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Effect of combination of a tissue-type plasminogen activator and an endothelin receptor antagonist, FR139317, in the rat cerebral infarction model

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Abstract

We were interested to investigate if a combination of a modified tissue-type plasminogen activator, SUN9216, which is constructed by modifying a single amino acid (Asn^{117}-Gln^{117}) to yield a tissue-type plasminogen activator lacking finger and growth factor domains with a long half-life in blood, and an endothelin receptor antagonist, FR139317, (*R*)2-[(*R*)-2-[(*S*)-2[[1-(hexahydro-1*H*-azepinyl)]carbonyl]amino-4-methyl-pentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)]propionyl}amino-3-(2-pyridyl)propionic acid, has greater thrombolytic efficacy than a thrombolytic agent alone in reducing the size of cerebral infarction. The thrombotic occlusion of the rat middle cerebral artery was induced by a photochemical reaction between rose bengal and green light, which causes endothelial injury followed by platelet adhesion and formation of a platelet-rich thrombus. SUN9216 (1 mg/kg) was injected intravenously 30 min after the middle cerebral artery occlusion and the time for reopening of the middle cerebral artery by SUN9216 was monitored for a 60-min period under an operating microscope. In the rats in which thrombolysis was achieved with SUN9216, the size of the cerebral infarction was significantly ($P < 0.05$) reduced as compared with that in the rats treated with saline and was comparable to the reduction produced by the combination doses. It is concluded that, under the present experimental conditions, endothelin may not be involved in the impaired local cerebral blood flow after thrombolysis.

Keywords: Middle cerebral artery thrombosis; Modified tissue-type plasminogen activator; SUN9216; Endothelin receptor antagonist; FR139317

1. Introduction

Endothelin-1 is known to be a potent vasoconstrictor (Yanagisawa et al., 1988). Cerebral microvessels which produce endothelin-1 (Hardebo et al., 1989) show marked sensitivity to this substance (Yoshimoto et al., 1990). In patients with acute ischaemic stroke, the plasma concentration of endothelin-1 is reported to increase significantly (Ziv et al., 1992), although its role in the initiation of stroke has not yet been understood. However, constriction of collateral vessels by endothelin-1 following stroke can further diminish blood flow into the ischaemic brain tissue and increase the size of cerebral infarction (Asano et al., 1989; Robinson and McCulloch, 1990; Robinson et al., 1990; Wilkins and Sauermelch, 1990; Macrae et al., 1991). In our previous studies (Umemura et al., 1993, 1994), a thrombolytic agent could induce the reopening of the middle cerebral artery occluded by photochemically induced thrombus in the rat. Thus, it is hypothesized that endothelin, which causes vasoconstriction, may be associated with hypoperfusion after thrombolysis in the middle cerebral artery. Therefore, we were interested to investigate whether or not a combination of a modified tissue-type plasminogen activator, SUN9216, and an endothelin-1 receptor antagonist, FR139317, had a greater efficacy in reducing the size of cerebral infarction than SUN9216 alone in a model of rat middle cerebral artery thrombosis.

FR139317, (*R*)2-[(*R*)-2-[(*S*)-2[[1-(hexahydro-1*H*-azepinyl)]carbonyl]amino-4-methyl-pentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)]propionyl}amino-3-(2-pyridyl)propionic acid, has been demonstrated to show a high affinity for endothelin ET_A receptor and a low

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affinity for endothelin ET_B receptor (Aramori et al., 1993), and to inhibit cerebral vasospasm in an experimental model of subarachnoid haemorrhage in the dog (Sogabe et al., 1992). Although a family of two endothelin receptor subtypes exists (Arai et al., 1990; Lin et al., 1991; Sakurai et al., 1990; Vane, 1990), endothelin ET_A receptors are predominantly on the vascular smooth muscle cells of a variety of tissues and endothelin-1 causes vasoconstriction through endothelin ET_A receptors (Sogabe et al., 1992).

In this study, the effect of a modified tissue-type plasminogen activator, SUN9216, was evaluated. SUN9216 is constructed by modifying a single amino acid (Asn^{117}-Gln^{117}) to yield a tissue-type plasminogen activator lacking finger and growth factor domains with a long half-life in blood. It was reported that SUN9216 is cleared from the blood about 20 times slower than native tissue-type plasminogen activator and is 8.6-fold more potent as a fibrinolytic agent than native issue-type plasminogen activator as studied in the rabbit following bolus injection (Larsen et al., 1991).

2. Materials and methods

2.1. Animal preparations

Wistar male rats weighing 240–260 g were used. Their body temperature was maintained at 37.5°C with a heating-pad (K-module Model K-20, American Pharmaseal Company, USA). The middle cerebral artery thrombosis model in the rat has been described previously (Umemura et al., 1993, 1994). In brief, under pentobarbital anaesthesia 50 mg/kg, intraperioneally) and spontaneous respiration, a catheter for the administration of rose bengal or thrombolytic agents was inserted into the femoral vein. The scalp and temporalis muscles were folded over and a subtemporal craniotomy was performed using a dental drill under an operating microscope. A 3-mm-diameter circular area of the window was illuminated with green light and the entire illuminated segment including the proximal end of the lenticulostriate branch became thrombotically occluded. Photo-illumination by green light (wave length, 540 nm) was achieved by using a xenon lamp (L4887: Hamamatsu Photonics, Hamamatsu, Japan) with a heat-absorbing filter and a green filter. The irradiation was directed by a 3-mm-diameter optic fiber mounted on a micromanipulator. The head of the optic fiber was placed on the window in the skull base at a distance of 2 mm above the vessel, delivering an irradiation dose of 0.62 W/cm^2. The incisions were closed after the 90-min observation period and a local anaesthetic was applied to the surgical wound every 3 h until the animals were killed. Twenty-four hours after the completion of the irradiation, the cerebrum was removed under anaesthesia by another investigator for subsequent analysis. The cerebrum was coronally sectioned into 1-mm-thick slices from the frontal lobe with a microslicer and six consecutive slices were stained with triphenyltetrazolium chloride (Katayama, Japan) and were then photographed. For each animal, infarction area was calculated using a computerized image analysis system. PO_2 and PCO_2 were determined before the injection of rose bengal and the mean arterial blood pressure was monitored with a pressure transducer during the experiments.

2.2. Administration of thrombolytic agents

SUN9216, 1 mg/kg, was administered as a single bolus intravenous injection in a volume of 0.5 ml, via the femoral vein 30 min after the thrombotic occlusion of the middle cerebral artery. The thrombosed middle cerebral artery was observed with an operating microscope for 60 min after the injection of SUN9216. The time taken for reperfusion to be established was determined by using an operating microscope. Twenty-four hours after the administration of SUN9216, the infarcted area of the cerebrum was assessed by using triphenyltetrazolium chloride. In separate experiments, the effect of a combination of SUN9216 and an endothelin-1 receptor antagonist, FR139317 (10 mg/kg), which was simultaneously injected via the femoral vein, followed by a further dose of FR139317 (32 mg/kg) administered subcutaneously 30 min after the first dose, was evaluated. The half-life of FR139317 is about 40 min when it is injected intravenously in rats. Thus, the subcutaneous dose was to achieve an anti-endothelin-1 effect within 24 h. The control animals were injected with an equal volume of saline for each treatment. SUN9216 alone or the combination of SUN9216 and FR139317 was administered at random.

2.3. Determination of immunoreactive endothelin-1 level in the brain

In separate experiments, the immunoreactive endothelin-1 level in the brain just before, 3 and 6 h after the middle cerebral artery occlusion was determined in nine animals. The brain was removed from the animals under pentobarbital anaesthesia and quickly frozen by dipping into liquid nitrogen and stored at −70°C until use. Tissue samples were prepared according to the previously described procedure (Matsumoto et al., 1989). Frozen tissues were homogenized in 10 volumes of 1 N AcOH containing 10 μg/ml of pepstain, using a Polytron homogenizer (Kinematika, Switzerland), and boiled for 10 min. The homogenates were centrifuged at 10000 × *g* for 20 min at 4°C, and the supernatant was stored at −70°C. The immunoreactive endothelin-1 was measured by radioimmunoassay after concentrat-

ing the stored supernatants by passing them through an Amprep C2 column (Amersham Japan, Tokyo, Japan) and lyophilization of the eluate. The test samples were redissolved in radioimmunoassay (RIA) buffer (0.1 M Tris-HCl, pH 8.2, containing 0.3% bovine serum albumin and 0.1% Tween 20). The amount of immunoreactive endothelin-1 was measured by RIA using rabbit anti-endothelin-1 antiserum (obtained from IBL, Gunma, Japan) and ^{125}I-labelled endothelin-1 (Amersham) as a tracer. The sensitivity of this RIA system was 5 pg per tube, and the crossreactivities to endothelin-1, endothelin-2, endothelin-3 and big endothelin-1 (rat) were 100277, < 0.01 and 80%, respectively.

2.4. Statistical analysis

Data are expressed as means ± S.E. Statistical analysis was made with the unpaired Student's *t*-test for comparisons between groups and the comparisons of more than three groups were made by analysis of variance. For the incidence of thrombolysis, groups were compared using Fisher's exact test. $P < 0.05$ was considered significant.

3. Results

Physiological variables following the operation were within the normal range ($PO_2 = 85.4 \pm 2.2$; $PCO_2 = 40.3 \pm 0.6$; pH = 7.41 ± 0.01). Mean arterial pressures before and 1 h after FR139317 injection were 116 ± 7.0 mm Hg and 96 ± 5.1 mm Hg (119 ± 7.6 mm Hg and 109 ± 5.2 mm Hg, control group), respectively. The middle cerebral artery was completely occluded by thrombus about 5 min after the administration of rose bengal, as monitored with an operating microscope. The left dorsolateral frontoparietal cortex and the left dorsolateral portion of the striatum were consistently infarcted 24 h after the middle cerebral artery occlusion.

Table 1
Effect of a combination of SUN9216 and an endothelin-1 receptor antagonist, FR139317, on the incidence of the reopening or the time of the reopening of the middle cerebral artery

	Number	Incidence of reopening [b]	Time of reopening (min)
Saline	14	0/14 (0%)	-
SUN9216	16	5/16 (31.3%) [a]	72.5 ± 5.8
SUN9216 and FR139317	16	6/16 (37.5%) [a]	65.8 ± 6.4

Data are expressed as means ± S.E.M. The time of reopening: thrombolysis time after thrombotic middle cerebral artery occlusion. [a] $P <$ 0.05 vs. saline. [b] The percentage of animals in a group in which thrombolysis was achieved.

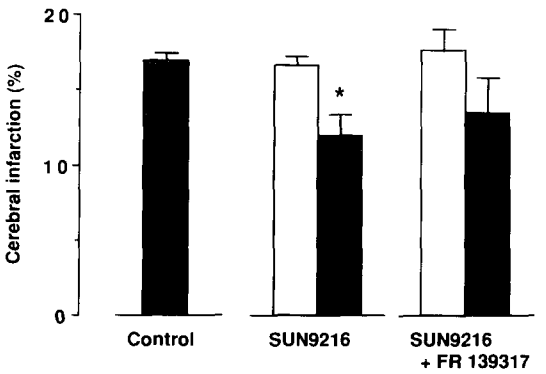

Fig. 1. The size of the cerebral infarction 24 h after the occlusion of the middle cerebral artery in animals treated with SUN9216 alone and the combination of SUN9216 and FR139317. Open and solid columns represent animals in which reperfusion was not and was established respectively. Data are expressed as means ± S.E.M. * $P < 0.05$ vs. control.

SUN9216 restored blood flow through the occluded middle cerebral artery by thrombolysis in 5 out of 16 animals (31.3%) as compared with 0 out of 14 animals treated with saline (Table 1). Furthermore, it significantly ($P < 0.05$) reduced the size of cerebral infarction as compared with that in the animals in which the reopening of the middle cerebral artery was unsuccessful or that in animals treated with saline (Fig. 1). Almost similar results were obtained in animals which had received a combination of SUN9216 and the endothelin-1 receptor antagonist, FR139317 (Table 1, Fig. 1).

The immunoreactive endothelin-1 level in the brain was measured just before, 3 and 6 h after the middle cerebral artery occlusion. The immunoreactive endothelin-1 level increased significantly ($P < 0.01$) 3 h after occlusion of the middle cerebral artery and continued to increase for a further 6 h after middle cere-

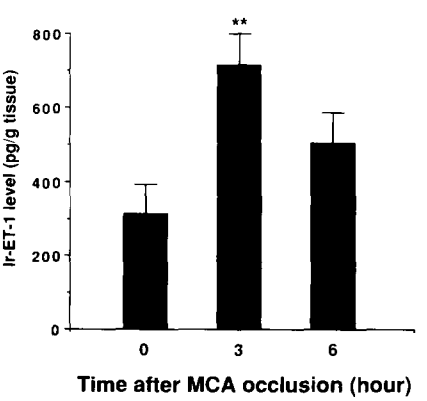

Fig. 2. The immunoreactive endothelin-1 (Ir-ET-1) level in the brain just before, 3 and 6 h after the middle cerebral artery occlusion. Animal numbers were 3 for just before and 5 for 3 and 6 h after the middle cerebral artery occlusion, respectively.

bral artery occlusion compared with the values just before the middle cerebral artery occlusion (Fig. 2).

4. Discussion

In this study, the thrombotic occlusion of the middle cerebral artery was induced by photochemical reaction between rose bengal and green light, which causes endothelial injury followed by platelet adhesion, aggregation and formation of a platelet-rich thrombus at the site of photochemical reaction. Using this model, we investigated whether or not endothelin-1 is associated with impaired local blood flow following reopening of the middle cerebral artery by thrombolytic agents. The combination of a modified tissue-type plasminogen activator, SUN9216, and an endothelin receptor antagonist, FR139317, did not enhance the efficacy of the thrombolytic agent in reducing the size of cerebral infarction.

In our previous study (Umemura et al., 1994), at 20 mg/kg, given as a bolus dose, SUN9216 could induce reopening of the occluded middle cerebral artery in 7 out of 10 animals (70.0%). In other experiments (unpublished observation), an agent which inhibited the release of glutamate due to cerebral ischaemia could reduce the size of cerebral infarction by 50% 1 h after middle cerebral artery occlusion. In the present experimental conditions, the dose of SUN9216 was not chosen to produce its optimum thrombolytic efficacy. In previous experiments, FR139317, which was injected intravenously, could inhibit the vasoconstriction induced by topical administration of a cerebrospinal fluid-blood mixture from outside the cat basilar artery (personal observation). On the basis of this action, the cerebral vasoconstriction induced by endothelin-1 via endothelin ET_A receptors can be inhibited by FR139317.

In our previous study (Umemura et al., 1993, 1994), a thrombolytic agent could reopen the thrombotically occluded middle cerebral artery in the rat. This resulted in a reduction in the cerebral infarction size. The reopening of the middle cerebral artery by thrombolytic agents may increase the production of thromboxane A_2 in the ischaemic area, because the activity of cyclooxygenase depends on oxygen (Gaudet and Levin, 1980; Gaudet et al., 1980). It has been reported that endothelin-1 also evokes the release of arachidonic acid in cultured microvascular endothelium derived from human brain (Stanimirovic et al., 1993). Thromboxane A_2, an enzyme product of arachidonic acid, induces platelet aggregation and vasoconstriction, leading to a reduction in cerebral microcirculatory blood flow (Sadoshima et al., 1989). It has been postulated that this contributes to postischaemic hypoperfusion (Chen et al., 1986). In a thrombotic middle cerebral artery occlusion model (Nakashima et al., 1988) which is very similar to our model, reopening of the occluded middle cerebral artery by the calcium antagonist, nimodipine, which was administered topically, reduced the size of cerebral infarction. In that study, local cerebral blood flow was determined using an autoradiographic technique and the reopening of the middle cerebral artery improved local blood flow but did not restore it to the preocclusion value. In that study (Umemura et al., 1993), the combination of the thrombolytic agent and a thromboxane A_2 receptor antagonist was not significantly more effective than the thrombolytic agent alone, in reducing the size of cerebral infarction. It therefore seemed likely that an alternative candidate for inducing postthrombolytic vasoconstriction is endothelin-1, which we investigated in the present study and found unlikely to be involved. It has been reported that the endothelin-1 pressor response is mediated almost entirely via the endothelin ET_A receptor and the vasodilator effect of this substance is due to activation of the endothelin ET_B receptor (McMurdo et al., 1993). However, some investigators have demonstrated that the vasoconstriction induced by endothelin-1 is mediated through endothelin non-ET_A receptor (ET_B?) (Williams et al., 1991; Bigaud and Pelton, 1992; Cristol et al., 1993). In this model, it is unclear whether or not the response mediated via an endothelin ET_B receptor may be associated with hypoperfusion after thrombolysis.

In acute ischaemic stroke, it was demonstrated that the plasma concentration of endothelin-1 is elevated (Ziv et al., 1992), but the mechanism for the rise in plasma endothelin-1 following stroke is as yet unclear. The brain immunoreactive endothelin-1 level was elevated 3 h after the middle cerebral artery occlusion in this model.

The production of endothelin by endothelial cells is stimulated by thrombin (Yanagisawa et al., 1988), transforming growth factor-β (Kurihara et al., 1989), interleukin-1 (Yoshizumi et al., 1990), angiotensin II (Emori et al., 1989) and shear stress (Yoshizumi et al., 1989). If these mediators stimulate the expression of endothelin-1 mRNA, the production of endothelin-1 may increase and cause vasospasm in cerebral vessels. However, as mentioned above, in this study, a combination of the thrombolytic agent and an endothelin ET_A receptor antagonist did not enhance the reduction in the size of cerebral infarction as compared with that elicited by the thrombolytic agent alone. These findings suggest that endothelin-1 response via an endothelin ET_A receptor may not be associated with hypoperfusion after the reopening of the occluded middle cerebral artery by a thrombolytic agent.

It is concluded that under the present experimental conditions, endothelin may not be involved in impaired local cerebral blood flow after the thrombolysis.

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