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Effects of Treatment with Nilvadipine on Cerebral Ischemia in Rats

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KAWAMURA, S., LI, Y., SHIRASAWA, M., YASUI, N. and FUKASAWA, H. *Effects of Treatment with Nilvadipine on Cerebral Ischemia in Rats.* Tohoku J. Exp. Med., 1998, **185** (4), 239-246 —— The protective effects of a Ca^{2+} antagonist, nilvadipine, on focal cerebral ischemia were studied in male spontaneously hypertensive rats. The animals received either nilvadipine ($3\ mg \cdot kg^{-1} \cdot day^{-1}$) or a vehicle subcutaneously. Group 1 ($n=11$) was treated for 7 days, and Group 2 ($n=11$) for 14 days. The middle cerebral artery was occluded on the 6th (Group 1) or 13th (Group 2) day of the treatment, and neuropathological outcomes were quantified 24 hours later. The mean arterial blood pressure was significantly reduced with nilvadipine to normal levels. The % infarct volumes of Groups 1 (37 ± 2) and 2 (34 ± 3) were significantly less than those of their controls (39 ± 3 [$n=11$] and 40 ± 4 [$n=12$], respectively), although the difference between Groups 1 and 2 was not significant. When infarct areas were compared in each of 8 coronal sections, the infarct size had decreased in the 5 posterior sections in Group 2, but only in 2 sections of Group 1. A significant decrease in the edema volumes was observed in Group 2, but not in Group 1. Thus, nilvadipine provided protective effects against cerebral ischemia in rats having chronic hypertension, and the effects were dependent on the duration of treatment. —— antihypertensive therapy; Ca^{2+} entry blocker; focal cerebral ischemia; spontaneously hypertensive rats

Chronic hypertension is one of the risk factors for stroke. Anti-hypertensive drug treatment has been shown to provide protective effects against experimental cerebral ischemia using hydralazine (Slivka 1993), angiotensin converting enzyme inhibitors (Fujii et al. 1992), and Ca^{2+} entry blockers (Sauter and Rudin 1989). These drugs are believed by some investigators to improve cerebral blood flow and metabolism in the ischemic brain (Tamaki et al. 1979; Jacewicz et al. 1990; Veniant et al. 1992).

Ca^{2+} entry blockers have been used widely for stroke research with spontane-

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ously hypertensive rats (SHR). However, the duration of treatment with them has been considerably shorter than that in previous studies using other drugs (about 3 months) (Fujii et al. 1992; Slivka 1993). In addition, to our knowledge there has been no study on whether the protective effects of Ca^{2+} entry blockers on cerebral ischemia are dependent on the duration of treatment.

The purpose of this present study is to investigate the effects of antihypertensive drug treatment with nilvadipine, a dihydropyridine Ca^{2+} entry blocker, on focal cerebral ischemia in SHR. Our intention was to pay special attention to whether or not the protective effects of nilvadipine on cerebral ischemia are dependent on treatment duration, even when a 2 week regimen is shortened to 7 days. We used a rat model of middle cerebral artery (MCA) occlusion (Kawamura et al. 1991), and assessed neuropathologic outcomes.

Materials and Methods

General preparation

Forty-five male SHR (Charles River Japan, Inc., Atsugi), 12 weeks old (230–310 g), with free access to food and water during all procedures, were used. Mean arterial blood pressures were measured without anesthesia by the tail-cuff method, using a Rat Manometer TK350 (Unicom, Inc., Chiba). The measurements were performed three times (before treatment, and on the 6th and 13th days of treatment—before MCA occlusion) for each animal, and mean values were calculated. Body weights were measured again before sacrifice.

Animal groups

Animals were randomly divided into four groups. Group 1 and Control 1 animals (11 rats per group) were treated with nilvadipine and a vehicle, respectively, for 7 days. They received MCA occlusion on the 6th day of the treatment. Group 2 ($n=11$) and Control 2 ($n=12$) animals were treated for 14 days with the drug and vehicle, respectively, and received MCA occlusion on the 13th day of the treatment.

Drug administration

Nilvadipine was dissolved in polyethylene glycol. Group 1 and Control 1 received nilvadipine (4.0 mg/ml) and the vehicle, respectively, administered subcutaneously at a rate of 10 μl/hour, using an osmotic pump (Alzet 2ML1; Alza Corporation, Palo Alto, CA, USA). Group 2 and Control 2 received the drug (8.0 mg/ml) and the vehicle, respectively, at a rate of 5 μl/hour, using another osmotic pump (Alzet 2ML2). The pump was implanted subcutaneously on the back under 1.5% halothane anesthesia. Both Groups 1 and 2 received nilvadipine with a dose approximately 3 mg/kg body weight/day.

Induction of ischemia

For surgical anesthesia, 4% halothane was used for induction, 1.5% halothane for maintenance during skin incision, and 1% halothane for maintenance during MCA occlusion. The animals were allowed to breathe a mixture of N_2O/O_2 (70%/30%) spontaneously. Rectal temperature was kept at 37°C with a heating pad. We occluded the left MCA using the method described previously (Kawamura et al. 1991). The left external carotid artery was ligated and cut. A 3-0 nylon thread was introduced into the proximal stump of the external carotid artery, and advanced into the internal carotid artery. When the tip of the thread entered the proximal segment of the anterior cerebral artery, the MCA origin was occluded by the thread. The skin incision in the neck was closed, and the animals were returned to their cages.

Neuropathology

Twenty-four hours after the MCA occlusion, animals were anesthetized with pentobarbital sodium (50 mg/kg body weight, i.p.), and transcardiac perfusion fixation was performed with 10% formalin. Coronal sections (5 μm thick) were obtained from the forebrains, stained with hematoxylin-eosin, and then examined by light microscopy. Area measurements were carried out at 8 coronal levels, chosen at 1.5 mm intervals, with the first level being 2.5 mm posterior to the frontal tip. Infarct areas in each section were measured separately in the striatum and the pallium (cortex and subcortical white matter) using an image analyzer. Infarct areas for the striatum and pallium were summed to give an infarct area for the hemisphere in each section. The infarct volume was calculated with a computer program by averaging the infarct areas of sequential sections, then multiplying the average by the total interval thickness between sections. The volume of both hemispheres between the 8 levels was also calculated. The infarct volume was expressed both in an absolute term (mm^3) and as a percent of the total cerebral volume (% infarct volume). In an attempt to partially characterize the infarct volume (secondary to swelling due to brain edema), edema volume was obtained by subtracting the volume of the right (non-ischemic) hemisphere from that of the left (ischemic) hemisphere (Brint et al. 1988). Prior to perfusion fixation, venous blood was aspirated from the right atrium of the heart, and the plasma concentration of nilvadipine (pCN) was measured using gas chromatography, as described previously (Kawamura et al. 1991). Nilvadipine was not detected in plasma obtained from control animals.

Statistical analysis

All data were expressed as mean$\pm$S.D. Intra-group comparisons of blood pressure before and after treatment were made using the paired t-test. Inter-group comparisons of blood pressure, infarct and edema volumes, and pCN values

were made using the Student's t-test. When infarct areas from each of the 8 coronal sections were analyzed, Controls 1 and 2 were combined into a single Pooled Control group, because the values of the two groups were similar (data not shown). Infarct areas in each coronal section of the three groups were compared by Scheffe's multiple comparison test. A p-value<0.05 was considered significant.

Results

Mean arterial blood pressure

Blood-pressure changes in each group are summarized in Table 1. The high blood pressure of controls was stable, but nilvadipine reduced the pressure to normal levels (60-to-70 mmHg decreases, $p<0.01$) in both Groups 1 and 2.

Neuropathological outcomes

Ischemic brain damage in each group after MCA occlusion is summarized in Table 2. The total infarct volumes (or the % infarct volumes) decreased in Groups 1 and 2, compared with their own controls ($p<0.05$): This decrease was primarily due to the decrease of pallium-infarction. The difference in the % infarct volumes between Groups 1 and 2 was not significant ($0.05<p<0.06$). Edema volumes of Group 2 were smaller than those of Control 2 ($p<0.05$), although the edema volumes were similar between Group 1 and Control 1. In

Table 1. *Mean arterial blood pressure (mmHg) in unanesthetized spontaneously hypertensive rats*

Group (n)	Control 1 (11)	Group 1 (11)	Control 2 (11)	Group 2 (12)
Pre-treatment	145±7	151±10	156±12	163±12
Post-treatment (Before MCA occlusion)				
Day 6	147±7	90±11**	152±8	94±11**
Day 13	—	—	157±14	98±12**

Values are mean±S.D. n=number of animals. MCA; middle cerebral artery. **$p<0.01$ vs. Pre-treatment (paired t-test), and vs. its own Control (t-test).

Table 2. *Ischemic brain damage 24 hours after middle cerebral artery occlusion*

Group (n)	Striatum (mm^3)	Pallium (mm^3)	Total (mm^3)	Infarct volume (%)	Edema volume (mm^3)
Control 1 (11)	51±7	190±13	241±18	39.0±2.6	60±17
Croup 1 (11)	49±6	174±16*	223±21*	36.6±2.4*	60±18
Control 2 (11)	47±10	195±20	242±27	39.6±3.5	62±13
Group 2 (12)	47±9	165±17**	212±24*	34.3±3.1**	49±15*

Values are mean±S.D. n=number of animals. *$p<0.05$, **$p<0.01$ vs. its own Control (unpaired t-test).

TABLE 3. *Mean ± S.D. infarct areas (mm^2) in the pallium in each coronal section*

Distance from frontal pole	Pooled Control ($n=22$)	Group 1 ($n=11$)	Group 2 ($n=12$)
2.5 mm	9.7±2.3	9.6±3.3	8.8±2.5
4.0 mm	13.7±1.1	13.3±1.4	12.8±1.9
5.5 mm	15.2±1.7	15.5±2.3	14.6±2.4
7.0 mm	18.7±1.5	17.4±1.5	16.7±1.6*
8.5 mm	19.0±1.6	18.0±1.6	17.5±1.8*
10.0 mm	19.2±2.0	17.9±1.6	17.4±1.7*
11.5 mm	19.5±3.5	16.4±2.0*	16.1±1.9*
13.0 mm	17.5±6.1	11.5±2.3*	9.6±3.9*

*$p<0.05$ vs. Pooled Control (Scheffe's test). Pooled Control = Controls 1 and 2.

order to show where the pallium-infarction decreased, infarct areas in the pallium of each coronal section are summarized in Table 3. The infarct areas decreased in the posterior (occipital) periphery of the pallium: The decreases in Group 2 were in 5 posterior sections, while in Group 1 they were in the 2 posterior sections only.

Values of pCN

The pCN of Group 1 (15.2±3.6 ng/ml) was greater than that of Group 2 (10.3±3.5 ng/ml; $p<0.01$). Before sacrifice, the body weight in Group 1 decreased to 256±13 g, and was less than the 283±18 g in Group 2 ($p<0.01$). The weights of the two groups had been similar before treatment (279±15 g for Group 1, and 290±12 g for Group 2), while Group-2 animals gained more weight during the additional 7-day period prior to MCA occlusion-induced weight loss.

DISCUSSION

Nilvadipine is photo-resistant even in solution, and has several distinguishing characteristics when compared to other Ca^{2+} entry blockers. It has more potent and highly selective Ca^{2+} entry blocking activity on the vascular smooth muscle, and exerts a longer-lasting antihypertensive effect with less tachycardia than nifedipine and nicardipine in various models of experimental hypertension (Ohtsuka et al. 1988; Fujii et al. 1992). We have shown previously that nilvadipine reduces infarct size dose-dependently following MCA occlusion in normotensive rats (Kawamura et al. 1991). However, knowledge about whether the drug has the additional benefit of reducing the consequences of a possible later stroke in hypertensive animals is of great value. This is because chronic hypertension is a major risk factor for stroke, and antihypertensive drugs, which have to be taken long-term, would be present at the time of a subsequent stroke onset. Therefore, the present study was performed using SHR with the particular objective to learn whether the protective effects of nilvadipine on cerebral is-

chemia are dependent on the duration of therapy.

Our results demonstrate that antihypertensive drug treatment using nilvadipine improves neuropathological outcomes following MCA occlusion in SHR. Edema volumes decreased in animals treated for 14 days, but not for those treated for 7 days, indicating that improvement in brain swelling was dependent on the duration of therapy. It is probable that brain swelling is mainly due to edema (increase in brain water content) at the acute stage of infarction. Thus, the duration-dependent protective effects against ischemic stroke could be demonstrated even in the absence of a statistically significant difference in infarct size when two-week treatment was shortened to seven days. Nevertheless, a statistical trend ($0.05 < p < 0.06$) in duration dependence in infarct size reduction was also demonstrated in the difference between Groups 1 and 2. Several investigators have shown protective effects of antihypertensive dihydropyridine Ca^{2+} entry blockers on focal cerebral ischemia in SHR, using, for example, isradipine (Sauter and Rudin 1989), nimodipine (Jacewicz et al. 1990), and nilvadipine (Shiino et al. 1991). However, the durations of treatment (< 7 days) are shorter than that in our study. In addition, while differences in the dose and/or timing of the drug-administration after stroke onset have been investigated, the effects of the duration of therapy have not been studied previously.

The plasma concentration of nilvadipine (pCN) in Group 2 was approximately 70% of that in Group 1, in spite of our attempt to apply the same dose for each group. The body-weight difference at the end of the experiment is the likely reason for the pCN difference. It is also possible that the osmotic pump may not have functioned as designed, or may have been surrounded gradually by connective tissue during the 14-day period despite surgical implantation by sterile methods, because it is a foreign body. Based on the results of blood-pressure measurements, however, both groups can be considered to have undergone similar antihypertensive drug treatment (Table 1).

The protective effects of Ca^{2+} entry blockers against ischemic brain injury may be related to inhibition of the Ca^{2+} influx into neuronal cells or the direct action of the drugs. Several possible mechanisms suggested in the literature include that such drugs ameliorate ischemic cerebral damage by affecting cerebral vasoconstriction (Brandt et al. 1983), platelet aggregation (Aragno and Doni 1976), and neuronal membrane and mitochondrial functions (Harris et al. 1981). Kawamura and Yasui (1994) have observed, using a closed cranial window technique in rats, that nilvadipine dilates pial arterioles in vivo. The arteriolar dilation can subsequently increase cerebral blood flow (Jacewicz et al. 1990; Furuichi et al. 1992). Our present study shows that infarct size decreased in the posterior periphery of the pallium. Nilvadipine probably increases the collateral blood flow from the (patent) posterior cerebral artery. The infarct size in the anterior periphery of the pallium (which could be perfused by the collateral flow from the anterior cerebral artery) did not decrease (Table 3). The reason for this

is unclear, but may be related to the fact that both the ipsilateral internal carotid and anterior cerebral arteries were also occluded in our rat model.

Chronic hypertension is known to be associated with a dysfunction of the vascular endothelium, characterized by an increased endothelium-dependent contraction and a decreased endothelium-dependent relaxation (Clozel et al. 1990; Heistad et al. 1990). The endothelial dysfunction may result in a decrease of the cerebral blood flow reserve. It is unknown whether nilvadipine can improve endothelial function. However, because a possible improvement in the cerebral blood flow alone can not explain the duration-dependent effects demonstrated in our present study, our results may encourage researchers to investigate the effects of Ca^{2+} entry blockers on the endothelial function. Arterial hypertension is also associated with hypertrophy of the medial layer of cerebral arteries and arterioles (Harper and Bohlen 1984; Johansson 1984; Baumbach and Heistad 1988). This histological vessel change, in turn, may be associated with changes in the cerebral blood flow. However, a reversal of the vascular morphology can not be expected to explain any results observed in our study, because the duration of therapy we used ($\leqq$2 weeks) was too short to induce such a morphological change. Thus, mechanisms of action of nilvadipine providing for duration-dependent protective effects on cerebral ischemia are still unclear.

Conclusion

Antihypertensive drug treatment with nilvadipine attenuates ischemic brain damage following MCA occlusion in SHR having chronic hypertension. Neuropathological findings indicate that the protective effects of the drug against cerebral ischemia are dependent on the duration of treatment. The results suggest that nilvadipine may reduce the consequences of a possible later stroke in hypertensive patients who receive long-term treatment.

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