Neuroprotective Effects of a Dual L/N-type Ca$^{2+}$ Channel Blocker Cilnidipine in the Rat Focal Brain Ischemia Model

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Although a blockade or lack of N-type Ca$^{2+}$ channels has been reported to suppress neuronal pathological processes in several animal models of pain and ischemic brain injury, information is still limited regarding the neuroprotective effects of a dual L/N-type Ca$^{2+}$ channel blocker, cilnidipine. In this study, we assessed the effects of cilnidipine in the rat focal ischemia model to analyze its potential utility for hypertensive patients with cerebral infarction. In an anesthetized rat model, cerebral vasodilator actions of cilnidipine were detected at hypotensive doses, which was less potent than those of an L-type Ca$^{2+}$ channel blocker, nilvadipine. In the rat focal brain ischemia model, an anti-hypertensive and anti-sympathetic dose of cilnidipine could reduce the size of cerebral infarction, whereas an equipotent hypotensive dose of nilvadipine failed to affect it. These results suggest that N-type Ca$^{2+}$ channel-blocking profile of cilnidipine may contribute its neuroprotective action in the animal focal brain ischemia model. Thus, treatment of hypertension with cilnidipine may prevent severe consequences after brain attack.

Key words: cilnidipine; N-type Ca$^{2+}$ channel; brain ischemia.

Voltage-dependent Ca$^{2+}$ channels have been divided into at least 5 types (L-, N-, T-, P- and Q-type) based on electrophysiological and molecular experimental evidences. Among them, N-type Ca$^{2+}$ channels have been widely recognized as an important regulator for the systemic cardiovascular tone, because the channels are extensively distributed on the sympathetic nerve endings and predominantly regulate catecholamine releases. Interestingly, several reports have suggested that a blockade or lack of N-type Ca$^{2+}$ channels can suppress the neuronal pathological processes of pain and ischemic brain injury in animal models. Therefore, drugs with an N-type Ca$^{2+}$ channel-blocking property would become beneficial for patients with neuronal diseases.

Cilnidipine is a Ca$^{2+}$ channel blocker with suppressive effects on L- and N-type Ca$^{2+}$ channels, and is currently used for the treatment of essential hypertension in Japan. Clinical evidences for its N-type Ca$^{2+}$ channel-blocking property can be observed by reduction of white coat effect, cold pressor stress-induced platelet aggregation, urinary catecholamine excretion and cardiac sympathetic overactivity in hypertensive patients. However, information regarding the effects of cilnidipine on ischemic brain injury is still lacking while its anti-nociceptive effect has been reported. In the present study, to clarify the potential utility of the drug for brain attack, we assessed effects of cilnidipine on focal brain ischemia in rats by comparing those of nilvadipine, which has been recognized as a Ca$^{2+}$ channel blocker that has high selectivity for cerebral vessels. First, we preparatively examined cardiovascular profiles of cilnidipine and nilvadipine in rats to better understand fundamental cardiovascular profile of cilnidipine and nilvadipine.

MATERIALS AND METHODS

All experiments were performed according to the Guidelines for Experiments of the Pharmaceutical Research Laboratories, Ajinomoto Co., Inc. (Kawasaki, Japan).

Hemodynamic Study. General Surgical Preparation
Male Sprague-Dawley rats (Charles River Japan, Yokohama, Japan) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The right femoral artery was cannulated for the measurement of mean blood pressure (MBP) with a pressure transducer (TP-400T, Nihon Kohden, Tokyo, Japan), and the right femoral vein was also cannulated for drug administration. A vertex craniotomy was performed with a high-speed drill for monitoring blood flow of the cerebral cortex (CBF) with a laser-doppler blood flowmeter (FLO-N1, Omegawave, Tokyo, Japan). The MBP and CBF were recorded using polygraph system (RM-6000; Nihon Kohden). Cerebral vascular resistance (CVR) was calculated with a basic equation; CVR = MBP/CBF. The body temperature was kept at 37—38 °C by a heating pad.

Experimental Protocol
After the stabilization period, 1 µg/kg of cilnidipine was intravenously administered through the cannula (n = 5), and changes in blood pressure and blood flow of the cerebral cortex were observed. Three to four minutes after its administration, the second dose (3 µg/kg) of cilnidipine was additionally administered, and hemodynamic changes were observed. The third, forth and fifth doses of cilnidipine (10, 30 and 100 µg/kg, respectively) was similarly administered, and hemodynamic changes were observed. The effects of nilvadipine (1, 3, 10, 30 and 100 µg/kg; n = 5) were also evaluated in the same manner.

Neuroprotective Effects of Ca$^{2+}$ Channel Blockers. General Surgical Preparation
The focal brain ischemia was performed using a method of tandem occlusion of the distal middle cerebral artery and ipsilateral common carotid artery, as previously described. Male spontaneously hypertensive rats (Charles River Japan, Yokohama, Japan), 8 weeks of age, were used. Cilnidipine (100 µg/kg), nilvadipine (100 µg/kg) or their vehicle (1 ml/kg) was administered intraperitoneally to the rat, and the animal was anesthetized with 2% isoflurane vaporized with 70% N2O and 30% O2. Body temperature was maintained at 37.0±0.5 °C with a heating pad during surgery. A 2-cm incision was made vertically midway between the left orbit and the left external auditory canal. The temporal muscle was reflected, and a subtemporal craniotomy was performed using a high-speed drill. A...
burr hole (3 mm in diameter) was made 1 mm rostral to the anterior junction of the zygoma and squamosal bones, and the main trunk of the middle cerebral artery was exposed. Twenty minutes after the drug administration, the left common artery was ligated with a thread, and the distal middle cerebral artery was permanently occluded just lateral to the olfactory tract by an electrocoagulation method. The incision was closed, and the animals were returned to their cages in the cabinet, where the room temperature and humidity were continuously maintained at 23±1°C and 55±10%, respectively (IE cabinet, KN-734, Natsume, Tokyo, Japan).

**Measurement of Injury Size** Twenty-four hours after the surgery, the animals were reanesthetized with pentobarbital sodium, and the brain was carefully removed and cut into 5-mm-thick coronal sections. The seven brain slices were immersed in 2% TTC (2,3,5-triphenyltetrazolium chloride, Sigma, St. Louis, USA), and preserved in 3.7% formaldehyde. The anterior surface of each section was photographed, and the infarct areas of 7 slices, indicated by a lack of staining, were calculated with a digital image analysis system (Macscope; Mitani Corporation, Fukui, Japan).

**Drugs** Cilnidipine was synthesized at Ajinomoto Co. Inc. (Kawasaki), and nilvadipine was extracted from commercially available tablets (Fujisawa, Osaka, Japan). Pentobarbital sodium and isoﬂurane were purchased from Tokyo Kasei (Tokyo, Japan) and Dai-Nippon Seiyaku (Osaka, Japan), respectively. Cilnidipine and nilvadipine were initially dissolved in 50% hydrogenated castor oil (HCO-60; Nikko Chemicals, Tokyo, Japan)–ethanol solution, which were diluted with saline, as previously reported.20) Pentobarbital sodium was dissolved in saline.

**Data Analysis** Data are expressed as the mean±S.E.M. The statistical significances within a parameter were evaluated by factorial analysis of variance (ANOVA) followed by Dunnett's test for mean values comparison. A *p* value <0.05 was considered statistically significant.

**RESULTS**

**Hemodynamic Study** The effects of the drugs on systemic and cerebral hemodynamics in anesthetized rats are summarized in Fig. 1. The basal values of mean blood pressure, blood flow of the cortex and cerebral vascular resistance were 92±5 mmHg, 14.5±1.0 ml/min/100 g (tissue) and 6.56±0.47 mmHg/(ml/min/100 g (tissue)), respectively (*n* = 10). Intravenous administration of cilnidipine significantly lowered the mean blood pressure at doses of 3—100 µg/kg, of which potency was similar to that of nilvadipine that decreased the mean blood pressure at doses of 3—100 µg/kg. The significant reduction of the cerebral vascular resistance was detected after the administration of cilnidipine at 3—100 µg/kg and nilvadipine at 1—100 µg/kg, in which the potency of cilnidipine was less great than that of nilvadipine. The significant increment of the blood flow of cerebral cortex was detected after the administration of nilvadipine at 3—100 µg/kg but not cilnidipine.

**Neuroprotective Effects** Figure 2 exhibits the typical photograph of the slice of brain showing that infarction area in a cilnidipine-treated rat assessed 24 h after the occlusion of the left middle cerebral artery was less than that in a control rat. Figure 3 summarizes the effects of cilnidipine (100 µg/kg, i.p.) and nilvadipine (100 µg/kg, i.p.) on cerebral infarction in the rat focal brain ischemia model. In the control group (*n* = 11), a percentage of infarction area to the left cerebral cortex obtained from the 7 slices was 31.9±1.9 (%). Cerebral infarction area in the cilnidipine-treated group (*n* = 7) was 22.9±3.6 (%), which was significantly different
from control values. In contrast, cerebral infarction area in the nilvadipine-treated group ($n=7$) was 30.8±2.9 (%), where there were no significant differences compared with control values.

**DISCUSSION**

The present study was designed to clarify the potential utility of cilnidipine for cerebral ischemia using the rat focal brain ischemia model with a tandem occlusion method, which has been reported to induce more stable and larger neocortical infarction than the middle cerebral arterial occlusion alone.\(^{19}\) We evaluated the effect of cilnidipine in a dose of 100 μg/kg, which has been confirmed to exert anti-hypertensive and anti-sympathetic actions in rats via the inhibition of L-type Ca\(^{2+}\) channels and N-type Ca\(^{2+}\) channels, respectively.\(^{20}\) Furthermore, we compared it to the effects of nilvadipine, which has been established to exert a potent cerebral vasodilator action.\(^{10}\)

To confirm the fundamental cardiovascular profiles of cilnidipine and nilvadipine, we first assessed the effects of these drugs on systemic and cerebral hemodynamic in rats. As shown in Fig. 1, equipotent hypotensive effects were observed after intravenous administration of cilnidipine and nilvadipine. Although both cilnidipine and nilvadipine decreased the cerebral vascular resistance, cilnidipine hardly affected the cerebral blood flow in contrast to nilvadipine. The result was in accordance with previous reports that anti-hypertensive doses of cilnidipine failed to change the cerebral blood flow in hypertensive patients and spontaneously hypertensive rats.\(^{21,22}\) Thus, these observations indicate that cilnidipine has a modest cerebral vasodilator action in comparison with nilvadipine.

The present study shows that cilnidipine, in contrast to nilvadipine, effectively suppressed neuronal damage in the focal brain ischemia model, suggesting that the neuroprotective effect is not simply associated with increment of cerebral blood flow by the drugs. In addition, it has been shown that the body temperature is not affected by supra-therapeutic doses of cilnidipine in rats,\(^{23}\) which supposes that cilnidipine directly acts on the ischemic neuronal cells in this study. While the cellular mechanisms of neuronal injury are analyzed extensively, cytosolic Ca\(^{2+}\) overload or excessive Ca\(^{2+}\)-dependent release of excitatory neurotransmitters including glutamate has been indicated as one of the important factors for the development of ischemic neuronal death. On the other hand, a role of N-type Ca\(^{2+}\) channels in pathophysiological processes of the brain ischemia has been reported using the rat permanent middle cerebral artery occlusion model, in which a peptide N-type Ca\(^{2+}\) channel blocker omega-conotoxin MVIIA decreased ischemia-induced glutamate releases.\(^{5}\) Since cilnidipine has been shown to inhibit an elevation of intrathecal glutamate concentration in a pain model,\(^{24}\) the N-type Ca\(^{2+}\) channel-blocking profile of cilnidipine may contribute its neuroprotective effect in the rat focal brain ischemia model. However, further extensive and careful examinations in vivo and in vitro should be planned since information, regarding the effects of cilnidipine on regional cerebral circulation, blood gases and brain temperature in animal models including the spontaneously hypertensive rats in addition to directly protective effects on damaging neuron, will be important to clarify mechanisms of its neuroprotective effects.

Recently, neuroprotective drugs such as a radical scavenger have been used for the treatment of acute brain ischemia in addition to improvement of cerebral circulation with anti-platelet drugs or tissue plasminogen activator after the ischemia occurred.\(^{25}\) Since we assessed neuroprotective effects of cilnidipine that was administered 20 min before brain ischemia in this study, it is still unclear whether cilnidipine can be applied to the treatment of brain ischemia in an acute stage. Thus, it should be clarified neuroprotective effects of cilnidipine that is administered after brain ischemia. However, the current results may provide new information that management of hypertension with cilnidipine prevents severe consequences after brain attack based on its N-type Ca\(^{2+}\) channel-blocking action.\(^{11,26}\)

In conclusion, an L- and N-type Ca\(^{2+}\) channel blocker cilnidipine reduced neuronal damage in the rat brain. Thus, the drug may be suitable for hypertensive patients with a risk of brain attack.

**REFERENCES**