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Effects of the Calcium Antagonist AE0047 on the Development of Neurological Deficit and Infarction after Middle Cerebral Artery Occlusion in Stroke-prone Spontaneously Hypertensive Rats

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Abstract

AE0047 [4-(4-benzhydrylpiperazino)phenethyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate dihydrochloride] is a new dihydropyridine calcium antagonist with protective effects against cerebral ischaemia and the occurrence of stroke in several animal models. We investigated the effects of AE0047 on focal ischaemia induced by middle cerebral artery occlusion in stroke-prone spontaneously hypertensive rats.

AE0047 at a dose causing 20 or 40% systemic hypotension (1 or 3 mg kg^{-1}) was given orally twice, 15 min and 24 h after occlusion. The neurological status of animals was investigated 2, 24 and 48 h after occlusion. Infarct area of brain was measured 48 h after occlusion. Middle cerebral artery occlusion resulted in the progressive deterioration of neurological status and large infarction in middle cerebral artery territories with 40% mortality. AE0047 dose-dependently attenuated the deterioration of neurological status, and reduced mortality to 0 or 10%. AE0047 significantly reduced infarct size and left/right hemispheric area ratio, an index of brain swelling.

These results suggest that AE0047 has the ability to ameliorate ischaemic cerebral stroke in hypertensive patients.

Hypertension is not only a major risk factor for stroke, but also an aggravating factor in the progression of brain damage after stroke in cases where ischaemic brain oedema occurs (Phillips & Whisnant 1990). In animals with focal cerebral ischaemia, infarct volume increases in line with blood pressure (Duverger & MacKenzie 1988), probably because collateral blood supply to ischaemic regions is impaired by increased arteriolar resistance resulting from structural or functional alterations of the vessel wall. Although long-term antihypertensive treatment before focal ischaemia has been found to decrease infarct size through the structural normalization of the cerebral vessels (Slivka 1991), it is generally accepted that antihypertensive treatment of the acute stage of stroke could risk reducing cerebral blood flow which might cause further reduction of blood flow in the so-called ischaemic penumbra (Astrup et al 1977; Symon et al 1977). On the other hand, severe hypertension could cause further damage by aggravating oedema, which indicates antihypertensive treatment.

AE0047 [4-(4-benzhydrylpiperazino)phenethyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate dihydrochloride] is a new dihydropyridine calcium antagonist which binds to the isradipine-binding site (Yamanaga et al 1997). AE0047 has a slow onset and long-lasting hypotensive effect in several animal models of hypertension (Ohtaki et al 1989; Uchida et al 1989). It also shows a selective vasodilating action on the cerebral vasculature (Nishikawa et al 1995) and protective effects in some animal models of cerebral ischaemia (Yamanaga et al 1992). In stroke-prone spontaneously hypertensive rats (SHRSP), AE0047 prevents stroke at a dose causing no significant reduction in blood pressure (Shinyama et al 1995). In addition, it has been shown in post-stroke SHRSP to suppress the progression of disease, especially of brain oedema (Shinyama et al 1997). AE0047 could thus be beneficial in the treatment of the acute stage of stroke. In the present study, to find out whether AE0047 has the potential to ameliorate ischaemic stroke, we examined the effects of AE0047 on neurological deficit and infarction in SHRSP subjected to middle cerebral artery occlusion.

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Materials and Methods

Animal preparation

Male SHRSP were purchased from Seiwa Experimental Animals (Fukuoka, Japan) and housed in the same room, given free access to water and fed an SP diet (SP, Funabashi Farm, Chiba, Japan). After surgery, animals were fed a powder SP diet. Animals (n = 30) aged 15–18 weeks were used for the experiment. The animals were initially anaesthetized with 3% halothane in oxygen. During surgery, the animals were placed on a sensor-controlled warming pad (model TH-200, Neuroscience, Tokyo, Japan) and rectal temperature was maintained between 37 and 38°C. The left femoral artery was cannulated to measure blood pressure (AP-601G, Nihon-Kohden, Tokyo, Japan) and heart rate (AT-601G, Nihon-Kohden, Tokyo, Japan) and collect arterial blood. Under anaesthesia with 1·5% halothane, 70% N_2O, and balance

oxygen, the animal was placed in the lateral position. Middle cerebral artery occlusion was performed as described previously (Matsui et al 1993) using an operating microscope (OME-NA, Olympus, Tokyo, Japan). Briefly, to expose the middle cerebral artery, we removed the temporal muscle and zygoma and retracted the jaw downward to allow access to the infratemporal skull. Under a magnification of $\times 20$, the mandibular nerve was followed to the forman ovale. Then, a $2{\cdot}5 \times 2{\cdot}5$ mm craniectomy was made with a microdrill anterior to the forman ovale. The dura was opened with a hooked 27 gauge needle. The left middle cerebral artery was electrocoagulated with bipolar microforceps at the level of the olfactory tract. The artery was divided with microscissors to ensure complete and permanent occlusion. Halothane anaesthesia was stopped, the craniectomy covered with a small piece of gel foam, the soft tissue allowed to fall back into place, and the skin sutured.

Arterial blood was obtained twice, before and 2 h after middle cerebral artery occlusion, to measure haematocrit and to analyse pH, PCO_2 and PO_2 using a pH/blood gas analyser (type 1306, Instrumentation Laboratory, Milan, Italy) and to determine plasma glucose concentration.

Drug treatment

Vehicle (0·03% ethanol and 0·01% Tween 80 in distilled water) or AE0047 (Green Cross, Osaka, Japan) at doses of 1 or 3 mg kg^{-1} was administered orally by gavage twice (15 min and 24 h after occlusion), at a volume of 10 mL kg^{-1}. The doses (1 and 3 mg kg^{-1}) of AE0047 were chosen with reference to previous studies which indicated their effectiveness in preventing and treating stroke in SHRSP (Shinyama et al 1995, 1997). At these two doses, maximum drop in blood pressure is found 2 h after oral administration.

Neurological status

Neurological status was evaluated three times (2, 24 and 48 h after occlusion), according to the method of Bederson et al (1986) with slight modifications: grade 0, no observable deficit; grade 1, forelimb flexion; grade 2, decreased resistance to lateral push and forelimb flexion; grade 3, same behaviour as grade 2, with circling toward the paretic side, or disappearance of response to lateral push; grade 4, death.

Histopathology

Forty-eight hours after middle cerebral artery occlusion, animals were anaesthetized with intraperitoneal injection of pentobarbital sodium (40 mg kg^{-1}). Thoracotomy was performed and the brain was perfused from the left ventricle of the heart with 150 mL of heparinized saline (pH 7·3), then fixed by perfusion with 200 mL of 4% phosphate-buffered formaldehyde (pH 7·3). The brain was removed and postfixed for at least 7 days in the same solution. The brains were coronally sectioned into 6 slices parallel to the plane of the optic chiasm at intervals of 2 mm. The second section was made at the optic chiasm. The slices were embedded in paraffin and further cut into pieces of 5-μm thickness and stained with haematoxylin and eosin. For each of the 5 coronal planes, total area (both hemispheres), area of left and right hemispheres and infarct area were determined under a CCD camera with low power magnification using an image analyser (MCID, Imaging Research, Ontario, Canada). The ratio of infarct area to total area, and the left (affected)/right hemispheric area ratio were calculated in each animal. As shown in Fig. 1, the anterior planes from the second to sixth slices were designated Planes 1, 2, 3, 4 and 5, respectively.

Statistical analysis

Vehicle-treated and AE0047-treated groups of SHRSP were compared by analysis of variance followed by Dunnett's method for multiple comparison. Neurological status between the groups was analysed by Steel's method. Results were considered significant at $P < 0{\cdot}05$. All data are expressed as means ± s.e.m.

Results

Physiological variables

Table 1 shows mean arterial blood pressure, heart rate, arterial blood gases and plasma glucose concentration immediately before and 2 h after middle cerebral artery occlusion. Immediately before occlusion, no significant differences were observed in any variable in any of the 3 groups. At 2 h after occlusion, AE0047 at doses of 1 and 3 mg kg^{-1} significantly reduced mean arterial blood pressure (by 20 and 40%, respectively). Variables of arterial blood gases were not significantly different among the 3 groups. AE0047 dose-dependently and significantly (at 3 mg kg^{-1}) reduced haematocrit.

Table 1. Physiological variables immediately before and 2 h after middle cerebral artery occlusion (MCA) in stroke-prone spontaneously hypertensive rats.

Treatment	(n)	MABP (mmHg)	HR (beats min^{-1})	Arterial blood PCO2 (mmHg)	Arterial blood PO2 (mmHg)	Arterial blood pH	Arterial blood Haematocrit (%)	Plasma glucose (mg dL^{-1})
Immediately before MCA occlusion (under halothane anaesthesia)								
Vehicle	(10)	160 ± 6	323 ± 11	48·1 ± 1·6	114 ± 8	7·33 ± 0·01	43 ± 2	196·7 ± 14·0
AE0047 1 mg kg^{-1}	(10)	161 ± 4	321 ± 8	46·5 ± 2·7	129 ± 6	7·32 ± 0·02	45 ± 1	180·7 ± 7·2
AE0047 3 mg kg^{-1}	(10)	163 ± 6	318 ± 11	46·5 ± 2·5	128 ± 7	7·32 ± 0·01	45 ± 1	173·6 ± 13·1
Two hours after MCA occlusion								
Vehicle	(10)	190 ± 6	416 ± 14	37·9 ± 1·7	104 ± 3	7·40 ± 0·01	48 ± 1	N.D.
AE0047 1 mg kg^{-1}	(10)	153 ± 9**	432 ± 12	37·6 ± 1·2	106 ± 2	7·40 ± 0·01	45 ± 2	N.D.
AE0047 3 mg kg^{-1}	(10)	113 ± 9**	437 ± 9	35·7 ± 1·8	112 ± 2	7·42 ± 0·01	42 ± 2*	N.D.

Values are means ± s.e.m. MABP, mean arterial blood pressure; HR, heart rate; N.D., not determined. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ compared with the value for vehicle-treated animals (Dunnett's method).

Table 2. Changes in neurological status of animals 2, 24 and 48 h after middle cerebral artery occlusion.

Treatment	Dose (mg kg^{-1})	n	Neurological status				
			0	1	2	3	4
2 h							
Vehicle		10	4	6	0	0	0
AE0047	1	10	5	5	0	0	0
AE0047	3	10	5	5	0	0	0
24 h							
Vehicle		10	2	1	3	3	1
AE0047	1	10	1	5	3	1	0
AE0047	3	10	4	1	4	1	0
48 h							
Vehicle		10	0	1	2	3	4
AE0047	1	10	0	1	4	5	0
AE0047	3	10	0	5	3	1	1

Vehicle, AE0047 1 mg kg^{-1} or AE0047 3 mg kg^{-1} was administered orally twice, 15 min and 24 h after occlusion. Grade 0, no observable deficit; grade 1, forelimb flexion; grade 2, decreased resistance to lateral push and forelimb flexion; grade 3, same behaviour as grade 2, with circling toward the paretic side, or disappearance of response to lateral push; grade 4, death. In the experiment with 3 mg kg^{-1} AE0047, there was a significant change in neurological status ($P < 0.05$) compared with vehicle treatment.

Neurological deficit

Table 2 shows changes in neurological status 2, 24 and 48 h after middle cerebral artery occlusion. At 2 h, there were no significant differences in the neurological status of animals in the 3 groups. In vehicle-treated animals, neurological status gradually worsened and 40% of animals were dead within 48 h. In contrast, treatment with AE0047 at doses of 1 and 3 mg kg^{-1} tended, though not significantly, to ameliorate neurological status 24 h after occlusion, and at 48 h, 3 mg kg^{-1} AE0047 significantly improved status. Only one animal was dead at 48 h.

Histopathological findings

Fig. 1 shows the schematic representation of the infarct area in the 5 coronal planes. In vehicle-treated animals, ischaemic damage was mainly located within the territory of the occluded middle cerebral artery, that is, in the frontoparietal (Planes 1–3), temporal (Plane 4) and occipital (Plane 5) cortex, and in the caudate putamen (Planes 1 and 2). AE0047 significantly reduced the infarct area of the frontal cortex (Planes 2 and 3), the caudate putamen (Plane 2) and the parietotemporal cortex (Plane 3). Quantitative data on total area (both hemispheres), infarct area, infarct area/total area and left/right hemispheric area ratio for the 5 coronal planes is shown in Table 3. There were no significant differences in the total area among the 3 groups. AE0047 significantly reduced infarct area and infarct area/total area in Planes 2, 3 and 4, and showed a tendency to reduce these values in Planes 1 and 5. Left/right hemispheric area ratio in vehicle-treated animals was over 1·0 in all except Plane 1, indicating the presence of brain swelling secondary to ischaemia. AE0047 tended to reduce left/right ratio in all coronal planes, but significantly so only in Plane 3.

Discussion

SHRSP show a high rate of both small-vessel and large-vessel disease, which result, respectively, in haemorrhagic and ischaemic (atherothrombotic) stroke. The cerebral lesions seen in the animal are similar to those seen in humans (Yamori et al 1976). It has also been indicated that this animal is particularly vulnerable to focal cerebral ischaemia because of decreased collateral supply and enhanced expansion of brain oedema, as seen in hypertensive patients (Duverger & MacKenzie 1988). This animal model was therefore considered suitable for evaluating the pathogenesis of stroke and improved therapies for stroke in hypertension.

Considerable evidence indicates that abnormal influx of Ca^{2+} into neuronal cells through calcium channels is a major trigger and a cause of ischaemic neuronal damage and that calcium antagonists are beneficial in the treatment of cerebral ischaemia (Siesjö & Bengtsson 1989; Lipton 1991). Most research into the possible mechanisms by which calcium antagonists prevent ischaemic brain damage has focussed on their cerebrovascular effects, i.e. facilitation of collateral blood supply through potent vasodilating action on the cerebral vasculature, and blocking action against abnormal Ca^{2+} influx into neurons through calcium channels (Meyer et al 1987; Siesjö & Bengtsson 1989; Lipton 1991). However, calcium antagonists have not been widely used for therapeutic purposes because they could aggravate brain oedema by increasing blood flow in cases where the blood-brain barrier function or ion homoeostasis of the membrane is disturbed (Harris et al 1982; Johshita et al 1985); they could also further reduce blood flow in ischaemic regions through a phenomenon known as intracerebral steal caused by strong vasodilating action (Vorstrup et al 1986).

In this study, middle cerebral artery occlusion in SHRSP resulted in the development of serious neurological deficit, with infarct in the caudate putamen and frontoparietal, temporal and occipital cortex larger than in normotensive animals (Bederson et al 1986). Neurological deficit in animals receiving vehicle was slight at 2 h after occlusion, but gradually progressed to serious levels resulting in a 40% death-rate within 48 h. Postischaemic administration of AE0047 at doses causing maximally 20 or 40% blood pressure reduction dose-dependently and significantly attenuated neurological deterioration. Histological examination revealed that AE0047 significantly reduced infarct size, which accounts for the clear

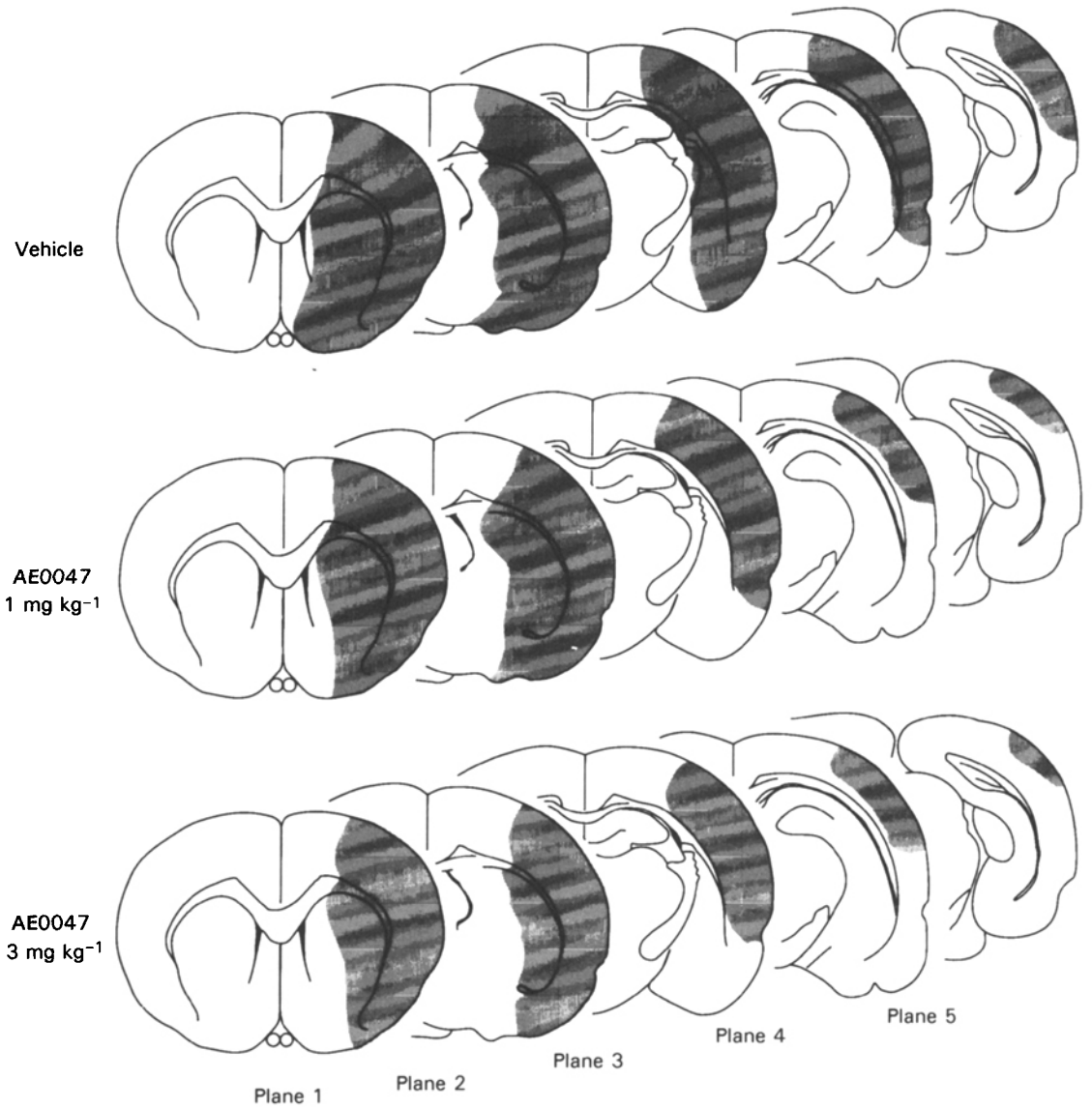

FIG. 1. Schematic representation of infarct area in the 5 coronal planes in SHRSP subjected to middle cerebral artery occlusion. Planes 1, 2, 3, 4 and 5 indicate planes at −2, 0, +2, +4 and +6 mm backward from the optic chiasm, respectively. Shaded areas show averaged infarct area. n = 10.

attenuation of neurological deterioration. These results indicate that the calcium antagonist AE0047 could have therapeutic effects on ischaemic cerebral stroke in hypertensive subjects.

In focal cerebral ischaemia, arterioles show heterogeneous response to the degree of residual blood flow (Brandt et al 1983; Meyer et al 1988). In severely ischaemic regions, after the initial vasodilation due to extracellular acidosis, more prolonged vasoconstriction is brought on by the rise in extracellular K^+, which prevents sufficient collateral blood supply to ischaemic regions. Since this vasoconstriction is reversed by calcium antagonists, it is most likely mediated by Ca^{2+} influx through calcium channels and subsequent Ca^{2+} accumulation in vascular smooth muscle cells (Brandt et al 1983; Meyer et al 1988). Another lipophilic calcium antagonist, nimodipine, is reported to distribute well in ischaemic tissue and to bind to calcium channels specifically using in-vivo binding technique (Hogan et al 1991). Since the lipophilicity of AE0047 is comparable to that of nimodipine (Yamanaga et al 1997), AE0047 may distribute in ischaemic regions and bind to the calcium channels of vascular smooth muscles, thereby relaxing the constricted arterioles. In cats subjected to middle cerebral artery occlusion, postischaemic administration of AE0047 at a dose causing 10% systemic hypotension does indeed increase cerebral blood flow in ischaemic penumbra regions (Shinyama et al 1994). In addition, AE0047 dose-dependently reduces haematocrit, which may result in the reduction of blood viscosity, although the mechanism involved is uncertain at present. Thus, AE0047 may improve cerebral blood flow in ischaemic regions through vasorelaxative or haemorheological actions, or both, thereby maintaining energy metabolism in neurons and reducing infarct size with accompanying neurological amelioration.

Ischaemia- or hypoxia-induced energy failure of neurons disrupts ion homoeostasis and is followed by an abnormal increase in intracellular Ca^{2+} (Ca^{2+} overload), which leads to neuronal death (Siesjö & Bengtsson 1989). In addition to beneficial effects on cerebral circulation, some calcium antagonists protect against neuronal necrosis directly by inhi-

Table 3. Total area, infarct area, infarct area/total area and left/right hemispheric area ratio in the 5 coronal planes of the rat brain 48 h after middle cerebral artery occlusion.

Treatment	Dose (mg kg^{-1})	(n)	Total area (mm^2)	Infarct area (mm^2)	Infarct/total (%)	Left/right
Plane 1						
Vehicle		(10)	62·8 ± 3·2	20·4 ± 1·6	32·7 ± 2·4	0·96 ± 0·05
AE0047	1	(10)	60·3 ± 1·6	19·1 ± 0·8	32·0 ± 1·5	0·99 ± 0·02
AE0047	3	(10)	62·8 ± 1·0	16·8 ± 1·6	27·0 ± 2·7	0·95 ± 0·03
Plane 2						
Vehicle		(10)	83·8 ± 3·1	25·8 ± 0·9	31·0 ± 1·1	1·05 ± 0·03
AE0047	1	(10)	82·4 ± 0·8	20·3 ± 1·6	24·8 ± 2·0	1·01 ± 0·02
AE0047	3	(10)	81·8 ± 1·7	15·2 ± 2·3**	18·9 ± 2·8**	0·98 ± 0·02
Plane 3						
Vehicle		(10)	98·8 ± 3·7	25·8 ± 2·1	26·4 ± 2·2	1·09 ± 0·02
AE0047	1	(10)	94·8 ± 1·4	13·7 ± 2·4**	14·7 ± 2·7**	1·06 ± 0·02
AE0047	3	(10)	96·0 ± 1·6	12·1 ± 2·8**	12·8 ± 3·0**	1·00 ± 0·01**
Plane 4						
Vehicle		(10)	99·8 ± 3·0	16·6 ± 2·3	16·9 ± 2·4	1·11 ± 0·03
AE0047	1	(10)	96·7 ± 2·1	8·0 ± 2·2*	8·4 ± 2·3*	1·07 ± 0·02
AE0047	3	(10)	100·9 ± 2·6	8·0 ± 2·1*	7·9 ± 2·0*	1·04 ± 0·02
Plane 5						
Vehicle		(10)	96·0 ± 2·2	6·4 ± 1·2	6·6 ± 1·2	1·08 ± 0·04
AE0047	1	(10)	95·1 ± 2·1	3·3 ± 1·3	3·4 ± 1·3	1·06 ± 0·02
AE0047	3	(10)	92·8 ± 4·2	3·0 ± 0·9	3·1 ± 0·9	1·06 ± 0·02

Values are means ± s.e.m. Vehicle, AE0047 1 mg kg^{-1} or AE0047 3 mg kg^{-1} was administered orally twice (15 min and 24 h after occlusion). Infarct/total, infarct area/total area; left/right, left/right hemispheric area ratio. *$P < 0.05$, **$P < 0.01$ compared with animals treated with vehicle (ANOVA followed by Dunnett's method).

biting Ca^{2+} influx, thereby limiting the extent of the infarction (Siesjö & Bengtsson 1989). It is, however, still unclear whether the neuronal protection conferred by AE0047 is attributable to the inhibition of Ca^{2+} overload in ischaemic/hypoxic cells by the blockade of calcium channels in neuronal or glial cells. To clarify these issues further studies are required.

In chronic hypertension, adaptive structural changes in the cerebral vessels, such as hypertrophy and remodelling, cause an upward shift in the range of cerebral blood flow autoregulation (Strandgaard 1976). In addition, autoregulatory capacity is impaired in severely ischaemic regions (Symon et al 1976; Dirnagl & Pulsinelli 1990), so that lowering of blood pressure could result in further reduction in cerebral blood flow in penumbra regions and thus the expansion of infarction. It might therefore be thought that AE0047-induced hypotensive action would further aggravate cerebral ischaemia, and indeed 3 mg kg^{-1} AE0047 appeared to reduce blood pressure sufficiently to take it below the lower limit of autoregulation. In our data, however, high-dose AE0047 caused neither deterioration of animals' condition nor expansion of infarct size. Since neurological status had not deteriorated 2 h after occlusion – the point at which blood pressure reduction was considered to peak – it seems likely that blood supply to ischaemic regions was not impaired.

On the other hand, hypertension enhances extravasation of blood constituents after 4–6 h of ischaemia. Histopathological examination also revealed that the left/right hemispheric area ratio in animals treated with vehicle was over 1·0, suggesting the presence of brain swelling secondary to ischaemia. Dysfunction of the blood-brain barrier has been demonstrated in experimental hypertension and in ischaemic brain tissue (Ito et al 1979; Kogure et al 1981). Brain oedema causes progressive microcirculatory compression, leading to aggravated primary ischaemic impact (Schuier & Hossmann 1980). In this model, brain oedema expands ischaemic lesions, leading to elevated intracranial pressure with herniation as the cause of death (Ohta et al 1986). In our current data, AE0047 showed a tendency to decrease, though significantly only in Plane 3, the left/right hemispheric area ratio, suggesting that it has a preventive effect against the development of brain oedema secondary to ischaemia. We speculate that the prolonged hypotensive effect of AE0047 accounts at least partly for its therapeutic effect in stroke by ameliorating the oedema linked to ischaemia or herniation through reduction of cerebral perfusion pressure and intracranial pressure.

The calcium antagonist flunarizine has been found to lower the increased permeability of Evans blue dye into the parenchyma elicited by arterial hypertension without significantly affecting blood pressure, therefore suggesting a direct action on cerebral vascular endothelial cells (Nag 1991). In contrast, neither nifedipine nor nimodipine has been demonstrated to have such a preventive effect against increased vascular permeability (Edvinsson et al 1983). We found that a single oral administration of AE0047 at 1 or 3 mg kg^{-1} suppressed the increased transfer of ^{125}I-labelled albumin into the parenchyma of the cortex of post-stroke SHRSP (unpublished observations). Since AE0047 has a structurally similar moiety to flunarizine, some mechanism(s) other than hypotensive effect may be involved in the ameliorating effect of AE0047 on brain oedema, especially in the vascular endothelium.

In conclusion, our results indicate that postischaemic administration of AE0047 attenuates neurological dysfunction and reduces infarct size after ischaemic stroke in hypertensive subjects. In addition, AE0047 appears neither to aggravate brain oedema associated with ischaemia, nor to further expand infarct area in ischaemic penumbra regions through the intracerebral steal phenomenon. AE0047 may have potential to remedy brain damage in cases where ischaemic cerebral stroke occurs during antihypertensive treatment.

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