



Neuroprotective properties of a protein kinase inhibitor against ischaemia-induced neuronal damage in rats and gerbils

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1 The neuroprotective properties of fasudil (HA1077), a novel protein kinase inhibitor, were evaluated in two animal models of cerebral ischaemia: transient bilateral carotid artery occlusion in Mongolian gerbils and cerebral microembolization in rats.

2 The cytoprotective effect of fasudil on delayed neuronal death in gerbils was compared with the effects of nimodipine, a calcium channel antagonist and ozagrel, a thromboxane A₂ synthetase inhibitor. The average of the neuronal cell density in the ischaemic control group was 17.8 ± 2.1 cells mm⁻¹, whereas fasudil (30 mg kg⁻¹) significantly diminished the loss of CA1 neurones with the average of the neuronal cell density of 101.0 ± 22.0 cells mm⁻¹; nimodipine (10 mg kg⁻¹) and ozagrel (30 mg kg⁻¹) did not significantly protect against the ischaemia-induced neuronal loss.

3 In the rat model, the effects of fasudil on the histological and neurological consequences of cerebral microembolization produced via the injection of microspheres were examined. Twenty-four hours after the injection of microspheres into the internal carotid artery, all animals in the control group showed typical symptoms of stroke. Neurological function was significantly improved in the fasudil-treated animals. In the controls, the infarcted area in a cortical slice selected to include the hippocampal area was 0.25 ± 0.01 cm² (mean \pm s.e.mean) ($43.9 \pm 2.4\%$ of cortical section of the half hemisphere); the difference was significant compared to the mean area of 32.7 ± 2.8 and $21.5 \pm 4.8\%$ observed in rats treated with fasudil (3, 10 mg kg⁻¹), respectively. Fasudil (10 mg kg⁻¹) significantly suppressed the increased water content in ischaemic brain tissues (saline-treated rats, $82.4 \pm 0.2\%$ vs fasudil-treated rats, $81.0 \pm 0.4\%$).

4 These results suggest that: (i) various protein kinases are involved in the pathogenesis of ischaemic injury; and (ii) the inhibition of protein kinases may be efficacious in preventing neuronal death, thus improving neurological function in the brain damage associated with ischaemic stroke.

Keywords: Cerebral ischaemia; fasudil (HA1077); protein kinase inhibitor; neuroprotection

Introduction

Ischaemic neuronal death may result from the activation of multiple cellular pathways. The activation of various protein kinases is thought to be involved in the regulation of cerebral circulation and in the pathogenesis of ischaemic injury. One of the important mediators of neurodegeneration during cerebral ischaemia may be protein kinase C. It has been reported that protein kinase C may play a role in the pathogenesis of ischaemic neuronal damage (Tanaka *et al.*, 1992), and treatment that decreases protein kinase C activity has been shown to exert a neuroprotective action against ischaemic injury (Hara *et al.*, 1990; Kawamura *et al.*, 1991). Protein kinase C also appears to be implicated in the activation of neutrophils and in the generation of oxygen free radicals by neutrophils (Arai *et al.*, 1993; Yoshida & Mohsenin, 1994), and furthermore it has been shown that leucocytes contribute to cerebral ischaemic cell damage and that stimulated neutrophils release oxygen free radicals that may promote cell death (Chopp *et al.*, 1994).

The activation of cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase may be another mechanism contributing to neuronal degeneration during ischaemia, since it has been shown that ischaemia, hypoxia and NMDA produce increases in the production of cellular cyclic AMP and cyclic GMP (Yoshida *et al.*, 1980; Siesjo, 1981). Ca²⁺-dependent myosin phosphorylation mediated by myosin light chain kinase (MLCK), which

shows a high level of activity in the cerebral arterial wall, is currently considered essential for smooth muscle contraction (Suzuki *et al.*, 1988).

Fasudil (1 - (5 - isoquinolinesulphonyl) - homopiperazine; HA1077) is a newly developed antivasospastic drug (Asano *et al.*, 1987; Shibuya *et al.*, 1990; Satoh *et al.*, 1992) that inhibits protein kinases, MLCK, cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase (Asano *et al.*, 1989) or protein kinase C (Seto *et al.*, 1995). Fasudil improves cerebral haemodynamics (Satoh *et al.*, 1991; Tsuchiya *et al.*, 1993), and inhibits the production of superoxide anions in neutrophils (Arai *et al.*, 1993).

The results of a double-blind placebo-controlled trial have indicated that fasudil is a safe and useful drug for patients with ruptured cerebral aneurysm; this was the first report of a placebo-controlled double-blind trial that demonstrated a significant reduction in angiographically revealed vasospasm by intravenous drug therapy (Shibuya *et al.*, 1992). Fasudil also reduced the incidence of low-density areas due to vasospasm seen on computerized tomography scans.

It has been reported that fasudil, but not the commonly used calcium channel antagonist, nicardipine, administered subsequent to ischaemia at relatively low doses (1 and 3 mg kg⁻¹ i.p.) reduced ischaemia-induced cell death in the hippocampal neurones of Mongolian gerbils (Asano *et al.*, 1991).

We examined the cytoprotective effects of nimodipine, a calcium channel antagonist, and ozagrel, a thromboxane A₂ synthetase inhibitor used in the treatment of acute ischaemic stroke, on delayed neuronal death in gerbils, and a higher dose (30 mg kg⁻¹) of fasudil was tested to examine the dose-de-

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pendence. We used a model of delayed neuronal death in gerbils as a preliminary screening for neuroprotective activity and only compounds that showed a positive effect were tested on a more severe model with neurological impairments.

We also examined the effects of fasudil on multiple cerebral infarctions in a rat model of microembolization stroke that produced neurological impairments. The experiments were designed to measure the effects in terms of attenuation of typical symptoms of stroke and reduction of infarct size 24 h after embolization.

Methods

Delayed neuronal death study

Male Mongolian gerbils, weighing 62 g to 82 g, were lightly anaesthetized with ether, and both common carotid arteries were exposed through a ventral midline incision. The arteries were then clamped with microclips. After 5 min, the clips were removed. Test drug or saline was given i.p. after re-circulation. Seven days later, the gerbils were anaesthetized with sodium pentobarbitone and perfused transcardially with buffered formalin. The brains were removed and coronal sections through the hippocampus were prepared and stained with haematoxylin and eosin. The degree of neuronal damage was assessed in the CA1 region of the hippocampus by quantifying the number of pyramidal cell bodies per millimetre.

Microembolization study

Rats, weighing 225 g to 445 g, were anaesthetized with pentobarbitone sodium (50 mg kg⁻¹, i.p.) and placed in the supine position; respiration was spontaneous. The left common, external and internal carotid arteries were exposed through a ventral midline incision. The left external carotid, occipital and pterygopalatine arteries were ligated, and a catheter was inserted via the left external carotid artery into the common carotid artery. Cerebral microembolism was produced by injecting 4,000 polymer microspheres (o.d. 50.0 ± 1.0 µm, Duke Scientific Corp.) into the internal carotid artery.

Twenty-four hours after embolization, the behaviour of the rats was scored on the basis of the severity of the following symptoms; truncal curvature, circling behaviour and rolling fit, all considered to be typical for stroke. The score consisted of 3 (severe), 2 (moderate), 1 (slight) and 0 (normal) for each symptom.

The rats were anaesthetized (pentobarbitone, 50 mg kg⁻¹, i.p.) and the brains were perfused with a 10% buffered formalin solution through the cardiac ventricle. The study was carried out on 32 rats, divided into 12 control, 10 fasudil (3 mg kg⁻¹) and 10 fasudil (10 mg kg⁻¹)-treated rats. The brains were dissected out and fixed in 10% buffered formalin solution until embedded in paraffin. Five coronal brain sections (5 µm) were prepared at 2 mm intervals and stained with Luxol fast blue–haematoxylin and eosin. Slice 3 was selected to include the hippocampal area. Infarct areas were quantified with a computerized image analysis system (Macintosh IICx, Image 1.27), and were expressed as a percentage of the coronal section of the half hemisphere.

Brain oedema was evaluated by determining water content in the cerebral hemispheres. The study was carried out on 24 rats, divided into 8 normal, 8 saline-treated/ischaemic and 8 fasudil-treated/ischaemic rats. Water content (g H₂O per 100 g of fresh brain weight) was determined by calculating the difference in weight of the tissue samples before and after drying.

Fasudil or saline was administered i.p. after the injection of microspheres.

Statistics

Values are expressed as mean ± s.e.mean. The significance of difference was calculated by Student's *t* test, Dunnett's test or

nonparametric Tukey-type multiple comparisons. *P* values of 0.05 or less were considered to represent significant differences.

Drug and chemicals

The drugs used were fasudil (Asahi Chemical Industry, Tokyo, Japan), nimodipine (Ferrer, Spain), ozagrel (Ono Pharmaceutical Co. Ltd., Osaka, Japan) and pentobarbitone sodium (Pittman-Moore, U.S.A.).

Results

Delayed neuronal death study

The 5-min bilateral occlusion of the carotid arteries resulted in a significant decrease in the number of cell bodies in the CA1 region of the hippocampus. In the sham-operated gerbils, the normal neurone count was 213 ± 5 cells mm⁻¹. The average neuronal cell density in the ischaemic control group was 17.8 ± 2.1 cells mm⁻¹ (*n* = 14), whereas fasudil (30 mg kg⁻¹) significantly diminished the loss of CA1 neurones with an average neuronal cell density of 101.0 ± 22.0 cells mm⁻¹ (*n* = 15, *P* < 0.01); nimodipine (10 mg kg⁻¹) and ozagrel (30 mg kg⁻¹) did not significantly protect against the ischaemia-induced neuronal loss (Figure 1).

Microembolization study

All animals in the control group showed typical symptoms of stroke such as truncal curvature, circling behaviour and rolling fits. Neurological function was significantly improved in the fasudil-treated animals (Table 1).

Twenty-four hours after the injection of the microspheres, there was swelling of the left embolized hemisphere secondary to the development of oedema. The coronal section of the brain showed multiple infarcts in the form of poorly stained areas involving the hippocampus, thalamus and cortex (Figure 2).

The coronal section of brain observed in rats treated with fasudil (10 mg kg⁻¹) showed multiple infarctions involving the hippocampus, thalamus and cortex, but the total size of infarctions was smaller than that in control rats (Figure 2). Treatment with fasudil (3, 10 mg kg⁻¹) dose-dependently reduced the infarct areas in slices 3, 4 and 5, compared with

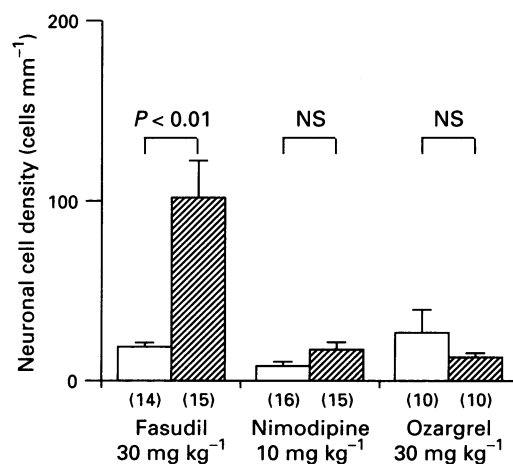


Figure 1 Effect of fasudil, nimodipine or ozagrel on ischaemia-induced cell loss in the CA1 area of the gerbil hippocampus. Saline-treated controls are shown as open columns and fasudil, nimodipine or ozagrel as hatched columns. Each column represents the mean ± s.e.mean of the number of experiments shown in parentheses. Statistical significance was assessed by Student's *t* test. NS indicates no significant difference.

Table 1 Protective effect of fasudil against the impairment of neurological function

Treatment	Dose	Stroke index score					Total	Significant difference (vs control)
		Score 0	Score 1	Score 2	Score 3			
Saline-treated/ ischaemic		0 (0.0)	3 (25.0)	8 (66.7)	1 (8.3)	12 (100.0)		
Fasudil-treated/ ischaemic	3 mg kg ⁻¹	0 (0.0)	4 (40.0)	4 (40.0)	2 (20.0)	10 (100.0)	NS	
Fasudil-treated/ ischaemic	10 mg kg ⁻¹	5 (50.0)	3 (30.0)	2 (20.0)	0 (0.0)	10 (100.0)	P<0.05	

Values are numbers of rats and percentages (in parentheses) of the total within each group. The significance of difference was calculated by nonparametric Tukey-type multiple comparisons. NS indicates no significant difference.

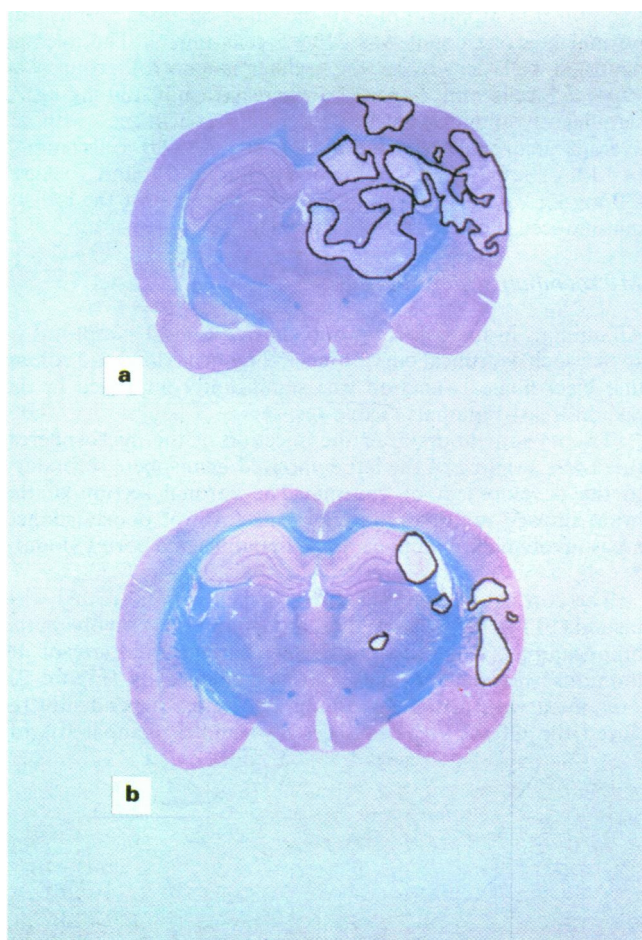


Figure 2 Coronal sections of saline-treated rat brain (a) and fasudil (10 mg kg⁻¹)-treated rat brain (b). Coronal brain sections were stained with Luxol fast blue-haematoxylin and eosin. The brain of saline-treated rats showed multiple infarctions in the form of poorly stained areas. Fasudil reduced the infarct area.

controls. The infarct area averaged $43.9 \pm 2.4\%$ ($n = 12$) of the cortical section of the half hemisphere (0.25 ± 0.01 cm²) of the cortical slice selected to include the hippocampal area (slice 3) in control rats; the difference was significant compared to the mean area of 32.7 ± 2.8 ($n = 10$, $P < 0.05$) and $21.5 \pm 4.8\%$ ($n = 10$, $P < 0.01$) observed in rats treated with fasudil 3 and 10 mg kg⁻¹, respectively (Figures 2 and 3).

Twenty-four hours after embolization, the water content of the ipsilateral hemisphere ($82.4 \pm 0.2\%$, $n = 8$, $P < 0.01$ vs normal) was significantly greater than that in control animals ($78.4 \pm 0.1\%$, $n = 8$). In the contralateral hemisphere, there was no change in water content (Table 2). In rats given fasudil

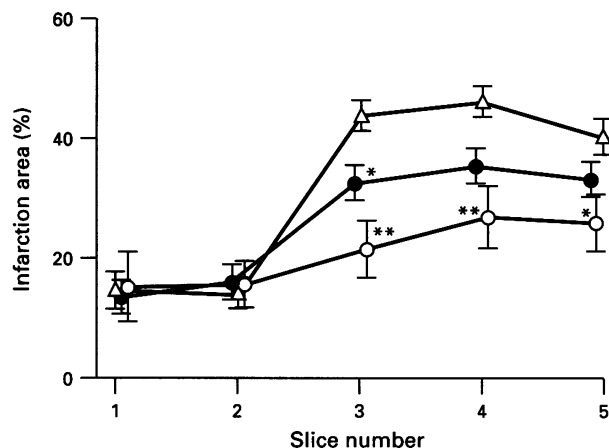


Figure 3 Effect of fasudil (△) control, $n = 12$; (●) 3 mg kg⁻¹, $n = 10$; (○) 10 mg kg⁻¹, $n = 10$ on the areas of ischaemic damage on 5 coronal slices. Coronal slices were prepared at 2 mm intervals and slice 3 was selected to include the hippocampal area. Each data point represents the mean \pm s.e. mean. The asterisks indicate a significant difference from the control (** $P < 0.01$, * $P < 0.05$: Dunnett's test).

(10 mg kg⁻¹), water content in the ipsilateral hemisphere ($81.0 \pm 0.4\%$, $n = 8$, $P < 0.05$) was lower than that in rats given saline.

Discussion

Multiple infarcts were produced in the cerebral hemispheres of rats by the injection of the 50 μ m microspheres into the left internal carotid artery and neurological impairment was evaluated 24 h later. The assessment of neurological function following cerebral microembolization is a subjective test and would only give an indication as to alterations in function, but all rats in the control group showed typical symptoms of stroke such as truncal curvature and forced circling during locomotion. Although distribution of the infarctions was unpredictable, the structures most frequently involved were the hippocampus, thalamus and cortex (Figure 2), and the total size of infarctions was comparatively constant.

In contrast to earlier studies of the beneficial effects of fasudil, we carried out this study with a microembolism model that is a multiple infarction model. This model should show more peripheral vessel occlusion than would occur by occluding at the origin of the middle cerebral artery (Ohtaki & Tranmer, 1994) or the common carotid artery (Asano *et al.*, 1991), and may be far closer to the sites of embolism in patients who have atherothrombotic strokes and lacunar infarctions (Nadeau *et al.*, 1993).

The principal finding of this study was that the administration of fasudil significantly improved the ischaemia-induced

Table 2 Effect of fasudil on cerebral water content 24 h after embolization

Treatment	Dose	Left hemispheres H ₂ O (%)	Right hemispheres H ₂ O (%)
Normal		78.4±0.1 (8)	78.4±0.1 (8)
Saline-treated/ ischaemic		82.4±0.2 (8)##	78.7±0.2 (8)
Fasudil-treated/ ischaemic	10 mg kg ⁻¹	81.0±0.4 (8)*	78.3±0.2 (8)

##*P*<0.01 vs normal group and **P*<0.05 vs control group, respectively.

impairment of neurological function, as well as impairment in the morphology. The neurological scores for the evaluation of posture and motor function were significantly lower in fasudil-treated animals than in non-treated controls. The morphological damage caused by microembolization was attenuated by fasudil treatment.

Some characteristics of this stroke model have been reported previously. Twenty-four hours after the injection of microspheres, regional cerebral blood flow in the embolized hemisphere reached 30–50% of the control value, depending on the brain area examined (frontal, parietal and occipital cortex; caudate nucleus; and thalamus) (Beley *et al.*, 1981). Other workers have found that embolization caused marked changes in energy metabolite levels, with decreases in phosphocreatine, ATP and ADP (Bralet *et al.*, 1979).

It has been reported that the i.v. infusion of fasudil increased local cerebral blood flow and glucose utilization at various sites, including the thalamus, cortex and hippocampus (Sako *et al.*, 1991; Tsuchiya *et al.*, 1993). These sites were consistent with the infarct areas in the present model. These results suggest that fasudil protects the brain from ischaemic infarction by improving haemodynamic and metabolic function, at least in part.

The normal human brain is 78% H₂O. A 2% increase in H₂O content appears to be associated with extensive oedema and increased intracranial pressure (Weir, 1987). Some investigators have noted an increase in blood brain barrier permeability in response to cerebral microembolization (Bralet *et al.*, 1979; Beley *et al.*, 1981), with the water level in the brain increasing significantly 6 h after embolization and showing a pronounced increase within 48 h. We noted a 4% increase in the water content of the ipsilateral hemisphere 24 h after embolization and the occurrence of cerebral oedema. When fasudil had been administered, there was a significant suppression of the elevation in water content in ischaemic brain tissue. But fasudil did not completely reverse the increased water content, thus other mechanisms, for example microspheres which can cause endothelial damage, may also be responsible for the increased water content.

In the presence of cerebral ischaemia, there is a massive influx of Ca²⁺ into neurones, the so-called final common pathway of cell death (Schanne *et al.*, 1979). The inhibition of Ca²⁺ entry into cells may possibly serve as a measure for the treatment of cerebral ischaemia (Meyer *et al.*, 1986; Germano *et al.*, 1987; Kucharczyk *et al.*, 1989; Kawamura *et al.*, 1991). The calcium entry blocker, nicardipine, administered before carotid artery occlusion in the Mongolian gerbil protected against the delayed neuronal death of CA1 pyramidal neurones (Alps *et al.*, 1988). On the other hand, nicardipine administered after bilateral carotid artery occlusion did not decrease the neurodegeneration (Asano *et al.*, 1991). In the

present study, the potent calcium entry blocker, nimodipine, failed to protect against ischaemia-induced neuronal loss in the hippocampus of gerbils. These discrepancies may be attributed to the different protocols (pre-ischaemia vs. post-ischaemia administration), and it is speculated that, when nicardipine or nimodipine was administered after an ischaemic event, the intracellular calcium concentration in nerve cells had already been elevated, thus, post-ischaemic treatment with nicardipine or nimodipine could not protect against neuronal damage.

Ozagrel, a thromboxane A₂ synthetase inhibitor, did not significantly protect against the ischaemia-induced neuronal loss. In a cerebral infarction model induced by the injection of arachidonate into the carotid artery in rabbits, ozagrel at doses of 0.3 and 1.0 mg kg⁻¹, i.v. decreased the incidence of cerebral infarction (Hiraku *et al.*, 1986). These workers considered that ozagrel exhibited a suppressive effect on cerebral infarction through the inhibition of thromboxane A₂ production. In the present study, ozagrel administered after bilateral occlusion did not decrease the neurodegeneration. This discrepancy may be attributed to the different methods employed to induce cerebral infarction (arachidonate vs. artery occlusion). We used delayed neuronal death model in gerbils as a preliminary screening for neuroprotective activity, so fasudil, a compound that showed a positive effect, alone was tested using a microembolism model in the rats.

Next, we considered why fasudil is effective in protecting against the delayed neuronal death in gerbils which is refractory to nimodipine and ozagrel, and in multiple infarctions in rats. Ischaemic neuronal death appears to result from the activation of multiple cellular pathways (Maiese *et al.*, 1994), with no single mechanism being responsible for the pathogenesis of ischaemic cerebral infarction. The activation of various protein kinases, such as protein kinase C, MLCK, cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase, are thought to be important factors involved in such multiple cellular pathways. Since fasudil potently inhibits protein kinase C, MLCK, cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase, this action may cause it to be effective in cases of ischaemic infarction.

The major therapeutic strategies in ischaemic stroke are suggested to be: (1) regulation of microcirculatory derangement; and (2) enhancement of neuronal ischaemic tolerance (Habel, 1994). Our present and previous findings suggest that various protein kinases are involved in the regulation of cerebral circulation and in the pathogenesis of ischaemic injury, and that inhibition of these protein kinases may be efficacious in preventing neuronal death, and thus improving neurological function in cases of brain damage associated with ischaemic stroke.

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