**Effect of the Intracellular Calcium Antagonist HA1004 on Cerebral Blood Flow in Rats**

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Abstract

The effect of a novel intracellular calcium antagonist, HA1004, on local cerebral blood flow (LCBF) was examined in rats. LCBF was measured in the striatum by the hydrogen clearance method. In the control rats, HA1004 (10 mg/kg, intravenously: IV) increased striatal LCBF by a maximum of 26% despite a transient drop in mean arterial blood pressure (MABP). The LCBF increased for a period of over 100 minutes after the infusion of HA1004. Nicardipine, a calcium entry blocker (0.1 mg/kg, IV), caused a decrease in MABP similar to that induced by HA1004 and increased LCBF by a maximum of 13% for up to 90 minutes after injection. In a second group of rats, the middle cerebral artery (MCA) was occluded by direct ligature of its proximal portion. After MCA occlusion, the LCBF rapidly dropped to 30% of the control value and gradually recovered to the control level during the next 60 minutes. However, in rats prophylactically treated with a small dose of HA1004 (1 mg/kg, IV), LCBF recovered more quickly than in untreated rats. The results of this study suggest that HA1004 increases LCBF by enhancing collateral blood flow to ischemic regions and may be useful for the treatment of ischemia.

Key words: intracellular calcium antagonist, HA1004, nicardipine, cerebral blood flow, cerebral ischemia

Introduction

It has been suggested\textsuperscript{1,15} that smooth muscle contractility of cerebral resistance vessels is more susceptible to the influx of calcium ions from extracellular fluid than is that of systemic blood vessels. Calcium entry blockers, which reduce the transmembrane movement of calcium ions, preferentially decrease cerebral vascular tone and increase cerebral blood flow (CBF). In fact, such calcium entry blockers as nimodipine or nifedipine reportedly increase CBF not only in the normal brain\textsuperscript{1,11,16,18,26,27} but also in the ischemic brain.\textsuperscript{6,10,12,25,30} However, in animal studies postsischemic administration of calcium entry blockers failed to avert calcium accumulation and did not improve CBF or neurological outcome following total cerebral ischemia.\textsuperscript{7,19,21} Reedt et al.\textsuperscript{24} found verapamil ineffective in restoring CBF following middle cerebral artery (MCA) occlusion in cats. On the other hand, Steen et al.\textsuperscript{29} and Milde et al.\textsuperscript{17} reported that nimodipine was effective even after complete cerebral ischemia. Most of the commonly used calcium antagonists are calcium entry blockers that act primarily by inhibiting the influx of extracellular calcium ions into cells through calcium channels in the cell membrane and do not directly affect the intracellular calcium ion concentration.\textsuperscript{23} It was recently reported that a novel intracellular calcium antagonist, \([N-(2-guanidinoethylamino)-5-isoquinolinesulfonamide] (HA1004) (Fig. 1), produced a dose-dependent increase in vertebral, coronary, and renal blood flow,\textsuperscript{2,3} and mitigated delayed cerebral vasospasm in dogs.\textsuperscript{21} To our knowledge, there have been no reports concerning the effect on
cerebral ischemia of intracellular calcium antagonists, which differ pharmacologically from calcium entry blockers and calmodulin antagonists.

In this study, we examined the effect of HA1004 on local cerebral blood flow (LCBF) in control and MCA-occluded rats. We also compared the effect of HA1004 with that of nicardipine, a calcium entry blocker.

Materials and Methods

HA1004 was synthesized by Asahi Chemical Industry, Tokyo, and nicardipine was obtained from Yamanouchi Pharmaceutical Company, Tokyo.

I. HA1004 and nicardipine administration to control rats

Male Sprague-Dawley rats weighing 200–250 gm were anesthetized with 3% halothane and tracheostomized. After injection of pancuronium bromide (2 mg/kg, intravenously: IV), the animals were mechanically ventilated with a 70% nitrogen/30% oxygen mixture containing 1% halothane. A polyethylene catheter was inserted into the right femoral artery to monitor arterial blood pressure (ABP) and to sample arterial blood for gas analyses. Another catheter was inserted into the right femoral vein to infuse HA1004 or nicardipine. Body temperature was measured rectally and was kept normal (36.5–37.5°C) by means of a heat lamp. LCBF was measured by the hydrogen clearance method, in which hydrogen gas is generated by hydroelectrolysis with an electrode (BE-ND, Bio-Medical Science Co., Ltd., Tokyo) placed stereotactically in the lateral part of the striatum. The upper incisor bar of the stereotactic frame was raised to 2.5 mm above the interaural line. The bregma was used as the rostral-caudal zero point. The coordinates for the left lateral portion of the striatum were A, +1.0 mm; L, +4.0 mm; and V, −4.0 mm. Finally, a reference electrode was placed subcutaneously in the scalp. After generation of the hydrogen gas, the polarographic current in the brain was measured for determination of the hydrogen clearance curve (RB-F-2, Bio-Medical Science Co., Ltd.). LCBF values were calculated with the BDA-1-2 computer system (Bio-Medical Science Co., Ltd.). Each absolute value was calculated by deducing the baseline value obtained after death by cardiac arrest. In each rat, normal LCBF was measured five times at 7-minute intervals prior to drug administration. Within 5 seconds of the last pretreatment measurement HA1004 (1.0 or 10 mg/kg, IV) or nicardipine (0.1 mg/kg, IV) was injected and measurement of LCBF was resumed at 7-minute intervals for the next 2 hours. Two arterial blood samples were taken from each rat for blood gas analysis, one immediately after drug injection and another after the final measurement of LCBF.

II. HA1004 administration to MCA-occluded rats

A left subtemporal craniectomy was performed according to the method described by Tamura et al. After the dura was opened, a 10-0 monofilament nylon suture 22 µm in diameter with an attached needle was passed behind the left MCA at a point above the olfactory tract and immediately proximal to the origin of a branch to the rhinal cortex (orbitofrontal branch) and then through a loop formed previously at a distal portion of the suture (Fig. 2 left). The MCA was completely occluded by suspension of a Scoville clip (0.24 gm) attached to the nylon suture.
that had been passed through the loop\textsuperscript{20} (Fig. 2 right). HA1004 (1.0 mg/kg, IV) was injected 2 minutes before the occlusion. LCBF was measured with electrodes stereotactically inserted into the lateral portion of the striatum on both sides. The measurements were made every 9 minutes, five times before and eight times after occlusion of the MCA. Cardiac arrest was induced by an intravenous injection of a KCl solution, and the baseline LCBF was then measured. In the untreated rats, LCBF was measured in the same fashion.\textsuperscript{20}

Student’s two-tailed t-test was used in the statistical analysis.

**Results**

1. **Effect of HA1004 and nicardipine on LCBF in control rats**

Table 1 lists the pre- and post-treatment values for pH, PaO\textsubscript{2}, and PaCO\textsubscript{2} in the control rats. Neither drug significantly affected these parameters. A small dose of HA1004 (1.0 mg/kg) had no significant effect on either LCBF or MABP. The latter fell from 89 ± 2 to 48 ± 5 mmHg immediately after injection of HA1004 at a dose of 10 mg/kg and gradually returned to the pretreatment level during the following 88 minutes. LCBF increased gradually, peaking at 63.3 ± 3.4 ml/100 gm/min, or 126% of the pretreatment level, 74 minutes after HA1004 injection and returned to the pretreatment level during the following 35 minutes (Fig. 3).

Nicardipine (0.1 mg/kg) lowered MABP immediately from 96 ± 2 to 52 ± 2 mmHg and increased LCBF to 57.4 ± 2.6 ml/100 gm/min, or 113% of the pretreatment level within 60 minutes. During the following 35 minutes, LCBF returned to the pretreatment level (Fig. 3).

![Graph showing the effects of HA1004 and nicardipine on LCBF and MABP](image-url)

**Fig. 3** The effects of HA1004 (1.0 mg/kg or 10 mg/kg) and nicardipine (0.1 mg/kg) on local cerebral blood flow (LCBF) in the striatum and mean arterial blood pressure (MABP) in control rats. The data are expressed as means ± SEM. *p < 0.05, **p < 0.025, ***p < 0.01, ****p < 0.005.

Table 1: Arterial blood gas analyses before and after HA1004 and nicardipine administration in control rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>pH</th>
<th>PaO\textsubscript{2} (mmHg)</th>
<th>PaCO\textsubscript{2} (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA1004</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1.0 mg/kg)</td>
<td>pre</td>
<td>5</td>
<td>7.440±0.014</td>
<td>103.3±8.6</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>5</td>
<td>7.416±0.013</td>
<td>96.1±5.9</td>
</tr>
<tr>
<td>HA1004</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10 mg/kg)</td>
<td>pre</td>
<td>5</td>
<td>7.428±0.031</td>
<td>96.6±4.6</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>5</td>
<td>7.427±0.037</td>
<td>108.2±2.1</td>
</tr>
<tr>
<td>Nicardipine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.1 mg/kg)</td>
<td>pre</td>
<td>5</td>
<td>7.415±0.025</td>
<td>115.6±8.5</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>5</td>
<td>7.393±0.029</td>
<td>108.9±4.8</td>
</tr>
</tbody>
</table>

The data are expressed as means ± SEM.
II. Effect of HA1004 on LCBF in MCA-occluded rats

Table 2 shows the results of the arterial blood gas analyses immediately after the final measurement of local cerebral blood flow in rats subjected to middle cerebral artery occlusion.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>pH</th>
<th>PaO₂ (mmHg)</th>
<th>PaCO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>5</td>
<td>7.419 ± 0.029</td>
<td>125.1 ± 4.3</td>
<td>31.5 ± 1.1</td>
</tr>
<tr>
<td>HA1004</td>
<td>4</td>
<td>7.431 ± 0.036</td>
<td>122.1 ± 7.3</td>
<td>35.4 ± 5.6</td>
</tr>
</tbody>
</table>

The data are expressed as means ± SEM.

![Fig. 4](image-url) The effect of HA1004 (1.0 mg/kg) on LCBF in the striatum of rats subjected to MCA occlusion. The data are expressed as means ± SEM. ○: occlusion alone, ●: HA1004 + occlusion, ▲: contralateral side.

II. Effect of HA1004 on LCBF in MCA-occluded rats

Table 2 shows the results of the arterial blood gas analysis immediately after the final LCBF measurement in the MCA-occluded rats. Blood gases did not significantly differ between the treated and untreated animals. A few minutes after the injection of HA1004 (1.0 mg/kg) MABP fell significantly (p < 0.005), from 74 ± 3 to 63 ± 3 mmHg; however, a fall of this magnitude may not have been reflected by LCBF. Figure 4 shows the changes in LCBF in the lateral part of the striatum after MCA occlusion, which caused an acute decrease in LCBF. The lowest value was similar in the treated and untreated groups (17.9 ± 1.5 and 17.2 ± 2.0 ml/100 gm/min, respectively); however, recovery of LCBF was faster in the treated than in the untreated rats. On the contralateral side, LCBF did not significantly differ between the treated and untreated animals.

Discussion

I. Effect of HA1004 and nicardipine on LCBF in control rats

Most calcium entry blockers are known to increase CBF by relaxing cerebral vascular smooth muscles by virtue of their ability to reduce transmembrane transport of Ca⁺ ions. Diltiazem (0.1 mg/kg) increased CBF by 30%²⁷ and nifedipine (0.5 mg/kg), in anesthetized control rats, by 33%.²⁸ Intravenous nimodipine infusion (1 μg/kg/min, 0.05 mg/kg) increased CBF by 18% and spinal blood flow by 41%.²⁹ However, there have been no reports on the effect on CBF of intracellular calcium antagonists, which directly affect the intracellular Ca⁺⁺ function of vascular smooth muscle.

We previously speculated that HA1004 is an intracellular Ca⁺⁺ antagonist, based on the observations that HA1004 inhibited the contractions produced by the Ca⁺⁺ ionophore A23187 in rabbit aorta and canine basilar artery as well as the phenylephrine-induced contractions of rabbit aortic strips in Ca⁺⁺ free solution.³¹ Furthermore, HA1004, like calcium entry blockers, competitively inhibited Ca⁺⁺-induced contraction of depolarized rabbit aorta. It also induced vasorelaxation without affecting the vasoconstrictor-stimulated increase in cytosolic Ca⁺⁺ in aortic smooth muscle, as shown by ⁴⁰Ca influx and efflux measurements.³² It is known that this compound potently relaxes blood vessels and increases blood flow in the vascular beds supplied by the vertebral, coronary, and renal arteries.³³ The dosage of HA1004 required for a 40% reduction in MABP is 3 mg/kg in spontaneously hypertensive rats, which corresponds to 0.03 mg/kg of nicardipine (unpublished data). In fact, the initial drop in MABP produced by 10 mg/kg of HA1004 (48 ± 5 mmHg) in our study was close to that induced by 0.1 mg/kg of nicardipine (52 ± 2 mmHg). Recovery of MABP after administration of 10 mg/kg of HA1004 was slower and the increase in CBF (26%) was larger than that occurred following injection of 0.1 mg/kg of nicardipine. These differences may reflect differences in mechanisms of action.

It is unclear whether or not the increase in LCBF induced by HA1004 was related to a change in cerebral metabolic rate, since we did not measure cerebral oxygen consumption or local glucose utilization in this study. However, the fact that HA1004 dilates cerebral vessels and increases LCBF means that it may promote vasodilation by the same mechanisms that inhibit the action or mobilization of intracellular Ca⁺⁺.
II. Effect of HA1004 on LCBF in MCA-occluded rats

We previously described the MCA occlusion model used in this study as a transient ischemia model.20 Others have accomplished MCA occlusion in rats by means of a small clip or bipolar cautery, whereas we ligated the MCA with a very fine nylon suture. We consider this method more efficient than the others because it damages fewer perforating arteries to the lateral portion of the striatum (lenticulostriate branches). Through retrograde flow, LCBF may recover early in this model, which is useful in the study of collateral flow and reperfusion.

Administration of 1.0 mg/kg of HA1004 did not alter LCBF on the nonoccluded side, but significantly enhanced recovery of LCBF on the MCA-occluded side. This effect is probably due to selective vasodilation within the ischemic region, where it is needed. It has been noted that calcium antagonists also dilate blood vessels in the nonischemic region (the 'steal' phenomenon) but we found no evidence for such an effect in this study.

In clinical practice, prophylactic administration of HA1004 may be beneficial when temporary clipping of a parent artery is necessary during surgery. Steen et al. found that the pretreatment with nimodipine improved CBF and neurological recovery in dogs after a period of complete cerebral ischemia. On the other hand, the administration of calcium entry blockers after complete ischemia has been established as has been reported ineffective in preventing intracellular calcium accumulation and improving CBF or the neurological outcome. However, Steen et al. and Milde et al. reported recently that delayed treatment with nimodipine after complete cerebral ischemia was effective in primates and in dogs respectively. The reasons for these discrepant results are unclear at present. Because of their unique mechanism of action, intracellular calcium antagonists, such as HA1004, may be useful in ameliorating the harmful effects of intracellular calcium accumulation, even after ischemia has been established.

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