Effect of Nilvadipine on the Development of Neurological Deficits in Stroke-Prone Spontaneously Hypertensive Rats

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Background and Purpose Several types of antihypertensive drugs have been reported to protect stroke-prone spontaneously hypertensive rats from stroke. However, the clinical relevance remains unclear. This study was performed to investigate the effect of nilvadipine, a calcium channel blocker, on the development of neurological deficits in stroke-prone spontaneously hypertensive rats. In addition, plasma levels of nilvadipine were measured to determine the clinical relevance.

Methods Salt-loaded stroke-prone spontaneously hypertensive rats were orally administered nilvadipine mixed with a powder diet (0.01% and 0.03%, wt/wt). Non-salt-loaded rats were maintained on tap water. Chronological changes in neurological deficit scores and systolic blood pressure were recorded. After 6 weeks of medication, measurement of plasma levels of nilvadipine, serum biochemical analysis, and pathological observation of both the brain and the kidney were performed.

Results In the salt-loaded control group, both severe hypertension and neurological deficit developed, and the final survival rate was 30%. Systolic blood pressure decreased significantly in the high-dose nilvadipine-treated group but not in the low-dose nilvadipine-treated group. However, the development of neurological deficit was almost completely inhibited in both nilvadipine-treated groups that had no deaths (P<.01). The mean plasma levels of nilvadipine in the low-dose group and in the high-dose group at the time of death were 0.21 ng/mL and 0.61 ng/mL, respectively.

Conclusions Nilvadipine inhibited the development of neurological deficit in stroke-prone spontaneously hypertensive rats at plasma concentrations lower than that in clinical use. Thus, nilvadipine might prevent cerebral vascular disorders at doses routinely used for essential hypertension. (Stroke. 1994;25:677-683.)

Key Words • calcium channel blockers • cerebrovascular disorders • hypertension • rats

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of antihypertensive drugs have been examined for the prevention of stroke.3-7 Because the plasma levels of drugs were not determined in this study, it is not clear whether any benefits occurred at clinically relevant doses.

Nilvadipine, an antihypertensive, dihydropyridine-type calcium channel blocker, has selective and lasting effects on cerebral arteries compared with other calcium channel blockers such as nicardipine, nifedipine, and diltiazem.12 The drug has a neuroprotective effect against ischemia with an ability to be well distributed in the brain.13 Nilvadipine was reported to be effective in several experimental models of cerebral ischemia.13-18

In this study we examined the effect of nilvadipine on the development of stroke, especially motor function, in salt-loaded SHRSP. Moreover, plasma levels of nilvadipine were measured in the rat to determine the clinical relevance.

Materials and Methods

Nilvadipine was synthesized in our laboratories and administered for 6 weeks mixed with a powder diet (CA-1, Clea Japan Inc) in concentrations of 0% (for both the non-salt-loaded group and the salt-loaded control group), 0.01% (for the low-dose group), and 0.03% (for the high-dose group) (wt/wt). Each diet contained 150 mg of hydroxypropylmethylcellulose 2910 and 320.1 mg of low hydroxypropylcellulose per 100 g of the powder diet to aid in the absorption of nilvadipine.
Thirty-nine male SHRSP (obtained from Professor Okamoto of Kinki University Medical School and bred at Fujisawa Pharmaceutical Co, Ltd) were maintained on a normal diet to 8 weeks of age. The SHRSP were divided into four groups based on systolic arterial blood pressure. One group was maintained on tap water ad libitum as the non-salt-loaded group that was fed with a diet containing 0% nilvadipine. The other three groups of 10 rats were given 1% NaCl solution instead of drinking water.

The measurements described below were performed in a single-blind manner. The average daily intake of the diet was measured at least three times per week. Systolic blood pressure was measured every 2 weeks by the tail-cuff method (blood pressure monitor MK-1000, Muromachi Kikai Co, Ltd). Body weight was recorded once a week. Neurological deficits, mainly a decrease of motor function, were observed three times per week at the same time (9 AM to 11 AM). The neurological deficits were evaluated according to a scoring system: 0, normal; 1, slight decrease of motor activity or slight excitement; 2, marked decrease in motor activity or hyperirritability; 3, no walking (decreased responsiveness); 4, inability to stand without support or paralysis of hind limbs; and 5, death.

Six weeks after starting the drug administration, animals were killed between 10 AM and 4 PM by bleeding under ether anesthesia. Blood samples were collected from the abdominal aorta. Serum was obtained from a part of each blood sample and used to measure several parameters with an automated blood analyzer (type 7150, Hitachi Co, Ltd). The measured parameters were total protein, total cholesterol, alkaline phosphatase activity, aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, creatinine, uric acid, Na⁺, K⁺, and Cl⁻. The rest of the blood sample was collected with a heparinized syringe for the determination of plasma levels of nilvadipine. The blood samples for the measurement of the drug levels were immediately cooled in an ice bath and centrifuged at 4°C. Bis(p-nitrophenyl)phosphate, an esterase inhibitor, was added to the plasma at a final concentration of 1 mmol/L to prevent degradation of nilvadipine. Nilvadipine levels in plasma were determined by gas chromatography with electron-capture detection.21

Immediately after the animals were killed, brain and kidney were dissected out and macroscopic observations were performed. Both organs then were fixed with 10% formalin. After the fixation, hemorrhage and/or infarction in the brain was confirmed visually. After embedding in paraffin, 4-μm sections were made for microscopic evaluation. The brain was stained by hematoxylin and eosin, and the kidney was stained by hematoxylin and eosin, periodic acid–Schiff, and azan.

Statistical analyses were performed by Wilcoxon's rank sum test or Fisher's exact probable method between the non-salt-loaded group and the salt-loaded control group, and Dunnett's-type multiple comparisons were used for assessing differences between the nilvadipine-treated groups and the salt-loaded control group. *P<.05 was considered statistically significant.

**Results**

The mean food intakes during the first 2 days after drug administration for the non-salt-loaded group, the salt-loaded control group, the low-dose group, and the high-dose group were 18.4, 19.4, 14.9, and 9.2 g per rat per day, respectively. After that all salt-loaded groups showed almost the same food intake as that of the non-salt-loaded group (range, 14.2 to 20.4 g per rat per day) until the 26th day. However, after that the salt-loaded control group rats showed a decrease in food intake. The mean drug intakes in the low-dose and the high-dose group were 5.4 to 9.1 mg/kg per day and 13.1 to 28.2 mg/kg per day, respectively.

Body weight in the salt-loaded control group began to decline 4 weeks after drug administration. Both nilvadipine-treated groups fared well and gained weight at a rate similar to that of the non-salt-loaded group (Fig 1). Before drug administration the systolic blood pressure was approximately 190 mm Hg in all groups. In the non-salt-loaded group the systolic blood pressure gradually increased with aging. However, the degree of increase in systolic blood pressure in the salt-loaded control group was greater than that in the non-salt-loaded group. The age-associated increase in systolic blood pressure was significantly inhibited only in the high-dose group at both the second and the sixth week compared with that of the salt-loaded control group (Fig 2).

In the salt-loaded control group, some rats had severe neurological deficits after the 26th day. The scores in both the nilvadipine-treated groups were almost the same or somewhat smaller than that in the non-salt-loaded group (Fig 3). Final survival rates in the non-salt-loaded group and in the salt-loaded control group

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**Fig 1.** Line graph shows effects of nilvadipine on body weight changes of stroke-prone spontaneously hypertensive rats. Salt-loaded rats were maintained on 1% NaCl drinking solution from the age of 8 weeks. Non-salt-loaded rats were maintained on tap water. Drug was given orally mixed with powder diet from 8 weeks of age. Numbers in parentheses indicate the number of rats for which body weight was obtained. Values are mean±SEM. *P<.05 compared with non-salt-loaded rats by Wilcoxon's rank sum test; †P<.05 compared with salt-loaded control group by Dunnett's-type multiple comparisons.
were 89% and 30%, respectively. In contrast, both nilvadipine-treated groups had no deaths (Fig 4).

The Table shows serum biochemical parameters. Aspartate aminotransferase, alanine aminotransferase, and sodium levels were almost the same in all groups (data not shown). Compared with the non-salt-loaded group, the salt-loaded control group showed significantly lower values of alkaline phosphatase activity, K⁺, and Cl⁻ and significantly higher values of total cholesterol, blood urea nitrogen, creatinine, and uric acid. These changes were ameliorated in both nilvadipine-treated groups almost equipotently.

No rat showed cerebral infarction and/or hemorrhage by macroscopic observation in the non-salt-loaded group or in either of the nilvadipine-treated groups. However, two of the three remaining rats in the salt-loaded control group showed edematous changes with cerebral infarction. The brains of the two rats showed mild swelling, and the vascular formation on the brain surface was not clear. The light microscopic observation of the thin sections obtained from these two rats indicated that one rat showed a slight to moderate degree of edema, necrosis, and hemorrhage, and the other rat showed a slight to moderate degree of hemorrhage and infiltration of inflammatory cells. No plaques were detected in the pial arteries. Preparations obtained from the rest of the rats showed no pathological findings.

Renal macroscopic observation showed no remarkable pathological finding in the non-salt-loaded group or in either of the nilvadipine-treated groups. However, in the salt-loaded control group all three rats showed a fine, pale granular appearance on the surface of the kidney. In these three rats, renal microsections showed fibrinoid necrosis in the arterioles and tubular degeneration and hyaline cast formation in tubules of moderate to severe degree. No plaques were detected in the renal arteries. In the non-salt-loaded group, only one rat showed these three pathological changes to a slight degree. Although two or three rats in each nilvadipine-
treated group showed some pathological changes, the degree was also slight. The differences between the result from the non–salt-loaded group and the salt-loaded control group and the differences between the salt-loaded control group and both nilvadipine-treated groups were statistically significant (P<.01).

The unchanged form of nilvadipine was not detected in blood samples from the salt-loaded control group. The blood nilvadipine levels of the low-dose group and of the high-dose group were 0.21±0.05 ng/mL and 0.61±0.08 ng/mL, respectively (mean±SEM).

Discussion

The results from this experiment clearly demonstrate that nilvadipine inhibited the development of neurological deficits, especially motor function, in salt-loaded SHRSP.

The group treated with a low dose of nilvadipine showed only a small tendency to inhibit the increase in blood pressure with age in SHRSP, but high-dose nilvadipine inhibited the blood pressure increase to a remarkable extent. However, neurological deficits, survival rate, pathological observations from both the brain and the kidney, and biochemical values in the serum in both nilvadipine-treated groups were almost the same as in the non–salt-loaded group. This suggests that nilvadipine inhibited the neurological deficits in SHRSP by some mechanism other than reduction in systolic blood pressure. However, even a small decrease in systolic blood pressure may play an important role in preventing neurological deficits. Several reports have demonstrated the effects of antihypertensive drugs such as β-blockers, calcium channel blockers, and angiotensin-converting enzyme inhibitor using SHRSP.4–7 Manidipine, betaxolol, and enalapril decreased systemic blood pressure and prevented cerebrovascular lesions or onset of stroke.4–6 In contrast, imidapril prevented the incidence of stroke without systemic blood pressure reduction.5 These reports suggest that reduction of systemic blood pressure is not always necessary to prevent the incidence of stroke.

Nilvadipine has been reported to have selective and long-lasting effects on the coronary and cerebral arteries in anesthetized dogs, and the IC90 value for basilar arterial strips was smaller than for peripheral arterial strips contracted by K+.12 These findings suggest that the drug might increase cerebral blood flow even at a dose that shows almost no effect on systemic blood pressure. In a study using anesthetized cats, the degree of increase in blood flow in the cerebral cortex was much larger than the decrease in systemic arterial pressure.22 In SHRSP, blood flow in the cerebral cortex

Influence of Long-term Treatment With Nilvadipine on Serum Biochemical Parameters of Stroke-Prone Spontaneously Hypertensive Rats

<table>
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<tr>
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<th>n</th>
<th>TP, g/dL</th>
<th>TC, mg/dL</th>
<th>AP, mU/mL</th>
<th>BUN, mg/dL</th>
<th>Creatinine, mg/dL</th>
<th>Uric acid, mg/dL</th>
<th>K+, mEq/L</th>
<th>Cl−, mEq/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non–salt-loaded group</td>
<td>8</td>
<td>6.6±0.1</td>
<td>54±3</td>
<td>452±21</td>
<td>26±2</td>
<td>0.5±0.02</td>
<td>2.0±0.3</td>
<td>4.5±0.2</td>
<td>104±0.7</td>
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<tr>
<td>Salt-loaded groups (nilvadipine treated)</td>
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<td></td>
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<td></td>
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<tr>
<td>0%</td>
<td>3</td>
<td>6.0±0.3</td>
<td>141±27</td>
<td>213±44*</td>
<td>37±3*</td>
<td>0.8±0.06†</td>
<td>4.4±0.4*</td>
<td>3.7±0.2*</td>
<td>97±2.9*</td>
</tr>
<tr>
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<td>6.8±0.1†</td>
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<td>485±34§</td>
<td>29±26</td>
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<td>2.4±0.3†</td>
<td>4.6±0.1‡</td>
<td>103±0.4§</td>
</tr>
<tr>
<td>0.03%</td>
<td>10</td>
<td>6.9±0.1§</td>
<td>63±1</td>
<td>462±17§</td>
<td>31±1</td>
<td>0.6±0.02‡</td>
<td>2.3±0.3§</td>
<td>4.8±0.1§</td>
<td>102±0.6</td>
</tr>
</tbody>
</table>

Values are mean±SEM. TP indicates total protein; TC, total cholesterol; AP, alkaline phosphatase; and BUN, blood urea nitrogen. *P<.05, †P<.01 compared with non–salt-loaded group (Wilcoxon’s rank sum test). ‡P<.05, §P<.01 compared with salt-loaded control (0%) group (Dunnett’s-type multiple comparisons).
decreases abruptly with severe hypertension, ie, when systolic blood pressure is greater than 200 mm Hg.23 Moreover, Fujishima et al24 reported that hypertensive rats were more susceptible to cerebral ischemia and the susceptibility was related to the degree of hypertension. Because the systolic arterial pressure in the salt-loaded group in our study was greater than 200 mm Hg, it is probable that the cerebral blood flow was decreased, resulting in oxygen deprivation. Treatment with nilvadipine at an early stage in the development of the pathological changes may act to increase or maintain the cerebral blood flow.

Calcium channel blockers may have a neuroprotective effect in models of cerebral ischemia through an inhibition of accumulation of intracellular calcium that may serve as a trigger of irreversible cellular injury.25-28 Nilvadipine inhibited calcium accumulation in neurons in cerebral hypoxic-ischemic damage in the rat (Satoh H, Moriguchi A, Mori J, Nakano K, Fujii T. 1990. Unpublished data) and has been reported to have neuroprotective properties in a rat model of global ischemia.13 The pharmacokinetic properties of nilvadipine, such as high brain-blood ratio of the drug and longer half-life in the brain compared with the blood,13 might contribute to its neuroprotective properties. Moreover, nilvadipine has been reported to suppress the influence of the superoxide radical in ischemia-reflow-induced paw edema in mice29 and to be antiatherosclerotic.28,29 Recently Arakawa et al30 demonstrated that nilvadipine reduced intimal thickening that developed after percutaneous transluminal coronary angioplasty in pigs. Because these animal experiments were performed at doses that might decrease blood pressure, these pharmacologic properties may play a role in the development of neurologic deficit in SHRSP by high-dose nilvadipine treatment. However, this cannot explain why low-dose nilvadipine treatment could prevent the development of neurologic deficits. As shown in our results, salt-loaded SHRSP showed an increase in plasma total cholesterol levels, which agrees with some published reports.31,32 However, obvious plaques in either the pial arteries or the renal arteries were not detected in this study. Although nilvadipine reduced the total cholesterol levels in plasma in salt-loaded SHRSP, it is uncertain whether this effect is related to the inhibition of the development of neurologic deficit.

On the other hand, close correlation between renal dysfunction and cerebral vascular disorders has been reported in SHRSP.5,7,23 Therefore, we investigated the degree of renal failure in this study. Increases in blood urea nitrogen and creatinine and hypokalemia in the salt-loaded control group suggested deterioration of renal function. Renal failure might induce an increase in uric acid and decreases in total protein, alkaline phosphatase activity, and Cl−. These changes were ameliorated in both the low-dose and the high-dose groups, suggesting that nilvadipine has the ability to prevent deterioration in renal function. In the renal pathological evaluation, the protective effect of nilvadipine was reconfirmed in salt-loaded SHRSP. Several effects of nilvadipine described above27,28 might contribute to the renal protective effect. Benidipine hydrochloride, a chemically related calcium channel blocker, attenuated the ischemic renal damage by inhibition of calcium overload,24 and therefore nilvadipine may also maintain renal function against glomerular ischemia by inhibiting calcium abnormalities. Such a renal protective effect of nilvadipine was observed in both nilvadipine-treated groups, suggesting that this effect might correspond to the inhibitory effect of nilvadipine on the development of neurologic deficit in SHRSP.

Kawamura et al19 reported that nilvadipine decreased ischemic neuronal injury in a dose-dependent manner. They also determined plasma nilvadipine levels 24 hours after subcutaneous nilvadipine administration. In their report, infarction volume in the high-dose group was significantly reduced at a mean plasma level of 1.67 ng/mL. In our study blood sampling for the determination of plasma levels of nilvadipine was performed from 10 AM to 4 PM. According to our preliminary study, using male rats that were orally administered nilvadipine mixed with powder diet for 30 days, peak plasma concentrations were obtained at night, when levels were twofold to threefold higher than those during the day. Thus, the plasma level of nilvadipine in the low-dose group was thought to be lower than that of the high-dose group of Kawamura et al.19 As shown in this study, lower plasma levels of nilvadipine might be effective for the prevention of neurologic deficits.

The mean plasma level of nilvadipine in the low-dose group was lower than that found 12 hours after the last dosing in a multiple-dosing study of healthy volunteers (4 mg BID)35; it was also lower than that found in daily clinical use for essential hypertension (4 mg BID).36 Therefore, nilvadipine might inhibit the onset of stroke or inhibit the development of neurologic deficit even at a dose that does not cause a significant decrease in blood pressure.

In conclusion, it was demonstrated that nilvadipine has the ability to inhibit the development of neurologic deficit in SHRSP in doses that minimally affect the blood pressure. The mechanism of action of nilvadipine may be renal protective effect; however, the possibility remains that other pharmacological profile(s) of nilvadipine such as cerebral vasodilative effect could play a role as well. The present study suggests that clinical use of nilvadipine for essential hypertension will also prevent the development of stroke.

Acknowledgments

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References

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22. Iwatsuka H, Suzuoki Z, Okamoto K. Genetic predisposition to mechanism(s) unrelated to the known beneficial effects of antihypertensives to reduce risk of stroke. Third, the efficacy of nilvadipine was obtained points toward the possibility that some calcium channel blockers might be beneficial for primary or secondary prevention of stroke. Second, the efficacy of the low dose of nilvadipine to prevent death and derangements without an antihypertensive effect points to mechanism(s) unrelated to the known beneficial effects of antihypertensiveness to reduce risk of stroke. Third, the efficacy of nilvadipine was obtained by an oral route of administration and at clinically relevant plasma levels, which are significant attractions for human use since it is unlikely that long-term

Editorial Comment

The accompanying article by Takakura et al provides remarkable data concerning the pharmacologic effect of nilvadipine, a dihydropyridine calcium channel blocker, in a rat model of stroke. The study is important in several aspects. First, prophylactic treatment for primary prevention of strokes has not been clearly established in humans, and the use of aspirin for this purpose is largely compassionate. Drugs available for secondary prevention of stroke thus far include only the antiplatelet class of aspirin and ticlopidine. In this context, the study with nilvadipine points toward the possibility that some calcium channel blockers might be beneficial for primary or secondary prevention of stroke. Second, the efficacy of the low dose of nilvadipine to prevent death and derangements without an antihypertensive effect points to mechanism(s) unrelated to the known beneficial effects of antihypertensiveness to reduce risk of stroke. Third, the efficacy of nilvadipine was obtained by an oral route of administration and at clinically relevant plasma levels, which are significant attractions for human use since it is unlikely that long-term
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