Attenuated Neuropathology by Nilvadipine After Middle Cerebral Artery Occlusion in Rats

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We investigated the effects of nilvadipine, a calcium antagonist, on cerebral ischemia in rats. Under halothane anesthesia, 30 rats had a 3-0 nylon suture introduced through the extracranial internal carotid artery to occlude the left middle cerebral artery. Nilvadipine was dissolved in polyethylene glycol 400. Immediately following occlusion, group 1 rats (n=10) were treated subcutaneously with vehicle and group 2 and 3 rats were treated with 1.0 (n=10) and 3.2 (n=10) mg/kg nilvadipine, respectively. Perfusion fixation was performed 24 hours later, and the histopathologic outcomes were quantified. In group 1 infarct volume was 28.2 ±11.4% of the total cerebral volume; in groups 2 and 3 infarct volumes were 25.5±11.6% (NS) and 13.9±9.2% (p<0.05 different from group 1), respectively. Nilvadipine decreased ischemic neuronal injury in a dose-dependent manner and may be of use in the treatment of cerebral ischemia. (Stroke 1991;22:51–55)

Cerebral ischemia causes massive influx of Ca$^{2+}$ into neurons, a so-called final common pathway of cell death. Thus, the inhibition of Ca$^{2+}$ entry into cells has been proposed as a therapeutic measure for cerebral ischemia, and recent evidence suggests that calcium entry blockers attenuate ischemic neuronal damage through two mechanisms: dilation of cerebral vessels and prevention of excessive Ca$^{2+}$ influx into cytoplasmic and mitochondrial compartments.

Nilvadipine (FR 34235) is a new dihydropyridine calcium entry blocker structurally related to nifedipine but 5–10 times more potent against KCl-induced vasoconstriction in dog coronary and basilar artery strips. Previous research has shown that nilvadipine has a potent vasodilator effect similar to that of nifedipine on norepinephrine-induced constriction in the basilar artery and thoracic aorta of rabbits. Moreover, nilvadipine was effective in reducing ischemia in a dog model of chronic coronary artery occlusion and may be a therapeutic agent for cerebral ischemia.

We investigated the effects of nilvadipine on focal cerebral ischemia. In an improved rat model of unilateral middle cerebral artery (MCA) occlusion, we used the intraluminal suture technique and assessed histopathologic outcomes.

Materials and Methods

Thirty adult male Sprague-Dawley rats weighing 250–340 g were housed at 22±2°C, 50±10% humidity, and a 12-hour light/dark cycle with free access to food and water. Halothane was used to induce (4% in a mixture of 75% N₂O and 25% O₂) and maintain (1%) anesthesia; 0.25 mg i.p. atropine sulfate was used for premedication. Each rat was allowed to breathe spontaneously, and rectal temperature was maintained at 37°C with a heating pad. The tail artery was cannulated for continuous monitoring of mean arterial blood pressure, as well as for repeated blood sampling for serial measurements of Pao₂, Paco₂, pH, hematocrit, hemoglobin concentration, and plasma glucose concentration. Gases were analyzed using the ABL 330 device (Radiometer, Copenhagen, Denmark); hemoglobin and plasma glucose concentrations were measured with Ektachem DT60 (Eastman Kodak Co., Rochester, N.Y.).

We occluded the left MCA using a modification of the method described by Zea Longa et al. Briefly, the left external carotid artery (ECA) was isolated via a ventral midline incision, and the only branch of the extracranial internal carotid artery (ICA), the pterygopalatine artery, was then isolated. The origin of the pterygopalatine artery was occluded with a microvascular clip so that the intraluminal suture was...
blood sampling. The heart was exposed via a midline incision, and a cannula was inserted into the ascending aorta via the left ventricle. The inferior vena cava and the tail artery again served for the continuous infusion of heparin and heat-sealed by a flame. The N2O/O2 mixture was switched to room air 20 minutes after MCA occlusion; groups 2 and 3 rats were never introduced erroneously into the pterygopalatine artery (we have since learned that the pterygopalatine artery can be clipped without dissecting the stylohyoid muscle). Next, the ECA was ligated and a 20-mm 3-0 monofilament nylon suture with its tip tapered and rounded with fine sand paper was introduced into the ECA stump. Approximately 17.5 mm of the nylon suture was advanced into the ICA, and faint resistance was then felt, indicating that the tip of the suture had entered the anterior cerebral artery. The origin of the MCA was thus occluded by the intraluminal suture, and blood flow from the ICA, the anterior cerebral artery, and the posterior cerebral artery to the MCA was blocked at the circle of Willis.

Nilvadipine was dissolved in polyethylene glycol 400. This drug is photoresistant in solution (unpublished data, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan). The rats were randomly divided into three groups of 10 rats each. Group 1 (control) rats were treated with 1 ml/kg s.c. vehicle immediately after MCA occlusion; groups 2 and 3 rats were treated with 1 ml/kg s.c. nilvadipine solution (1.0 and 3.2 mg/ml, respectively). The skin incision was then closed. The catheter in the tail artery was filled with heparin and heat-sealed by a flame. The N2O/O2 mixture was switched to room air 20 minutes after MCA occlusion, and the rats were then returned to individual cages. No rat showed signs of distress or pain during the postoperative period.

Twenty-four hours after MCA occlusion, the rats were anesthetized with 40 mg/kg i.p. pentobarbital sodium. Each rat was breathing spontaneously, and its rectal temperature was controlled at 37°C. Heparin (1,000 IU/kg) was administered intravenously, and the tail artery again served for the continuous monitoring of mean arterial blood pressure and serial blood sampling. The heart was exposed via a midline incision, and a cannula was inserted into the ascending aorta via the left ventricle. The inferior vena cava was occluded with an aneurysm clip, and the right atrium was then incised. Perfusion fixation was performed with 10% buffered formalin. The brain was removed from the skull and stored in 15% buffered formalin.

The forebrain was embedded in paraffin wax. Coronal sections 5 μm thick were obtained and stained with hematoxylin and eosin. Area was measured in eight coronal sections 1.5 mm apart, the first section 2.5 mm posterior to the frontal tip. Areas of the hemisphere and areas of ischemic neuronal death were plotted on tracings from projections of the coronal sections. Areas of infarction were then measured using an image analyzer (Texture Analyzing System, Leitz, Wetzlar, F.R.G.) separately for the striatum, pallium, and each hemisphere. The volume of infarction was calculated with a computer program using numerical integration of the areas of infarction for the eight coronal sections and the distances between them. At the same time, the volume of each hemisphere was calculated using the hemispheric areas measured. Infarct volume was expressed both as a percentage of the total cerebral volume and in absolute terms as cubic millimeters.

Prior to perfusion fixation, blood was aspirated from the right atrium of the heart, immediately cooled in an ice bath, and then centrifuged at 3,000 rpm and 4°C for 10 minutes. To inhibit enzymatic degradation of nilvadipine, 10^-3 M bis-p-nitrophenyl phosphate (Nakarai, Kyoto, Japan), an esterase inhibitor, was added to the plasma obtained. This plasma (2.5 ml) was stored at -20°C until the plasma concentration of nilvadipine (pCN) was measured using gas chromatography.15

We calculated mean, standard deviation (SD), and standard error of the mean (SEM) for all data. For between-group comparisons, we used Scheffé's multiple-comparison procedure. For within-group comparisons of physiological values, we used repeated-measure analysis of variance and Dunnett's test. We compared...
pCN between groups 2 and 3 using Student’s unpaired t test. We considered $p<0.05$ to be significant.

**Results**

In groups 1 and 2 mean arterial blood pressure remained stable (Table 1). However, in group 3 blood pressure decreased by approximately 25 mm Hg after the administration of nilvadipine ($p<0.05$) and was lower than that in groups 1 and 2 ($p<0.05$). Several rats developed mild hypercapnia due to surgical anesthesia. All other values were within normal ranges.

Prior to sacrifice, several rats again exhibited mild hypercapnia under anesthesia (Table 1). All other values were within normal ranges. Groups 2 and 3 had similar mean arterial blood pressures, both lower than that of group 1. However, these values did not differ significantly among the groups.

There was a remarkably consistent pattern of ischemic brain damage, and typical examples in rats from groups 1 and 3 are shown in Figure 1. Lesions were seen in the dorsolateral frontoparietal cortex, the caudoputamen, and the globus pallidus. The boundaries between areas of infarction and adjacent normal areas were sharply delineated.

Absolute and percentage infarct volumes in each group are shown in Figures 2 and 3, respectively. In group 1, mean±SD absolute infarct volume was $42±10$ mm$^3$ in the striatum and $124±65$ mm$^3$ in the pallium, for a total of $166±73$ mm$^3$; mean±SD
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FIGURE 2. Bar graphs of mean±SEM absolute infarct volume in striatum (top), pallium (middle), and total brain (bottom) of vehicle-treated control rats (open bars), rats treated with 1.0 mg/kg nilvadipine (shaded bars), and rats treated with 3.2 mg/kg nilvadipine (filled bars). Ten rats in each group. *p<0.05 by Scheffe's multiple-comparison procedure.

FIGURE 3. Bar graphs of mean±SEM percentage infarct volume of vehicle-treated control rats (open bars), rats treated with 1.0 mg/kg nilvadipine (shaded bars), and rats treated with 3.2 mg/kg nilvadipine. Ten rats in each group. *p<0.05 by Scheffe's multiple-comparison procedure.

per centage infarct volume was 28.2±11.4%. In group 2, mean±SD absolute infarct volume was 41±12 mm³ in the striatum and 107±64 mm³ in the pallium, for a total of 148±71 mm³; mean±SD percentage infarct volume was 25.5±11.6%. All of these values for group 2 were lower than those for group 1, but not significantly so.

On the contrary, in group 3 the total absolute infarct volume (77±50 mm³) was less than that of group 1 (p<0.05). Absolute infarct volume in the striatum (31±16 mm³) was less than, but not significantly different from, that of group 1. Absolute infarct volume in the pallium (46±37 mm³) was also less than that of group 1 (p<0.05). Therefore, the significant difference in total absolute infarct volume resulted from a decrease in absolute infarct volume in the pallium. As shown in Figure 1, bottom, infarct volume decreased in the peripheral area of the frontoparietal cortex. The percentage infarct volume of group 3 was 13.9±9.2% (p<0.05 different from group 1).

No nilvadipine was detected in plasma obtained from group 1 rats. Mean±SD pC₀ in group 2 was 0.38±0.27 ng/ml, and that in group 3 was more variable (1.67±2.00 ng/ml). There was no significant difference between groups 2 and 3 (p<0.06).

Discussion

Our results clearly demonstrate that nilvadipine reduces infarct volume in rats in a dose-dependent manner when given just after MCA occlusion. A significant decrease in infarct volume was observed in the periphery of the pallium. In addition, nilvadipine lowered mean arterial blood pressure 15 minutes after its administration in a dose-dependent manner. Twenty-four hours after the administration of a single dose, nilvadipine was detected in the systemic circulation.

Calcium entry blockers are widely used in the treatment of such cardiovascular diseases as coronary artery disease, arrhythmias, cerebral vasospasm, and hypertension. The therapeutic effects of these agents have been attributed to inhibition of the influx of Ca²⁺ into cells in both myocardial tissue and vascular smooth muscle. However, this inhibitory action may induce adverse side effects on cardiac functions (e.g., negative inotropy and chronotropy). Nilvadipine was developed to minimize these undesirable effects; it shows high specificity for vascular smooth muscle and has long-lasting effects.

In spite of extensive investigation, the mechanism of action of calcium entry blockers on cerebral ischemia is still unclear. Several possible mechanisms suggest that such drugs ameliorate ischemic cerebral damage by affecting cerebral vasoconstriction, platelet aggregation, and neuronal membrane and mitochondrial functions. Recent investigations into brain ischemia in several animal species and stroke patients have shown that calcium entry blockers may improve cerebral blood flow and metabolism. Improvements in neurologic and neuropathologic outcomes have also been observed in other studies. The results of Germano et al indicate that nimodipine improved neurologic outcome and decreased the size of infarcts when administered up to 6 hours after MCA occlusion in rats. On the other hand, dihydropyridine calcium entry blockers may increase the susceptibility of brain tissue to ischemic damage in baboons and did not attenuate brain damage after focal cerebral ischemia in cats. The reasons for these differences in the effects of dihydropyridine calcium entry blockers are unclear, but they may be due to differences in species or the methods of inducing cerebral ischemia.

We observed a decrease in infarct volume in the periphery of the pallium, where there was collateral...
circulation from the anterior and posterior cerebral arteries. This area appears to correlate anatomically with the ischemic penumbra, the area of nonfunctioning but structurally preserved brain tissue at the periphery of infarction. Infarct volume in the ischemic core (i.e., the basal ganglia and internal capsule) did not decrease significantly (Figure 2) although infarct volume tended to decrease in the medial areas of the striatum (Figure 1).

Our results suggest that nilvadipine provides a possible measure for the treatment of cerebral ischemia. However, we administered nilvadipine only immediately following an ischemic insult. Further study is needed to determine whether this treatment has beneficial effects when initiated up to several hours following the insult.

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References


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