases of pial collateral flow into ischemic brain regions. Takayasu and Dacey\textsuperscript{36} have shown that AT877 may produce a greater vasodilatory effect on parenchymal arterioles, which are the most distal resistant vessels, compared with the conventional calcium channel antagonists such as nimodipine and nifedipine. Thus, it is possible that AT877 can improve cerebral microcirculation in ischemic brain as well. In addition, this agent could potentially act on the basilar or contralateral carotid system feeding into the circle of Willis. Dilation of these large arteries would act to increase circle of Willis pressure and thereby provide a greater pressure head for perfusing collateral arteries. AT877 is an intracellular calcium antagonist that has the potential to delay ischemia-induced intracellular calcium accumulation.\textsuperscript{20} This direct antagonism of the adverse actions of excess intracellular calcium may also play an important part in the neuroprotective effect of AT877 against ischemia.\textsuperscript{33}

In our rat model of transient focal ischemia we used moderate halothane-induced hypotension to generate a more consistent and widespread lesion of cerebral damage to examine the neuroprotective effects of new agents. In our preliminary studies some normotensive rats, even those undergoing 180 minutes of MCA occlusion, showed no or little ischemic damage in the neocortex. However, the potential effects of general anesthesia with halothane on the cerebral vasculature should be considered before interpreting our experimental data. In previous investigations, no significant difference in the size of areas of profound ischemia that occurred after MCA occlusion in the rat have been demonstrated with several hypotensive regimens, including deep vol-

Evidence it presumably involves a vasodilatory effect on cerebral arteries, resulting in an increase of local CBF in areas of brain ischemia. AT877 was supplied by Asahi Chemical Industry, Tokyo, Japan.

Acknowledgment

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References

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