Prophylactic Effect of Imidapril on Stroke in Stroke-Prone Spontaneously Hypertensive Rats

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Background and Purpose: It has been reported that some angiotensin converting enzyme inhibitors can prevent stroke-prone spontaneously hypertensive rats from stroke at much higher doses than clinical doses used for hypertension therapy. This study was performed to investigate the prophylactic effectiveness of imidapril against stroke in comparison with enalapril.

Methods: Salt-loaded stroke-prone spontaneously hypertensive rats were orally given imidapril (0.5, 1, 2, and 5 mg/kg per day), enalapril (2 and 5 mg/kg per day), or hydralazine (5 mg/kg per day). Stroke signs were scored, and blood pressure, protein concentration, and N-acetyl-b-D-glucosaminidase activity in urine were measured. After 2 weeks of medication, angiotensin converting enzyme activities in the aorta were measured 24 hours after dosing.

Results: In the control group, severe hypertension developed, and all rats died within 12 weeks because of stroke. Imidapril and enalapril dose-dependently decreased the stroke-related mortality, and both agents at 5 mg/kg per day showed excellent prophylaxis, although they did not inhibit hypertensive development. Imidapril at 0.5 mg/kg per day significantly prevented stroke to almost the same extent as enalapril at 2 mg/kg per day or hydralazine at 5 mg/kg per day. Imidapril dose-dependently suppressed the elevation of the two urinary indexes, which was followed by stroke. Imidapril inhibited enzyme activity in the aorta more strongly than did enalapril at the same dose.

Conclusions: Imidapril prevented the incidence of stroke in stroke-prone spontaneously hypertensive rats at a dose of 0.5 mg/kg per day or more by amelioration of kidney dysfunction. Reduction of blood pressure is not necessary, although enzyme inhibition in the vasculature may partly relate to the effect.

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Key Words • angiotensin converting enzyme inhibitor • hypertension • kidney • rats

Hypertension is a leading risk factor for cerebrovascular disorders.1 Although antihypertensive therapy is reported to be efficacious in reducing the incidence of these disorders,2 stroke still remains a major complication of hypertension.

Some angiotensin converting enzyme (ACE) inhibitors, such as enalapril,3,4 captopril,5 delapril,6 and cilazapril,7 have been reported to have preventive effects on stroke in stroke-prone spontaneously hypertensive rats (SHRSP),8 but the effective doses were much higher than those that produced antihypertensive effects in spontaneously hypertensive rats (SHR)9-12 and were extremely higher than clinical doses.

Enalapril at 15 mg/kg per day did not reduce blood pressure in salt-loaded SHRSP,3 although delapril at 10 mg/kg per day did.6 An antihypertensive effect is not always necessary for ACE inhibitors to prevent stroke. Enalapril reduced urinary protein excretion and increased the glomerular filtration rate and tubular reabsorption of water.3 Delapril reduced urinary protein excretion, left ventricular weight, and blood pressure.6

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Cilazapril attenuated the increase in distensibility of pial arterioles.7,13 Thus, there are two possible mechanisms of the stroke-preventive action of ACE inhibitors in SHRSP: 1) reduction of blood vessel damage induced by high angiotensin II levels and 2) amelioration of kidney dysfunction.

Imidapril, a newly synthesized ACE inhibitor, lowers blood pressure in two-kidney, one clip renal hypertensive rats and SHR14,15 and inhibits ACE activity in serum, lung, aorta, kidney, and heart (Y. Hashimoto, T. Sugaya, R. Ishida, unpublished observations) of rats at a potency almost equal to that of enalapril.

In the present study, we examined the minimum effective dose of imidapril on stroke in salt-loaded SHRSP compared with enalapril; in addition, we examined the effects on kidney dysfunction and vascular ACE activity to elucidate the mechanisms involved.

Materials and Methods

Study 1 was performed as follows. SHRSP were originally obtained from K. Okamoto at Kinki University and bred at Tanabe Seiyaku Co., Ltd. At the age of 10 weeks, 80 male SHRSP were divided on the basis of systolic blood pressure measured by the tail-cuff method (blood pressure monitor MK-1000; Muromachi Kikai Co., Ltd., Tokyo) into the following six groups: control

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(n=20), imidapril 2 and 5 mg/kg (n=15 and 10, respectively), enalapril 2 and 5 mg/kg (n=15 and 10, respectively), and hydralazine 5 mg/kg (n=10). When the rats were 11 weeks old, tap water and a normal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo) were replaced by 1% NaCl solution and a special diet\textsuperscript{16} (Funabashi SP; Funabashi Farm Co., Ltd., Chiba, Japan). The replacement resulted in a diet characterized by high sodium and a low protein content. After this time, imidapril hydrochloride and enalapril maleate (both synthesized at the Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co., Ltd.) and hydralazine hydrochloride (Tokyo Kasei, Tokyo) dissolved in distilled water were administered daily to the rats by gavage at a volume of 5 ml/kg for 18 weeks. Thereafter, the drugs were withdrawn and the rats were kept under the same conditions until they were 38 weeks old.

We daily observed the behavior of the SHRSP to detect stroke signs according to the criteria of Okamoto et al.,\textsuperscript{8} and the number of rats with stroke signs was scored. Body weight was also measured daily. In the animals without stroke signs, systolic blood pressure and heart rate were measured indirectly 24 hours after dosing, once a week for the first 6 weeks and at 5-week intervals thereafter.

After 9 weeks of medication, analysis of urine stored for 24 hours was carried out with an automated clinical analyzer (XA-18; Japan Tectron Instruments Corp., Tokyo). Urinary protein concentrations were measured by the dye-binding method\textsuperscript{17,18} (Bio-Rad Laboratories, Richmond, Calif.) using bovine serum albumin as a standard. Urinary N-acetyl-\textbeta-D-glucosaminidase activity was colorimetrically assayed using sodio-\textalpha-m-cresolsulphonphthaleinyl N-acetyl-\textbeta-D-glucosaminide (Shionogi & Co., Ltd., Osaka, Japan) as the substrate.\textsuperscript{19} To 10 µl of urine sample, 400 µl of the substrate solution was added, and the mixture was incubated for 13 minutes at 37°C. The reaction was terminated by adding sodium carbonate, and the absorbance at 600 nm was measured. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 µmol of \textalpha-m-cresolsulphonphthalein per minute.

When the rats became comatose, they were killed with ether. After the animals died naturally or were euthanized, their brains were removed as soon as possible for macroscopic observation to detect cerebrovascular hemorrhage. Brains without surface hemorrhages were fixated with 10% neutral buffered formalin solution and cut into 1-mm-thick slices for macroscopic observation.

For the assay of ACE activity, other male SHRSP were divided into the following three groups: control (n=8), imidapril 2 mg/kg (n=7), and enalapril 2 mg/kg (n=8). They were treated for 2 weeks according to the protocol described above. Twenty-four hours after the final drug administration, the thoracic aortas in the euthanized rats were removed and rinsed with saline. The tissue was homogenized with 9 vol of ice-cold 10 mM tris(hydroxymethyl)aminomethane (Tris)-buffered saline (pH 7.4)−0.2% sucrose at 0°C, followed by centrifugation (2,000 rpm×10 minutes, 4°C). Fifty microliters of the supernatant was added to 400 µl of 1 mM Hippuryl-His-Leu (Sigma Chemical Co.) in 50 mM Tris–NaCl buffer (pH 7.4) and incubated at 37°C for 1 hour. The reaction was stopped by adding 2 ml of methanol, and reaction tubes were chilled in an ice-water bath. Hippuric acid was measured by a high-performance liquid chromatography method.\textsuperscript{20} ACE activity was expressed as picomoles of hippuric acid formed per milligram of wet tissue per minute (pmol/min per mg tissue).

In study 2, we examined the effects of lower doses of imidapril on the parameters used in study 1 to clarify the minimum effective dose of imidapril and the relation between kidney dysfunction and stroke. Sixty-one male SHRSP were divided into the following three groups: control (n=24), imidapril 0.5 mg/kg (n=19), and imidapril 1.0 mg/kg (n=18); they were medicated according to the protocol described above for study 1, excluding the withdrawal period. Urine and blood pressure were examined every other week. For urinalysis, we selected half of the rats in each group so that the mean value of systolic blood pressure would not change.

The survival rate, estimated by the Kaplan-Meier method, and the incidence of stroke signs were statistically analyzed by the log rank test or \textit{χ²} test. Other values were analyzed by Scheffe’s multiple comparison. A value of \textit{p}<0.05 was considered significantly different.

\textbf{Results}

The survival rate in study 1 is shown in Figure 1. In the control group, deaths occurred after the second week, and all rats died within 12 weeks. During the medication period, all the rats in the imidapril 5 mg/kg
and the enalapril 5 mg/kg group showed stroke-related neurological signs. No rats treated with 5 mg/kg imidapril showed stroke-related neurological signs.

and the enalapril 5 mg/kg group showed stroke-related neurological signs. All of the 19 rats in the control group and four of the five rats in the hydralazine group had cerebrovascular hemorrhage. Thus, almost all the dead rats with cerebral lesions in these groups had also shown stroke signs during their lives. In the group treated with enalapril 2 mg/kg, however, five of 11 dead rats showed stroke signs only. We decided that the animals that showed stroke signs and/or had cerebrovascular hemorrhage during the medication period were judged to be suffering from stroke. All the control rats were suffering from stroke (100%; 20 of 20).

Figure 2 illustrates the incidence of stroke signs during the medication period in study 1. Stroke signs were shown by 19 of 20 rats (95%) in the control group, no rats in the imidapril 5 mg/kg group, one (7%) in the imidapril 2 mg/kg group, and one (10%) in the enalapril 5 mg/kg group. The number of rats with stroke signs in the enalapril 2 mg/kg and hydralazine groups increased gradually, and the values of the incidence reached 80% and 70%, respectively, at the end of the medication period. There were statistical differences among the values of the incidence of stroke signs ($\chi^2 = 83.87, df = 5; p = 0.0001$), and the same three clusters as in the life-span study were identified.

The relation between stroke signs and cerebrovascular hemorrhages in the animals that died spontaneously or were euthanized during the medication period is shown in Table 1. Nineteen of 20 dead rats in the control group and five of six dead rats in the hydralazine

<table>
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<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Rats that died (n)</th>
<th>SS (n)</th>
<th>CVH (n)</th>
<th>SS+CVH (n)</th>
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SS, rats showing stroke signs; CVH, rats showing cerebrovascular hemorrhage; SS+CVH, rats fitting both criteria.

One stroke-prone spontaneously hypertensive rat in the enalapril 2 mg/kg group died with neither stroke signs nor cerebrovascular hemorrhage.
incidence of stroke in both the enalapril 2 mg/kg and hydralazine groups was 80% (12 of 15 and eight of 10, respectively; two rats each in the two groups did not die but showed stroke signs during the medication period). The values of incidence in the imidapril 2 and 5 mg/kg groups and the enalapril 5 mg/kg group were 13% (two of 15), 0%, and 10% (one of 10), respectively, and were significantly different from the control group by the \( \chi^2 \) test (\( p<0.01 \)).

Blood pressure increased sharply 2 weeks after the beginning of the experiment in all groups treated with the ACE inhibitors as well as in the control group, and these high blood pressure levels were maintained thereafter. The hydralazine group, in contrast, showed marked hypotension soon after the beginning of medication. Heart rate in all the ACE inhibitor–treated groups increased slightly after the beginning of medication but did not markedly exceed that of the control group. Reflex tachycardia as a result of hypotension was observed in the hydralazine group (Figure 3).

Control rats gradually lost body weight beginning at 3 weeks after the beginning of the experiment, whereas animals treated with the ACE inhibitors showed reasonable weight gain. Hydralazine-treated rats showed little increase in body weight during the first several weeks (Figure 4).

Urinary protein excretion and N-acetyl-\( \beta \)-D-glucosaminidase activity in the imidapril 2 and 5 mg/kg and the enalapril 5 mg/kg groups were lower than in the control group at 19 weeks of age. Both parameters in the enalapril 2 mg/kg and the hydralazine groups were slightly lower than those in the control group, but there were no significant differences (Figure 5).

ACE activities in the aorta were determined by using other salt-loaded SHRSP. The values were 23.7±3.7, 11.4±0.8, and 17.7±1.4 pmol/min per mg tissue in the control, imidapril 2 mg/kg, and enalapril 2 mg/kg groups, respectively (\( n=7-8 \)). Imidapril inhibited ACE activity significantly (\( p<0.01 \)) versus the control group, whereas enalapril inhibited ACE activity slightly.

The survival rate and incidence of stroke signs in study 2, which involved lower doses of imidapril, are shown in Figure 6. Changes in the control group were almost identical to those in study 1. The \( \chi^2 \) values between these two controls were 0.37 (\( df=1; p=0.5445 \)) for survival rate and 0.14 (\( df=1; p=0.7074 \)) for stroke
signs. Imidapril at 0.5 and 1.0 mg/kg increased life span and decreased the incidence of stroke signs in a dose-dependent manner. Systolic blood pressure rose equally in the three groups in an age-related manner, as in study 1 (data not shown).

Urinary protein excretion and N-acetyl-β-D-glucosaminidase activity increased in an age-related manner in the control group. Imidapril suppressed the elevation in a dose-dependent manner. Individual changes indicated that stroke signs occurred after increases in these two indexes. Animals in which the levels of these indexes remained low were not prone to stroke in the imidapril groups (Figures 7 and 8).

**Discussion**

The present study demonstrated that imidapril dose-dependently protected salt-loaded SHRSP from stroke-related death at daily doses of 0.5 mg/kg or more. Since the two control groups in study 1 and study 2 showed almost equal changes in both survival rate and incidence of stroke signs, we statistically analyzed all nine groups. The values of $\chi^2$ were low: 0.18 ($df=2; p=0.9120$) for the incidence of stroke signs and 1.69 ($df=2; p=0.4286$) for the survival rate among the three groups of enalapril 2 mg/kg, hydralazine, and imidapril 0.5 mg/kg. The statistical analysis revealed that the protective effect of imidapril at 0.5 mg/kg on stroke was as potent as that of enalapril at 2 mg/kg. Thus, imidapril is four times as potent as enalapril in stroke prevention. Compared with enalapril, imidapril has an equal potency of antihypertensive effect, an equal absorption rate, and one third of the bioavailability value. The efficacy of ACE inhibition is equipotent between imidapril and enalapril in serum, lung, aorta, kidney, and heart in rats 1 hour after oral administration (Y. Hashimoto, T. Sugaya, R. Ishida, unpublished observations). Taken together, the different efficacy of the two agents in regard to stroke prevention may depend on factors other than the potency of antihypertension and ACE inhibition.
Imidapril or enalapril at 5 mg/kg per day is reported to be sufficient for suppressing genetic development of hypertension in SHR, which is the low-renin-type model of hypertension, as well as in SHRSP. In this experiment, however, no hypotensive effect was observed at the dose in salt-loaded SHRSP, because the increases in body sodium and blood volume depress the renal release of renin and excessively elevate the systemic blood pressure.

Various antihypertensive treatments with hydralazine,7 nifedipine, manipidine,24 betaxolol,24 or hydrochlorothiazide26 prolonged life span in SHRSP. The authors of these studies pointed out that the reduction of vascular stress was important to protect SHRSP from stroke. In the present study, hydralazine, at the dose that suppresses hypertensive development, significantly exerted a preventive effect on stroke compared with the control, but the action was less efficient than that of ACE inhibitors. These findings indicate that hypotension slightly contributed to prolongation of the life span in SHRSP. In our study, the only systolic blood pressure was measured by the indirect method, and therefore hypotension remains to be confirmed by the direct method.

In the control group, almost all rats that had shown stroke signs also had cerebrovascular hemorrhage. However, this relation was obscure in the groups treated with enalapril. Antihypertensive drugs have been reported clinically to not only reduce stroke morbidity and mortality27,28 but also to keep cerebrovascular lesions to a minimum even if patients have had cerebrovascular hemorrhages, thus bringing about a favorable prognosis.29 It seems that the stroke signs observed in the enalapril group were possibly induced by a minimal cerebral lesion. These results suggest that judgment of positive evidence of stroke should be based on the observation of both stroke signs and cerebrovascular hemorrhages in the SHRSP kept on medication.

Shibota et al30 reported that the incidence of stroke in SHRSP relates to an augmented renin-angiotensin system caused by renovascular damage, because salt-loading in SHRSP results in suppression of plasma renin activity, thereafter histopathological disorders in the renovascular elevation and proteinuria with high plasma renin activity and angiotensin II levels, and, finally, stroke signs. In cases of kidney dysfunction, N-acetyl-β-D-glucosaminidase, an enzyme present in tubular epithelial cells, often leaks into the urine, and thus its presence in the urine can be used clinically as an index of kidney dysfunction. Although proteinuria is reported to occur in the salt-loaded SHRSP,30,31,32 the elevation of N-acetyl-β-D-glucosaminidase activity has not yet been reported. We found that N-acetyl-β-D-glucosaminidase activity was also elevated by salt-loading in SHRSP.

Imidapril ameliorated the renal dysfunction shown by these indexes in the same dose-dependent manner as it prevented stroke. The individual analysis revealed that stroke signs followed severe renal disorder, and rats with low levels of urinary indexes were able to resist stroke for a long period. Thus, the protective effect of imidapril on the kidney preceded that against stroke. ACE inhibitors are known to produce differential effects on efferent and afferent arterioles and to reduce internal pressure in the kidney.31 Imidapril probably ameliorated kidney dysfunction in salt-loaded SHRSP in a manner similar to that of other ACE inhibitors.

The preventive effects of ACE inhibitors against stroke and kidney dysfunction are probably related to both potency and duration of ACE inhibitory activity in both cerebral and renal arteries, since ACE activity in the vascular wall is higher in hypertensive than in normotensive animals.32 High levels of angiotensin II are implicated in vascular damage and hypertensive complications.13,33 It is reported that the ACE inhibitory activity of imidapril in the vasculature lasted more than 24 hours and longer than in plasma.34 Thus, the measurement of ACE inhibitory activity in the vasculature is suitable to clarify the different efficacy of imidapril and enalapril in regard to stroke prevention. Because the first animal with stroke signs was observed after the
second week, we compared ACE activities in the aorta at that time. The ACE inhibitory activity of enalapril was less potent than that of imidapril 24 hours after dosing, corresponding to the potency of the preventive effect against stroke. Considering that ACE inhibition in the aorta could be applied to that in cerebral and renal vessels, suppression of vasculotoxic angiotensin formation could be a factor responsible for protection of the cerebral and renal vascular wall.

After withdrawal, the rats that had been treated with ACE inhibitors did not show the sharp increase in mortality that was observed in the control group in the early stage. These data suggest that chronic ACE inhibition brought about some organic changes that resisted stroke in the vasculature of these rats.

In conclusion, imidapril postponed the onset of stroke and prolonged life span in salt-loaded SHRSP, even if it failed to reduce blood pressure at doses of 0.5 mg/kg per day or more. It appears that this effect of imidapril is primarily due to systemic improvements resulting from the amelioration of kidney dysfunction; the duration of ACE inhibition in the vasculature may play a role in the mechanism underlying this effect.

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It has been known for many years that antihypertensive therapy reduces the risk of stroke in hypertensive patients. The study by Ogiku et al suggests that treatment of stroke-prone hypertensive rats with imidapril or enalapril, which are angiotensin converting enzyme (ACE) inhibitors, or hydralazine reduces the incidence of stroke-related mortality. ACE inhibitors probably protect hypertensive subjects against stroke by reduction in arterial pressure, but the authors suggest that another mechanism may also contribute to protection against stroke.

The authors suggest that ACE inhibitors may reduce the incidence of stroke in SHRSP by an indirect mechanism related in some way to amelioration of renal dysfunction. This putative "renal protective" effect of ACE inhibitors currently is under intensive study.

An alternative possibility is that ACE inhibitors may augment endothelium-dependent relaxation. Many studies (e.g., Reference 1) indicate that endothelial function is impaired by chronic hypertension. Endothelial dysfunction in hypertension may contribute to both vasomotor abnormalities and increased adherence of platelets or leukocytes to endothelium. Several studies (e.g., Reference 2) suggest that ACE inhibitors improve endothelium-dependent relaxation during hypertension even without a reduction in arterial pressure.

The hypothesis that low doses of ACE inhibitors may protect against stroke, even without reduction in blood pressure, is intriguing and potentially important.

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