Protective Effects of Benidipine on Arachidonic Acid-Induced Acute Cerebral Ischemia in Rats

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ABSTRACT — Acute cerebral ischemia was produced in rats by injection of arachidonic acid (AA) into the internal carotid artery. Evans blue (EB) was intravenously injected and its extravasation into the brain was determined as an indicator of disturbances in the blood-brain barrier and endothelial cells. Control animals showed severe cerebral edema and marked blue staining of the brain. Benidipine (30 μg/kg, i.p.) suppressed the increase in cerebral water content and the extravasation of EB. Similarly, nicardipine (100 μg/kg, i.p.) suppressed the elevation of water content and the extravasation of EB. Furthermore, both benidipine (30 μg/kg, i.p.) and nicardipine (100 μg/kg, i.p.) improved the neuronal injuries following AA-injection. An antiplatelet agent, ticlopidine (100 mg/kg, i.p.), and a thromboxane A_2 synthetase inhibitor, OKY-1581 (3 mg/kg, i.p.), also suppressed the elevation of cerebral water content. A lipoxygenase inhibitor, AA-861 (200 mg/kg, p.o.), and a cyclooxygenase inhibitor, indomethacin (10 mg/kg, i.p.), did not prevent the increase in cerebral water content. Neither benidipine (3–30 μg/kg, i.v.) nor nicardipine (100 μg/kg, i.v.) inhibited the $AgNO_3$-induced thrombus formation of the abdominal aorta, whereas ticlopidine (100 mg/kg, p.o.) and OKY-1581 (3 mg/kg, i.v.) prevented the thrombus formation. From the present results, it is suggested that benidipine, as well as nicardipine, may protect against AA-induced acute cerebral infarction via a mechanism independent of antithrombotic action.

Keywords: Benidipine, Arachidonic acid, Cerebral ischemia (acute), Nicardipine, Cerebral edema

It has recently been reported that calcium antagonists have ameliorating effects on cerebral damages in various animal models of acute cerebral ischemia (1–4). Clinically, nicardipine and nimodipine have been reported to be beneficial in patients with subarachnoid hemorrhage and to reduce the ischemic damage resulting from reduction of cerebral blood flow (5). Arachidonic acid (AA) is one of the polyunsaturated fatty acids which are present in membrane phospholipids. It is a potent stimulator of platelet aggregation and is known to induce endothelial damage and edema in the brain (6–9). Thus, the cerebral infarction induced by the infusion of AA into the internal carotid artery has been used as an experimental thrombo-embolic stroke model. However, the morphological changes and the mechanisms involved have not been fully clarified in the AA-induced cerebral infarction.

It has been reported that antiplatelet agents have ameliorating effects on AA-induced cerebral damage in rabbits (10, 11). In contrast, there are few reports concerning the effect of calcium antagonists on AA-induced cerebral damage (12). Benidipine is a newly-developed 1,4-dihydropyridine calcium antagonist (13) that has long-lasting antihypertensive and antianginal activities (14, 15). In the present study, we investigated the possible protective effects of benidipine on AA-induced acute cerebral infarction in rats, and also examined whether platelet aggregation is related to the pathogenesis of cerebral infarction induced by AA.

MATERIALS AND METHODS

Animals

Male Wistar rats, weighing 250–300 g, were used for the experiment. Prior to the experiment, all animals had free access to standard rat chow and water.

AA-induced cerebral infarction

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital at 50 mg/kg. A polyethylene cannula was inserted from the right external carotid artery to the origin of the internal carotid artery. At a fixed time after the treatment with a test compound, 0.5 mg/kg of AA was injected rapidly through the cannula. Ca-antagonists such as benidipine and nicardipine, OKY-1581 or indomethacin were peritoneally injected at 30 min before the injection of AA. AA-861 and ticlopidine were orally administered at 2 and 4 hr before the injection of AA, respectively.

Cerebral water content

Three hours after the injection of AA, the animal was sacrificed by decapitation. The whole brain was removed from the skull, and the wet weight (W) of the left and right cerebral hemisphere was measured. Both cerebral hemispheres were then dried in an oven heated at 90°C for 3 days and then weighed again to obtain the dry weight (D). The cerebral water content was calculated as follows:

$$\text{Cerebral water content } (\%) = (W - D)/W \times 100$$

Extravasation of EB

In some experiments, 5 min after the AA-injection, Evans blue (EB) (2% in saline, 0.1 ml per 100 g body weight) was injected intravenously. Three hours after the injection of AA, the animal was sacrificed. The whole brain was removed from the skull, and the wet weight (W) of the left and right cerebral hemisphere was measured. Each cerebral hemisphere was dissolved in 5 ml of 1 N KOH, to which 9 ml of phosphoric acid-acetone mixture was added, mixed and centrifuged at $1000 \times g$ for 10 min to extract the dye. The absorbance of the supernatant was determined at the wavelength of 620 nm using a spectrophotometer, and the amount of dye-leakage was determined from a previously prepared calibration curve.

In another series of experiments, the extravasation of EB was determined in rats not injected with AA, in order to examine the effects of AA and the drug per se.

Histological examination

For histologic analysis, 6 to 13 rats of each group were prepared. Thirty minutes after the administration of benidipine (30 μg/kg, i.p.) and nicardipine (100 μg/kg, i.p.), AA was injected intravenously. Three hours after the injection of AA, the cerebrum of each rat was removed and fixed in buffered 10% formaldehyde (pH 7.25). The cerebrum was cut coronally into 2–3 mm slices including the corpus mamill, and then each slice was embedded in paraffin using a standard technique. The paraffin sections were stained with hematoxylin and eosin to determine the quality and extent of cerebral lesions. Cerebral lesions were assessed using the following grading criteria: +, focal and mild changes; ++, more extensive but not diffuse and moderate changes; +++, diffuse and moderate changes; ++++, diffuse and severe changes.

Experimental thrombosis in rats

Thrombus was produced in rats by a slight modification of the method described by Zimmermann et al. (16). Under sodium pentobarbital (50 mg/kg, i.p.) anesthesia, the abdominal aorta and jugular vein were carefully cleared of surrounding connective tissues. The jugular vein and femoral artery were cannulated for injection of either vehicle or drug and for recording femoral arterial blood pressure, respectively. Vessel wall lesions were induced by perivascular application of a solution of 30% silver nitrate ($AgNO_3$) for 4 min. For standardization, the vessel was kept in a plastic grove (10-mm length). The injury to the vessel wall was stopped by washing out the artery with saline. The thrombotic occlusion of the abdominal aorta decreased the blood pressure of the femoral artery almost to 0 mmHg. Test compounds, except for ticlopidine, were intravenously injected 5 min before exposing the artery to $AgNO_3$. Ticlopidine was orally administered 4 hr before exposing the artery to $AgNO_3$.

Drugs

Benidipine (hydrochloride, KW-3049), nicardipine (hydrochloride), OKY-1581 (17), a thromboxane A_2 synthetase inhibitor (TXSI) and AA-861 (18), a 5-lipoxygenase inhibitor, were synthetized in our laboratories. Indomethacin and AA (sodium salt) were purchased from Sigma Chemical Co. Ticlopidine was purchased from Ricerchimica. Benidipine and nicardipine were dissolved in saline containing 5% Tween-80, and OKY-1581 and indomethacin were dissolved in 0.05 N NaOH-saline. The stock drug solutions were then diluted with saline for intravenous or intraperitoneal administrations, so that the injection volume would be 0.1 ml solution per 100 g of animal's body weight. For oral administration, ticlopidine and AA-861 were suspended in 0.3% carboxymethyl cellulose solution so that the administration volume would be 1 ml suspension per 100 g of animal's body weight. Since indomethacin at a dose of 2 mg/kg (i.v.) is reported to inhibit platelet aggregation ex vivo (19), a dose of 10 mg/kg (i.v. or i.p.) was used in this experiment.

Statistical method

Data of brain water content and Evans blue extravasation are expressed as means ± S.E.M. Statistical significance of these data was determined by Duncan's test. Thrombosis data were evaluated by the χ^2 test. Histological data was evaluated by Mann-Whitney's *U*-test. P values of 0.05 or less were considered to indicate statistically significant differences.

RESULTS

Brain water content

Time course of the elevation of water content in the right hemisphere following AA-injection is shown in Fig. 1. The cerebral water content gradually increased with time after AA-injection, and the peak response was attained after 4 to 6 hours. Benidipine (30 μg/kg, i.p.) inhibited the increase in cerebral water content, the effect being statistically significant (Table 1). Similarly, nicardipine (100 μg/kg, i.p.) had an ameliorating effect on the increase in cerebral water content. Ticlopidine (100 mg/kg, p.o.) and OKY-1581 (3 mg/kg, i.p.) also inhibited the elevation of cerebral water content (Table 2). In contrast, the cyclooxygenase inhibitor in-

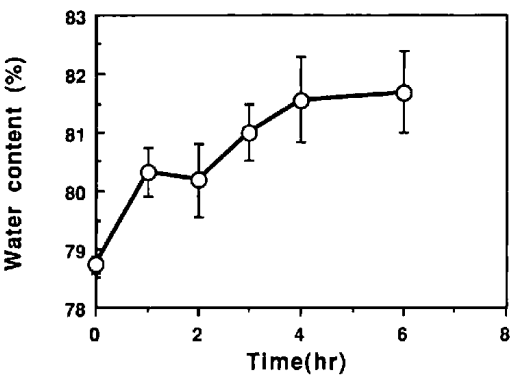

Fig. 1. Time course of changes in right cerebral water content of rats after the injection of AA. All values are means ± S.E.M of 6 rats.

Table 1. Effects of benidipine and nicardipine on cerebral water content 3 hr after the injection of arachidonic acid

Drugs	Dose (μg/kg)	Route	(N)[a]	Cerebral water content (%) left	Cerebral water content (%) right
Normal	–	i.p.	(6)	78.73 ± 0.16	78.83 ± 0.10
Control	–	i.p.	(5)	78.67 ± 0.50	81.92 ± 0.66[b]
Benidipine	30	i.p.	(5)	78.55 ± 0.36	79.41 ± 0.56**
Nicardipine	100	i.p.	(5)	79.05 ± 0.22	80.67 ± 0.24*

Drugs were administered 30 min before the injection of arachidonic acid (AA). The rats in normal and control groups were given the vehicle without and with the injection of AA, respectively. Values are expressed as the mean ± S.E.M. [a]: Number of animals examined. [b]: $P < 0.01$ vs. left cerebral water content. *and** : $P < 0.05$ and $P < 0.01$ vs. control group, respectively.

Table 2. Effects of various drugs on cerebral water content 3 hr after the injection of arachidonic acid

Drugs	Dose (mg/kg)	Route	(N)[a]	Cerebral water content (%) left	Cerebral water content (%) right
Normal	–	p.o.	(6)	78.73 ± 0.15	78.88 ± 0.00
Control	–	p.o.	(6)	79.13 ± 0.27	81.64 ± 0.10[b]
Ticlopidine	100	p.o.	(5)	78.64 ± 0.10	80.89 ± 0.23*
OKY-1581	3	i.p.	(5)	78.87 ± 0.22	80.48 ± 0.27*
AA-861	200	p.o.	(5)	79.15 ± 0.46	82.04 ± 0.24
Indomethacin	10	i.p.	(5)	79.12 ± 0.70	82.32 ± 0.12

Ticlopidine and AA-861 were administered 4 hr and 2 hr before the injection of arachidonic acid (AA), respectively. OKY-1581 and indomethacin were administered 30 min before the injection of AA. The rats in the normal and control groups were given vehicle without and with the injection of AA, respectively. Values are presented as the mean ± S.E.M. [a]: Number of animals examined. [b]: $P < 0.01$ vs. left cerebral water content. *: $P < 0.05$ vs. control group.

domethacin (10 mg/kg, i.p.) and the lipoxygenase inhibitor AA-861 (200 mg/kg, p.o.) had no effect on the increase in cerebral water content.

Effects on extravasation of EB

When AA was not injected, the extravasations of EB were 0.40 ± 0.07 μg/brain, 0.38 ± 0.11 μg/brain and 0.37 ± 0.05 μg/brain in the vehicle-, nicardipine (300 μg/kg, i.p.)- and benidipine (100 μg/kg, i.p.)-treated groups, respectively. These values were not significantly different among these three groups and were very low compared with that of the control group injected with AA (56.31 ± 2.79 μg/brain).

Benidipine (3–100 μg/kg, i.p.) decreased the extravasation of EB following AA-injection in a dose-dependent manner. The inhibitory effects of benidipine were statistically significant at doses of 10 μg/kg and greater. While nicardipine at a dose of 100 μg/kg prevented the extravasation of EB, 300 μg/kg of it did not (Fig. 2). The protective potency of benidipine of AA-induced extravasation of EB was approximately 3 times higher than that of nicardipine. Ticlopidine (100 mg/kg, p.o.) and OKY-1581 (3 mg/kg, i.p.) also inhibited the extravasation of EB (data not shown).

Histological examination

Three hours after the injection of AA, the neuronal neurons were observed in the cerebral cortex, hippocampus, thalamus and hypothalamus. The lesions included congestion of vessels, perivascular edema, extravasated hemorrhage, edematous and spongiose neuropil, and cell shrinkage or karyopyknosis of neurons. Some lesions were observed even in the neocortex of the hemisphere contrarateral to that injected with AA. The benidipine (30 μg/kg, i.p.)-treated group exhibited less neuronal injury compared with the control group (Figs. 3 and 4). The treatment with nicardipine (100 μg/kg, i.p.) also ameliorated the lesions similarly to that with benidipine (Table 3).

Effects on $AgNO_3$-induced thrombus formation

Within 60 min following $AgNO_3$-application, the abdominal aorta was almost completely filled with thrombi and the fall of femoral arterial blood pressure was detected in all rats of the control group. As shown in Table 4, neither benidipine (10, 30 μg/kg, i.v.) nor nicardipine (100 μg/kg, i.v.) inhibited the thrombus formation in the abdominal aorta. While ticlopidine (100 mg/kg, p.o.) and OKY-1581 (3 mg/kg, i.v.) prevented the thrombus formation, indomethacin (10 mg/kg, i.v.) did not.

DISCUSSION

AA is a potent inducer of platelet aggregation and has been demonstrated to produce cerebral infarction in rabbits and rats when injected into the carotid artery (6–8, 20). There have been several reports indicating that antiplatelet agents exert beneficial effects in this model (10, 11, 21). The interesting finding of the present study is that the Ca-antagonist benidipine, which did not show any antithrombotic effects, exhibited beneficial effects of AA-induced cerebral infarction. Therefore, the mechanism for the protective effects of benidipine on AA-induced cerebral infarction seems to

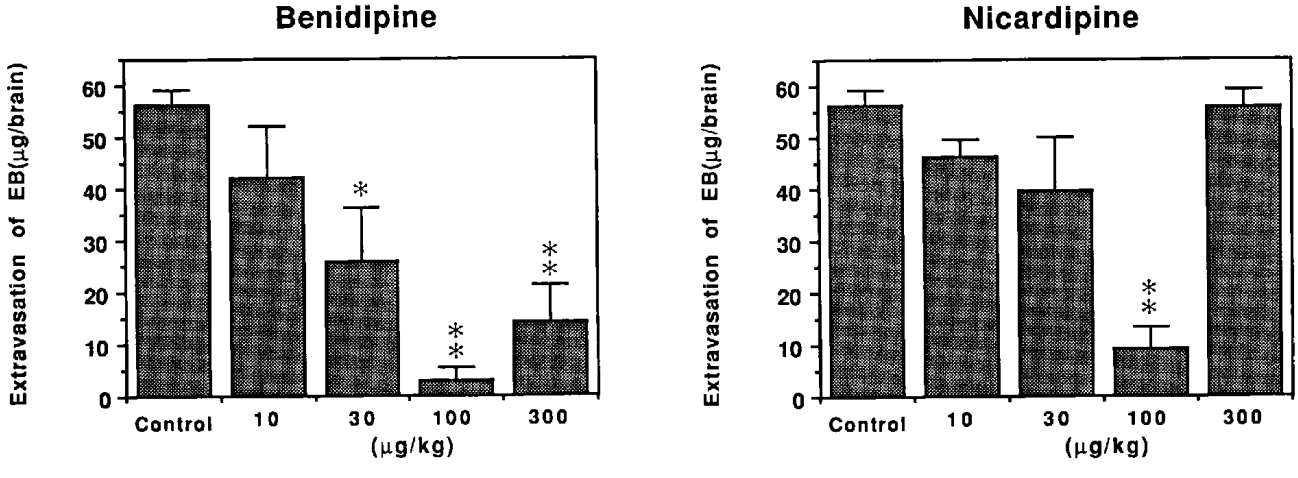

Fig. 2. Effects of benidipine and nicrdipine on extravasation of EB 3 hr after the injection of AA in rats. The drugs were administered 30 min before the injection of AA. Column heights represent the mean values ± S.E.M of 6 rats. *and**: significantly different from the control at $P < 0.05$ and $P < 0.01$, respectively.

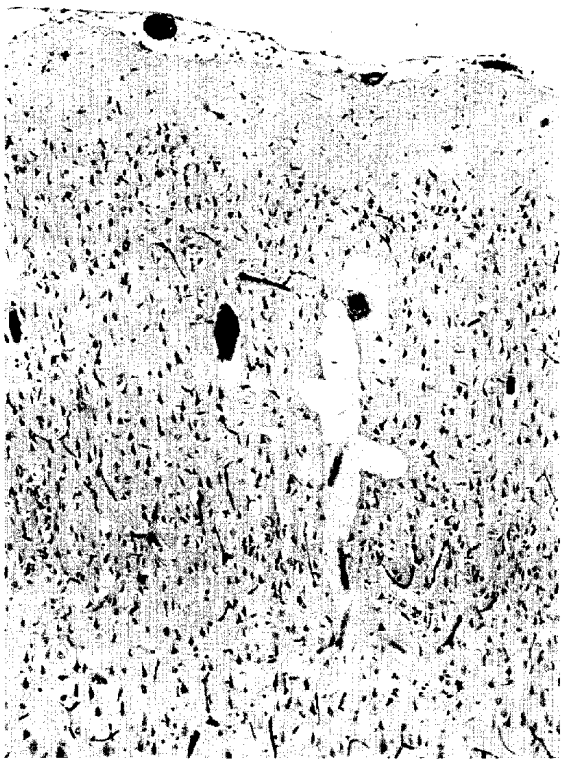

Fig. 3. Photomicrograph of the neocortex of the cerebrum from a control rat challenged with arachidonic acid. Cerebral lesions charactrized by cell shrinkage, karyopyknosis of neurons, perivascular edema and congestion are apparent.

Fig. 4. Photomicrograph of the neocortex of the cerebrum from a rat treated with benidipine (30 μg/kg, i.p.), followed by the challenge with arachidonic acid. Note that the cerebral lesions are ameliorated by the pretreatment with benidipine.

be different from those of an antiplatelet agent like ticlopidine.

In our preliminary electronic microscopic study, intravascular platelet thrombus formation associated with pronounced cerebral damage was observed in some areas of the brain injected with AA. Moreover, ticlopidine (100 mg/kg, p.o.), known as an antiplatelet drug (22), not only prevented the formation of arterial thrombus induced by $AgNO_3$ but also reduced the cerebral damage induced by the injection of AA. Therefore, it seems that platelet aggregation is one of the important deleterious factors responsible for vascular injuries in AA-induced cerebral damage and that the protective action of ticlopidine is mediated via its antiplatelet effect.

In AA-induced cerebral infarction, vascular permeability increasing factors originating from activated platelets such as serotonin and thromboxane A_2 may be important (23). OKY-1581 inhibited $AgNO_3$-induced thrombus formation in the abdominal aorta in a dose-dependent manner, and the effect of a dose of 3 mg/kg (i.p.) was statistically significant. Additionally, OKY-1581 (3 mg/kg, i.p.) prevented the extravasation of EB as well as the increase in cerebral water content. The present results are in agreement with the previous report (21) that OKY-046, a TXSI, ameliorates the AA-induced cerebral infarction in rats. These findings suggest that thromboxane A_2 may be involved in the elevation of vascular permeability as well as the thrombus formation, both of which could finally lead to cerebral damage. However, the cyclooxygenase inhibitor indomethacin (10 mg/kg, i.p.) did not suppress the increase in cerebral water content induced by injection of AA. Indomethacin inhibits the formation of both thromboxane A_2 and PGI_2, the latter of which is involved in so called cytoprotection or membrane stabilization (24–26). Therefore, the reduction of PGI_2 seems to be the reason for the inability of indomethacin to prevent cerebral infarction.

Minamisawa et al. (27) reported that the lipoxygenase inhibitor AA-861 (200 mg/kg, p.o.) suppressed the increase in water content in a rat model of transient ischemia with reperfusion and suggested that the increase in leukotriene C_4 levels was causally related to the increase in cerebral water content. In addition, Dempsey et al. (28) reported that the lipoxygenase inhibi-

Table 3. Histopathological findings of arachidonic acid-induced cerebral lesions in rats

Findings	Grade	Control (n = 13)[a]	Benidipine 30 μg/kg (n ± 11)	Nicardipine 100 μg/kg (n = 6)
Congestion	−	0	3	3
	+	4	4	2
	++	5	3*	0*
	+++	4	1	1
Extravasated hemorrhage	−	2	3	3
	+	6	7	1
	++	3	1	2
	+++	1	0	0
	++++	1	0	0
Perivascular edema	−	0	7	3
	+	8	1*	2*
	++	5	3	1
Karyopicnosis of neurons in neocortex	−	0	1	0
	+	0	2	3
	++	0	3**	1**
	+++	4	4	1
	++++	9	1	1
Karyopicnosis of pyramidal cells in hippocampus	−	0	2	1
	+	2	4	3
	++	3	3**	2**
	+++	7	2	0
	++++	1	0	0
Edematous and spongiose neuropil	−	0	3	1
	+	9	4	4
	++	4	4	1

Drugs were administered 30 min before the injection of arachidonic acid (AA). The control was treated with vehicle. Cerebral lesions were graded from − to ++++ and numbers of animals graded are listed in the table. *and**: $P < 0.05$ and $P < 0.01$ vs. control group, respectively. [a]: Number of animals examined.

Table 4. Effects of benidipine and nicardipine on $AgNO_3$-induced thrombus formation

Drugs	Dose (μg/kg)	Route	No. obstruction / No. tested
Control	−	i.v.	24/26
Benidipine	3	i.v.	3/4
	10	i.v.	6/9
	30	i.v.	3/4
Nicardipine	100	i.v.	4/5
OKY-1581	1	i.v.	4/6
	3	i.v.	0/4**
Indomethacin	10	i.v.	3/4
Control	−	p.o.	7/7
Ticlopidine	30	p.o.	4/4
	100	p.o.	0/4**

Drugs except for ticlopidine were intravenouly administered 5 min before the treatment with $AgNO_3$. Ticlopidine was orally administered 4 hr before the treatment with $AgNO_3$. The rats in the control group were given vehicle. **: $P < 0.01$ vs. control group.

tor nordihydroguaeretic acid limited the formation of ischemic cerebral edema. In the present study, however, AA-861 (200 mg/kg, p.o.) failed to protect against the increase in cerebral water content induced by the injection of AA. It is not likely, therefore, that the metabolites of the 5-lipoxygenase pathway play a major role in the elevation of water content in the present model of AA-induced cerebral infarction.

The Ca-antagonists benidipine and nicardipine did not suppress the $AgNO_3$-induced thrombus formation in abdominal aorta, indicating that these drugs do not have substantial antiplatelet or antithrombotic effects in rats in vivo. Moreover, our unpublished observation indicates that benidipine does not inhibit platelet aggregation induced by collagen in vitro in rats. Nevertheless, both drugs exhibited the beneficial effects on AA-induced cerebral infarction. These findings suggest that the protective mechanism of benidipine and nicardipine is different from those of antiplatelet drugs.

In our present study, while 100 μg/kg (i.p.) of nicar-

dipine significantly ameliorated the extravasation of EB, 300 μg/kg (i.p.) was ineffective. An excessive reduction of blood pressure can induce a decrease in cerebral blood flow and finally promote carebral ischemia in damaged brain. Thus, the deleterious effect of nicardipine at the high dose of 300 μg/kg seems to be due to the excessive reduction (about 50 mmHg) of blood pressure.

Hladovec and DeClerck (29) previously showed that calcium antagonists inhibit endothelial cell injury in rats subjected to perturbations of plasma Ca balance in vivo. Recently, Karasawa et al. (30) have demonstrated that benidipine protects against endothelial cell damage resulting from ischemia and reperfusion. These observations raise the possibility that the protective action of benidipine against AA-induced cerebral infarction might be due to the protection against the endothelial cell damage. This hypothesis seems to be supported by the fact that benidipine prevented the extravasation of EB, an indicator of a disturbance in the blood brain barrier (31, 32), which is formed by the endothelial linings of cerebral vasculatures.

Fujimoto et al. (8) recently reported that when AA was injected into the cerebral artery, marked vascular injuries were observed even in the absence of circulating platelets. The authors suggested that the cerebral vascular injuries induced by AA may not always be due to the platelet aggregation. In our electron microscopic studies of the cerebrum of rats injected with AA, endothelial denudation of small vessels and perivascular edematous changes were observed even without mural platelet thrombi. In some cases, deendothelialization was observed in accord with thrombus formation (data not shown). Since AA is known to have a detergent effect (8, 33), the vascular injuries induced by AA may be caused, at least partly, by this effect. Endothelial cell damage produced by the detergent effect of AA could, in turn, induce platelet adhesion and accelerate the permeability of venules within the brain tissues (8), resulting in more exagerated cerebral injuries like edema. Thus, AA could induce endothelial damage either by its detergent effect or by its platelet aggregatory effect, leading to the formation of thromboxane A_2. In any case, calcium antagonists seem to have protective effects against endothelial cell damage, although the mechanism involved is not fully understood.

Another possible explanation for the protection by benidipine is that Ca-overload into brain cells was inhibited by benidipine. A rise in cytosolic free calcium has been postulated to be an important cause of irreversible cell injury in ischemia (34, 35). Recently, benidipine has been reported to have beneficial effects in ischemic organ failures of diverse origins (36, 37). Therefore, benidipine might protect against the progression of cerebral injury following the obstruction of cerebral vasculature.

In conclusion, our present results demonstrated that benidipine, which does not have antiplatelet and antithrombotic action, protects against AA-induced acute cerebral infarction. The beneficial effect of benidipine may involve its protective effects on endothelial cells. The present results also suggest that benidipine may have some beneficial effects on cerebral infarction.

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