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. During cerebral ischemia the massive influx of calcium into the arteriole smooth muscle cells is considered the cause of early vasocostriction of cortical conducting vessels in the core areas of brain ischemia. 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.

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 nimodipine, nifedipine, and nicardipine, have been expected to be the most promising agents because of their direct vasodilating effects against ischemic damage of brain tissue.

Results A continuous infusion of AT877 significantly improved local cerebral blood flow during ischemia. The treated animals showed a better neurological outcome after a 24-hour observation period, and a significant reduction in ischemic brain injury resulted in both the neocortex (149±20 versus 41±14 mm³, P<.01) and the striatum (80±5 versus 46±8 mm³, P<.05). The size of the neocortical infarct was reduced, with increasing mean ischemic cerebral blood flow in the control and treated animals (r=.923, P=.0001).

Conclusions AT877 pretreatment was effective in preventing brain injury during transient focal cerebral ischemia and improving neurological status. This beneficial effect seems to be mediated, in part, by its primary action of increasing cerebral blood flow.

Key Words calcium antagonists cerebral blood flow cerebral ischemia focal rats

See Editorial Comment, page 1240.

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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, augments local CBF, and reduces delayed neuronal degeneration in the CA1 subfield of the gerbil hippocampus after temporary ischemia. AT877 has a...
high predilection for acting on intracranial blood vessels, which has a minimal influence on arterial blood pressure.

The present study was designed to investigate the neuroprotective effect of preischemic treatment with AT877 in a model of transient focal cerebral ischemia in the rat. We assessed postischemic neurological outcome daily and infarct size 48 hours after the ischemic event. Local CBF was also measured to determine whether the agent exerted a vasodilatory effect in the ischemic brain.

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 O<sub>2</sub>/N<sub>2</sub>O. The rats were then intubated and mechanically respired with a mixture of 50% O<sub>2</sub>, 50% N<sub>2</sub>O, 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 PaCO<sub>2</sub> constant at 35 to 40 mm Hg and PaO<sub>2</sub> 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 (1304 pH/Blood Gas Analyzer, Allied Instrumentation Laboratory) in parallel. 

Hypotension was established before induction of ischemia and consequently a physiological normograde blood pressure. Two electrodes were placed into the cortex within the ipsilateral MCA territory: 6 mm lateral to midline and 1 mm anterior to bregma (ischemic brain region A) and 3 mm lateral to midline and 4 mm posterior to bregma (ischemic brain region B). Hydrogen gas was introduced to the breathing mixture for 2 to 3 minutes: the hydrogen concentration of the inhaled gas was approximately 5%. Local CBF values were calculated from their clearance curves using the initial slope index.

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: grade 0, neurologically normal observation; grade 1, flexion of contralateral forelimb; 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.

Forty-eight hours after surgical intervention, the rats were reanesthetized with halothane and then decapitated. The fresh brains were sectioned coronally into 1.8-mm slices, after which the brain slices were immersed in a buffered solution of 1% 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co) at 37°C to 38°C for 30 minutes. The white- and pale-stained areas, considered to represent infarcted brain tissue in the present study, were measured on both the anterior and posterior surfaces of each slice, using an imaging analysis system (Jandel Video Analysis, Jandel Scientific). Infarct volumes were calculated from the sum of the average of the infarcted areas seen on both surfaces of each slice, multiplied by its thickness.

All data are expressed as mean±SEM. The changes in physiological parameters and local CBF in each group were analyzed with ANOVA followed by Dunnett's test. The significance of each measurement of the above data at individual times and infarct volume between the control group and the AT877-treated group was evaluated by two-tailed Student's t test. A regression analysis of individual data points between mean local CBF during ischemia and the neocortical infarct volume in both groups was used to determine their relation. The difference of neurological outcome scores between groups was analyzed by the χ<sup>2</sup> test. A significant difference in the statistical results was defined as P<.05.

Results

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>Paco₂, mm Hg</th>
<th>Blood Glucose, mmol/L</th>
<th>Hematocrit, %</th>
<th>Rectal Temperature, °C</th>
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</thead>
<tbody>
<tr>
<td><strong>Control (n=10)</strong></td>
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<td></td>
<td></td>
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<td></td>
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</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</td>
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<tr>
<td><strong>AT877 (n=10)</strong></td>
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<tr>
<td>Before occlusion</td>
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<td>5.5±0.2</td>
<td>45±1</td>
<td>37.2±0</td>
</tr>
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</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<.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

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**Fig 1.** Line graphs show local cerebral blood flow changes in ischemic brain regions A (a; top) and B (b; bottom) (see "Materials and Methods") of control and AT877-treated groups after middle cerebral artery occlusion (MCA-O) and reperfusion. Vertical bars represent SEM. *P<.05, **P<.01 significantly different from cerebral blood flow values of corresponding regions in the control group.
less than that in the control group at $229\pm 23\ mm^3$ ($P<.01$); total infarct volume was reduced by more than 60% with AT877 pretreatment. Moreover, as shown in Fig 3, significant reductions in ischemic injury in both the neocortex ($149\pm 20$ versus $41\pm 14\ mm^3$; $P<.01)$ and the striatum ($80\pm 5$ versus $46\pm 8\ mm^3$; $P<.05$) were found in the AT877-treated group.

To clarify whether increased local CBF in the ischemic MCA territory had a beneficial influence on modifying ischemic brain damage in the treated animals, the average values of all recorded local CBF measurements during the ischemic period were plotted for points of neocortical infarct volumes of the corresponding rats. As can be seen in Fig 4, a reduction in neocortical infarct volume with increasing ischemic local CBF was demonstrated. The relation between mean ischemic local CBF and neocortical infarct volume was well described by a quadratic equation, which was generated from individual data points: $y=595.05-20.28x+0.17x^2$ ($r=.923, P=.0001$). The symbols $\bullet$ and $\circ$ 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
Evidence it presumably involves a vasodilatory effect on cerebral arteries, resulting in an increase of local CBF in areas of brain ischemia.

Mies et al. showed that the ischemic threshold for the depletion of ATP, i.e., 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. 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. 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. This reversal effect on ischemia-induced vasospasm should result in increases of pial collateral flow into ischemic brain regions. Takayasu and Dacey 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 blockers 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. 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.

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 volatile anesthesia, hypovolemia with phlebotomy, and the use of vasodilating agents. We chose an MAP of 60 mm Hg during transient ischemia to maintain cerebral perfusion within the normal range in the area of the brain with intact autoregulation. Volatile anesthetic agents such as halothane and isofluorane have been shown to increase CBF in both ischemic and nonischemic brain areas and also to affect cerebral autoregulation. It has also been postulated that halothane may exert a deleterious influence on the function of the blood-brain barrier. Thus, this halothane-induced opening of the blood-brain barrier may in fact enhance the effects of AT877 on regional CBF and intracellular calcium accumulation in areas of focal ischemia. However, the results of previous experimental studies have suggested that these effects of halothane occur at concentrations greater than 2% to 4%, and in the present study both groups of animals were anesthetized with identical concentrations of halothane of less than 1.5%. Thus, it seems unlikely that halothane used in this experiment significantly altered the blood-brain barrier function or local CBF.

To our knowledge, this is the first study that uses AT877 as a cerebral protector in an MCA occlusion stroke model. Therefore, our initial efforts have focused on pretreatment with AT877. Although preischemic treatment with AT877 may be considered impractical in the clinical setting of stroke therapy, there appear to be a number of other applications for pretreatment, such as the management of patients with crescendo transient ischemic attacks or the temporary vascular occlusion of major vessels during aneurysm surgery or carotid endarterectomy. Also, we may anticipate the efficacy of early postischemic treatment with AT877 against brain ischemia even if the influx of calcium into the cell has already begun because of its ability to antagonize intracellular actions of calcium. Moreover, another merit of early stroke intervention with this agent may be restoration of critical blood flow in areas of brain ischemia. Because the mechanism of action of AT877 differs from that of calcium channel blockers, these two agents may prove to be additive in their protective effect against cerebral ischemia. Accordingly, further investigation of the neuroprotective effect of AT877 during cerebral ischemia is needed to establish its clinical validity.

Acknowledgment

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References


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