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Effect of TTC-909 in a middle cerebral artery thrombosis model in stroke-prone spontaneously hypertensive rats

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Abstract

We investigated the effect of TTC-909, a preparation of the stable prostaglandin I_2 analogue clinprost (isocarbacyclin methylester; methyl 5-{(1*S*,5*S*,6*R*,7*R*)-7-hydroxy-6-[(*E*)-(*S*)-3-hydroxy-1-octenyl] bicyclo[3.3.0]oct-2-en-3-yl} pentanoate) incorporated into lipid microspheres, on infarct volume 24 h after photochemically induced thrombotic occlusion of the middle cerebral artery in stroke-prone spontaneously hypertensive rats (SHR). Under anesthesia, the photosensitizing dye rose bengal (20 mg/kg) was administered intravenously and photoirradiation with green light (wavelength 540 nm) on the middle cerebral artery above the rhinal fissure was achieved using a xenon lamp for 10 min. Infarct volume 24 h after the photochemically induced thrombotic occlusion of the middle cerebral artery was significantly larger in stroke-prone SHR than in Wistar rats. When TTC-909 in doses of 100, 300 and 900 ng/kg/h was intravenously infused for 3 h, starting immediately after the end of the 10-min photoirradiation, the infarct volume was dose-dependently reduced and was statistically significant at a dose of 900 ng/kg/h ($p < 0.05$). Ozagrel, a thromboxane A_2 synthetase inhibitor, significantly reduced the infarct volume. The model of photochemically induced thrombotic occlusion of the middle cerebral artery in stroke-prone SHR is very useful, because the cerebral infarction is large enough and reproducible. TTC-909 may be effective for the treatment of acute ischemic stroke.

Keywords: Stroke; Cerebral ischemia, focal; Thrombosis, photochemically induced; Middle cerebral artery occlusion; SHR (Stroke-prone spontaneously hypertensive rat); Prostacyclin

1. Introduction

Platelet aggregation, which plays an essential role in arterial thrombosis, is widely implicated in the pathogenesis of thrombotic stroke. Photochemically induced thrombotic occlusion of the rat middle cerebral artery is a thrombotic stroke model based on thrombus formation as a result of a photochemical reaction between rose bengal and green light (Watson et al., 1985). This reaction between rose bengal and green light causes endothelial injury followed by platelet adhesion, aggregation and formation of a platelet and fibrin-rich thrombus at the site of the reaction (Umemura et al., 1993).

In hypertensive rats, middle cerebral artery occlusion give rise to much larger infarcts than in normotensive strains. Distal middle cerebral artery occlusion also produces the largest and most reproducible infarcts in stroke-prone spontaneously hypertensive rats (SHR) (Coyle and Jokelainen, 1983; Coyle and Heistad, 1991; Fujii et al., 1992; Okuyama et al., 1991). Yao et al. (1996) showed that the infarct in spontaneously hypertensive rats (SHR), produced by photochemically induced occlusion of the distal middle cerebral artery, was moderate in size and localized reproducibly with an acceptable coefficient of variation (Cai et al., 1998).

The generation of prostacyclin (prostaglandin I_2) by endothelial cells is crucial for maintaining homeostasis in the blood stream, because prostacyclin counteracts the

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thromboxane generated by platelets (Vane and Bergstrom, 1979). Because of its highly potent vasodilating and antiplatelet activity, prostacyclin has been used to treat various types of thrombotic disorders such as ischemic cerebrovascular diseases (Gryglewski and Stoch, 1987). Prostacyclin has proven useful in open trials (Gryglewski et al., 1983; Miller et al., 1984), but not in double-blind trials (Hsu et al., 1987; Huczynski et al., 1985; Martin et al., 1985). The inconsistency of prostacyclin efficiency may be accounted for by the instability of prostacyclin: the half-life of prostacyclin in vivo is about 3 min (Moncada, 1983). In addition, the hypotensive effect of prostacyclin might reduce collateral blood flow in the ischemic area and offset any direct beneficial effects (Awad et al., 1983).

Isocarbacyclin methylester (clinprost) (methyl 5-{(1*S*,5*S*,6*R*,7*R*)-7-hydroxy-6-[(*E*)-(*S*)-3-hydroxy-1-octenyl] bicyclo[3.3.0]oct-2-en-3-yl} pentanoate) and its active metabolite, isocarbacyclin (TEI-7165; 5-{(1*S*,5*S*,6*R*,7*R*)-7-hydroxy-6-[(*E*)-(*S*)-3-hydroxy-1-octenyl] bicyclo[3.3.0]oct-2-en-3-yl} pentanoate), are chemically stable prostacyclin analogues. TTC-909 is a preparation of clinprost incorporated into lipid microspheres (Fig. 1). The hypothetical sequence of events for TTC-909 to exert pharmacological effects is as follows: the lipid microsphere delivers clinprost selectively to the disease area, where clinprost would be released gradually from the lipid microsphere and then be hydrolyzed to TEI-7165 by esterase action, to exert its pharmacological activity. Both clinprost and TEI-7165 inhibit platelet aggregation and platelet adhesion in vitro and suppress the prostaglandin $F_{2\alpha}$-induced contraction of isolated canine arteries (Sawada et al., 1995). TTC-909 also has vasodilative and antiplatelet activity in vivo, similar to that of prostacyclin (Inoue et al., 1995a,b). TTC-909 improved changes in microcirculation and glucose utilization following permanent occlusion of the middle cerebral artery in stroke-prone SHR (Shima et al., 1995).

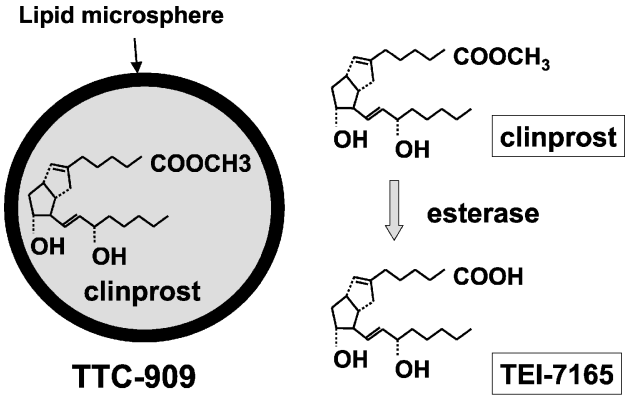

Fig. 1. Chemical structures of clinprost, isocarbacyclin (TEI-7165) and a model of lipid microsphere formation (TTC-909). TTC-909 is a drug preparation of clinprost (isocarbacyclin methyester) incorporated into lipid microspheres with a diameter of 0.2 μm. Clinprost is hydrolyzed to TEI-7165 by esterase.

In this study, the cerebral infarction 24 h after photochemically induced thrombotic occlusion of the middle cerebral artery in stroke-prone SHR was compared with that in normotensive rats, and the effects of TTC-909 on cerebral infarction in stroke-prone SHR were investigated. We also examined the effects of a thromboxane A_2 synthetase inhibitor, ozagrel, on the cerebral infarction produced by photochemically induced thrombotic occlusion of the middle cerebral artery in stroke-prone SHR.

2. Materials and methods

2.1. Animals

Ten male Wistar rats 11 weeks of age (Japan SLC) and 111 male stroke-prone SHR 10–12 weeks of age (Taisho Pharmaceutical, Tokyo, Japan) were housed in an air-conditioned room at 22 °C with a 12-h light–dark schedule (lights on at 7:00 a.m.). The animals were provided with a standard diet for Wistar rats and OA-2 diet (Clea, Japan) for stroke-prone SHR with free intake of tap water. There were no signs of spontaneous stroke in these stroke-prone SHR. The systolic blood pressure in each conscious stroke-prone SHR was measured using a rat tail sphygmomanometer system (KN-210, Riken Kaihatsu, Japan). All the stroke-prone SHR we used had a systolic blood pressure of over 160 mm Hg.

All experimental procedures were performed under the guidelines of the Animal Experiment Committee of Taisho Pharmaceutical.

2.2. Drugs

TTC-909 was from Taisho Pharmaceutical. Intralipid 10%® (vehicle) was purchased from Otsuka Pharmaceutical (Tokyo, Japan). TTC-909 was diluted with intralipid 10% as vehicle. Ozagrel (Xambone injection®) was purchased from Kissei Pharmaceutical (Matsumoto, Japan) and was dissolved in saline as the vehicle. TTC-909, ozagrel or each vehicle was infused through the tail vein in a volume of 1 ml/kg/h.

Rose bengal was purchased from Wako and dissolved in saline.

2.3. Preparation of middle cerebral artery occlusion model

Rats were anesthetized with 2% halothane and were maintained with 1% halothane in room air; body temperature was kept at 36.5 ± 1 °C using a heat pad. Under an operating microscope (MD-II, Nagashima), the left middle cerebral artery was exposed through a burr-hole craniectomy (2–3 mm in diameter) performed via the transtemporal route, without damage to the zygomatic bone. The

middle cerebral artery was observed through the dura matter. The photosensitizing dye rose bengal (20 mg/kg) was administered intravenously and photoirradiation with green light (wavelength 540 nm) on the middle cerebral artery above the rhinal fissure was achieved by using a xenon lamp (L4887: Hamamatsu Photonics, Japan) for 10 min. The disruption of blood flow in the middle cerebral artery was made sure under the microscope. After the photoirradiation, the skin was sutured. The rats were weaned from the respirator for anesthesia and set up in Bollmann cages for the infusion of drugs.

2.4. Infarct size

At 24 h after thrombotic occlusion of the middle cerebral artery, the rats were anesthetized with ether, and the brains were perfused with saline and then with a 10% buffered formalin solution given through the left cardiac ventricle. The brains were dissected out and fixed in 10% formalin solution. Serial sections (about 5-μm thick) were cut coronally from the paraffin block of the whole brain. Thirty sections were prepared and stained with hematoxylin and eosin. Of all the sections prepared, 15 sections taken at the same intervals were selected.

The infarct area was serially measured on each slide (15 slides/rat), using a microcomputer imaging analyzer (MCID: Imaging Research), by an investigator who was blinded as to the pharmacological treatment given the animals. The infarct volume of each rat was calculated as follows:

V (Infarct volume: mm^3) = S (total infarct area: mm^2) × D (distance between each section: mm).

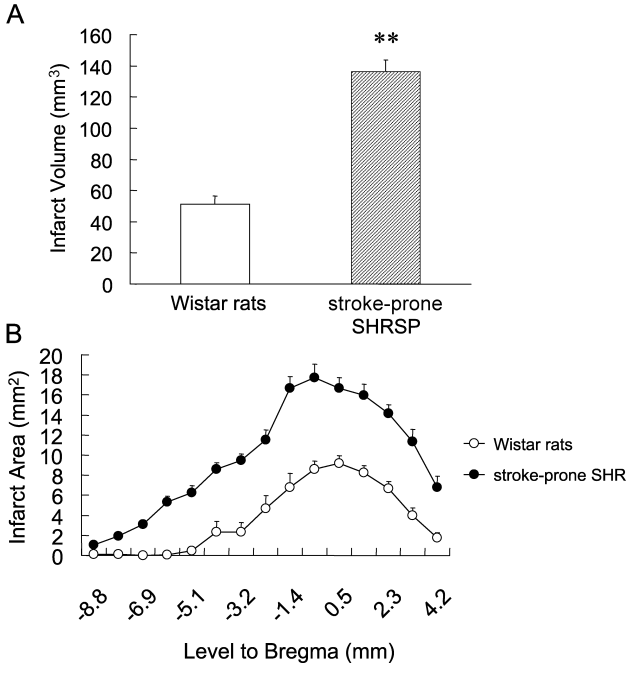

Fig. 2. Infarct volume (mm^3) (A) and the area (mm^2) of each section (B) 24 h after photochemically induced thrombotic occlusion of the middle cerebral artery in stroke-prone SHR and Wistar rats. The infarct area was measured in 15 sections stained with hematoxylin and eosin ($n = 10$). $^{**}p < 0.01$, significantly different from Wistar rats group (Student's t-test).

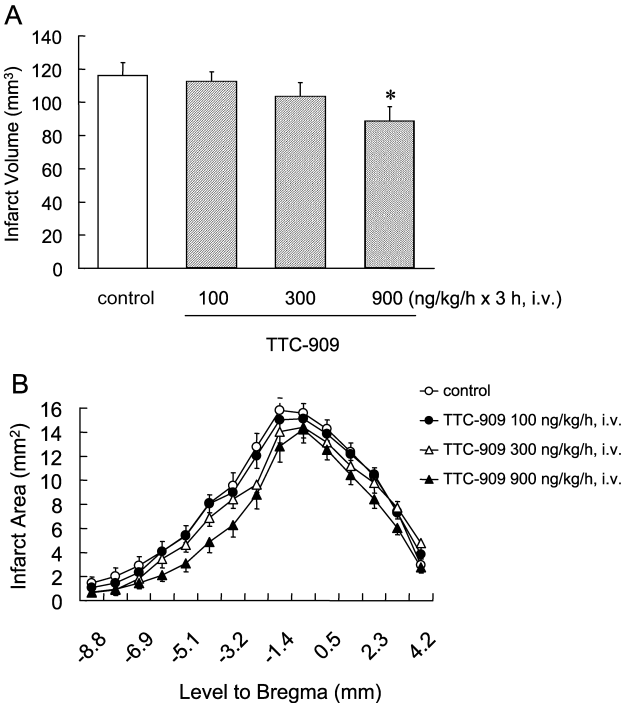

Fig. 3. Effect of TTC-909 on infarct volume (A) and infarct area of each section (B) 24 h after photochemically induced thrombotic occlusion of the middle cerebral artery in stroke-prone SHR. Vehicle or TTC-909 in doses of 100, 300 and 900 ng/kg/h was intravenously infused for 3 h, starting immediately after the end of 10 min of photoirradiation. Each point represents the mean ± S.E.M. ($n = 13$). $^{*}p < 0.05$, significantly different from vehicle-treated group (Dunnett's test).

2.5. Physiological parameters

Physiological parameters before and after the infusion of TTC-909 were measured in thrombotic middle cerebral artery occluded animals other than those used for histopathological examination. Blood pressure was measured via an arterial cannula inserted into the carotid artery, using a pressure transducer (AP-621G, Nihon Kohden). Hematological parameters (hematocrit (%), pH, pO_2 (mm Hg), pCO_2 (mm Hg)) were measured using hematological analyzer i-STAT (I-STAT).

2.6. Statistical analysis

Regarding differences between Wistar rats and stroke-prone SHR in infarct volume, induced by thrombotic occlusion of the middle cerebral artery, statistical analysis was made using Student's t-test following an F-test for analysis of variance. For evaluation of the effect of TTC-

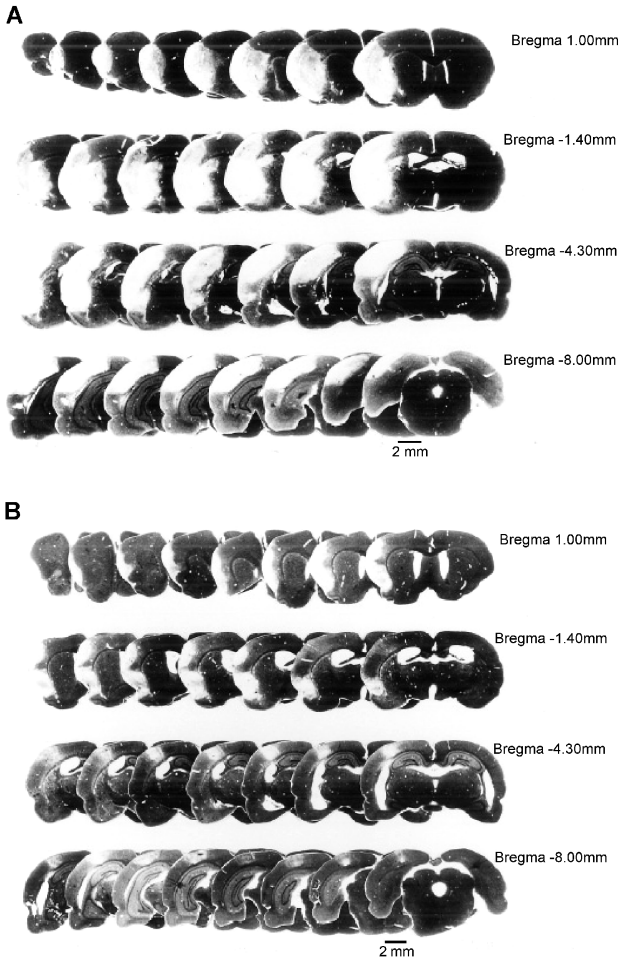

Fig. 4. Representative serial coronal sections of the whole brain in vehicle-treated animals (A) and TTC-909 (900 ng/kg/h)-treated animals (B). The decreased intensity of the hematoxylin and eosin stained region is the infarction. The numbers show the stereotaxic level relative to Bregma (mm), according to the atlas of Paxinos and Watson (1997).

909 and ozagrel on infarct volume induced by thrombotic occlusion of the middle cerebral artery, Bartlett's test followed by Dunnett's test were used. For physiological variables, Student's *t*-test between the vehicle-treated and TTC-909-treated groups at each time of measurement was used. *p* values less than 0.05 were regarded as statistically significant.

3. Results

3.1. Cerebral infarction in Wistar rats and stroke-prone SHR

Infarct volume and infarct area in each section are shown in Fig. 2. In Wistar rats, the cerebral infarction was limited to the cerebral cortex, the infarct volume being 51.3 ± 5.2 mm^3. In stroke-prone SHR, cerebral infarction was induced in the cerebral cortex and striatum 24 h after thrombotic occlusion of the middle cerebral artery, the infarct volume being 136.0 ± 7.8 mm^3.

3.2. Effects of TTC-909 and ozagrel on cerebral infarction

The effect of TTC-909 on cerebral infarction 24 h after photochemically induced thrombotic occlusion of the middle cerebral artery in stroke-prone SHR is shown in Fig. 3. It is known that prostacyclin decreases blood pressure in high doses. While TTC-909 did not have a hypotensive effect in doses that were used in this study (100–900 ng/kg/h), TTC-909 in a higher dose (2000 ng/kg/h) had a slight hypotensive effect (in house data). It is assumed that the fall in blood pressure, reduced of cerebral blood flow, resulted in aggravation of the cerebral infarction. Therefore, we used TTC-909 doses of up to 900 ng/kg/h, doses which did not cause hypotension, in this study.

When the vehicle in a volume of 1 ml/kg/h was intravenously infused for 3 h, starting immediately after the end of the 10-min photoirradiation, cerebral infarction was induced in the cerebral cortex and striatum, and the infarct volume was 116.2 ± 7.8 mm^3. When TTC-909 in doses of 100, 300 and 900 ng/kg/h was intravenously infused for 3 h, the infarct volume was dose-dependently reduced, an effect

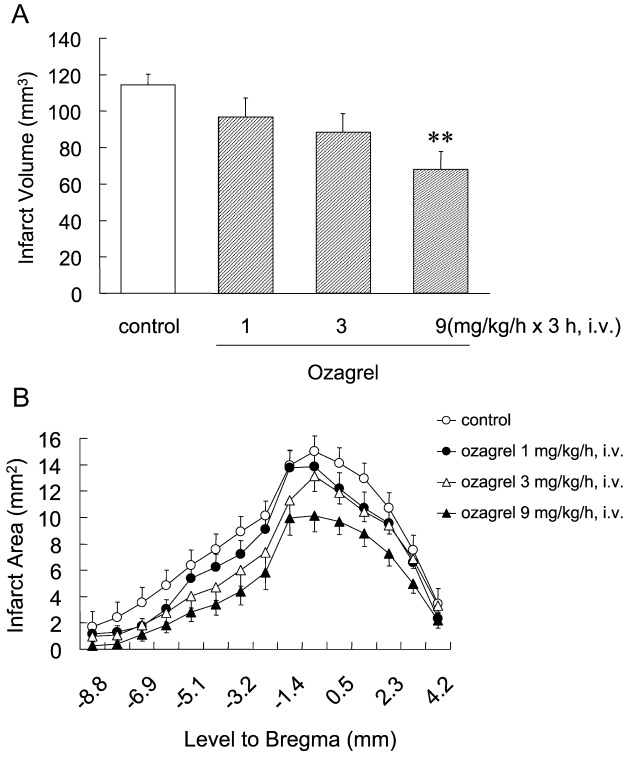

Fig. 5. Effect of ozagrel on infarct volume (A) and infarct area of each section (B) 24 h after photochemically induced thrombotic occlusion of the middle cerebral artery in stroke-prone SHR. Vehicle or ozagrel in doses of 1, 3 and 9 mg/kg/h was intravenously infused for 3 h, starting immediately after the end of 10 min of photoirradiation. Each point represents the mean ± S.E.M. ($n = 15$). $^{**}p < 0.01$, significantly different from vehicle-treated group (Dunnett's test).

Table 1
Physiological variables before infusion and at the end of 3 h infusion of vehicle or TTC-909 in stroke-prone SHR subjected to photochemically induced thrombotic occlusion of middle cerebral artery

	Before the infusion		At the end of the infusion	
	Control	TTC-909	Control	TTC-909
MABP (mm Hg)	207 ± 4	208 ± 7	214 ± 6	209 ± 10
RT (°C)	35.9 ± 0.3	35.8 ± 0.1	37.3 ± 0.1	37.1 ± 0.4
Hct (% PCV)	49.5 ± 0.6	49.7 ± 0.5	53.3 ± 0.7	54.2 ± 0.9
pH	7.422 ± 0.004	7.420 ± 0.003	7.411 ± 0.010	7.402 ± 0.011
pO_2 (mm Hg)	68.2 ± 2.0	68.0 ± 1.9	90.7 ± 2.7	87.7 ± 2.4
pCO_2 (mm Hg)	48.0 ± 0.5	46.9 ± 0.5	35.5 ± 1.2	37.4 ± 1.4

Each value represents the mean ± S.E.M. ($n = 6$).
MABP: mean arterial blood pressure.
RT: rectal temperature.
Hct: hematocrit.

that was statistically significant with a dose of 900 ng/kg/h (88.6 ± 8.6 mm^3, $p < 0.05$) in the TTC-909-treated group. Fig. 4 shows representative serial coronal sections of the whole brain in vehicle-treated animals (A) and TTC-909 (900 ng/kg/h)-treated animals (B).

The effect of ozagrel on cerebral infarction 24 h after photochemically induced thrombotic occlusion is shown in Fig. 5. When the vehicle in a volume of 1 ml/kg/h was intravenously infused for 3 h, starting immediately after the end of 10-min photoirradiation, the infarct volume was 114.4 ± 6.0 mm^3. When ozagrel in doses of 1, 3 and 9 mg/kg/h was intravenously infused for 3 h, the infarct volume was dose-dependently reduced and was statistically significant with a dose of 9 mg/kg/h (67.9 ± 10.2 mm^3, $p < 0.01$).

3.3. Physiological parameters

Physiological parameters before and after the infusion of TTC-909 were measured in thrombotic middle cerebral artery-occluded animals. All parameters in the TTC-909-treated group were not significantly different from those in the vehicle-treated group, before or after the infusion of TTC-909 (Table 1).

4. Discussion

Cerebral infarction 24 h after photochemically induced thrombotic occlusion of the middle cerebral artery above the rhinal fissure was investigated in Wistar rats and stroke-prone SHR. While cerebral infarction was limited to cerebral cortex in Wistar rats, it occurred not only in the cerebral cortex but also in the striatum in stroke-prone SHR. The infarct volume was larger in stroke-prone SHR than in Wistar rats. It has been reported that the lower limit of cerebral blood flow autoregulation is shifted to a higher level and that cerebrovascular resistance is increased in SHR (Fujishima and Omae, 1976a) and in stroke-prone SHR (Coyle, 1987). Furthermore, a markedly decreased cerebral perfusion pressure after bilateral carotid occlusion in SHR resulted in ischemic brain energy metabolism (Fujishima et al., 1975; Fujishima and Omae, 1976b) and ischemic infarction (Ogata et al., 1976). In focal ischemia, the narrow anastomoses between the middle cerebral artery and the anterior or posterior cerebral artery at cortical arterial boundary zones in stroke-prone SHR compared with normotensive rats restrict blood flow into the territory of the occluded middle cerebral artery, resulting in large infarcts (Coyle and Heistad, 1991). Our present findings are consistent with these results, showing 1.5 times larger infarction in stroke-prone SHR than in Wistar rats. These results suggest that the photochemically induced thrombotic occlusion model of middle cerebral artery in stroke-prone SHR is very useful, because the cerebral infarction is stable, large enough and reproducible.

Our previous study showed that cerebral infarction was produced only in the ipsilateral cerebral cortex but not in the ipsilateral basal ganglia when the middle cerebral artery above the rhinal fissure was occluded permanently with a microbipolar coagulation in stroke-prone SHR (Okuyama et al., 1991). In contrast, cerebral infarction was produced not only in the cerebral cortex but also in the striatum when the same region of middle cerebral artery was occluded by thrombus formation in stroke-prone SHR. In the model of photochemically induced thrombotic occlusion of the middle cerebral artery, thrombus is formed by a photochemical reaction between rose bengal and green light (Watson et al., 1985). This reaction causes endothelial injury followed by platelet adhesion, aggregation and formation of a platelet and fibrin-rich thrombus at the site of the reaction (Umemura et al., 1993). Once platelets are activated and aggregated, alpha and dense granules release packaged products such as serotonin and platelet factor 4, which can activate surrounding platelets to promote further platelet adhesion and aggregation (Weksler, 1992). Thus, in the present thrombotic stroke model, thrombus might be formed even in the middle cerebral artery ventral to the rhinal fissure which sends blood to the striate branches.

Takamatsu et al. (1998, 2000) reported that a reperfusion-like phenomenon may be involved in the progress of brain damage in the model of photochemically induced throm-

botic occlusion of the middle cerebral artery. When the blood flow was monitored for 4 and 24 h after the photoirradiation in this thrombotic stroke model, blood flow was not restored within 4 h, but for 24 h after photoirradiation (in house data). It is thought that reperfusion disrupts the blood–brain barrier and exacerbates edema formation (Yang and Betz, 1994). Reoxygenation during reperfusion provides oxygen as a substrate for numerous enzymatic oxidation reactions that produce reactive oxidants (Chan, 1994, 1996). Therefore, the production of a larger infarction in the thrombotic model than in the permanent middle cerebral artery occlusion model might be attributed to the reperfusion phenomenon.

TTC-909 has a vasorelaxant effect on the contraction induced by prostaglandin $F_{2\alpha}$ and U-46619, a thromboxane A_2 receptor agonist, in basilar, coronary, renal, mesenteric and femoral arteries in vitro (Sawada et al., 1995). Shima et al. (1995) showed that TTC-909 improved changes in the microcirculation in postischemic tissues, particularly in the ischemic rim and the surrounding area, and prevented the development of ischemic edema following permanent occlusion of the middle cerebral artery in stroke-prone SHR. From these findings, the inhibitory effect of TTC-909 on the cerebral infarction produced by photochemically induced thrombotic occlusion of the middle cerebral artery is thought to be due to improvement in cerebral blood flow in the ischemic penumbra. However, Kawai et al. (1995) reported that microthrombi were generated in the surrounding ischemic region following thrombotic occlusion of the middle cerebral artery, leading to the progression of ischemic legions. Microthrombi may be formed from circulating platelets that are activated or aggregated in the damaged middle cerebral artery vessels, and may contribute to secondary brain damage. There is a possibility that TTC-909 reduces the formation of microthrombi, since clinprost, its active metabolite TEI-7165, and TTC-909 inhibit platelet aggregation and platelet adhesion in vitro (Inoue et al., 1995b) and ex vivo (Inoue et al., 1995a), respectively.

In addition to the vasorelaxant and antiplatelet effects of TTC-909, it also has direct protective effects against neuronal damage induced by cerebral ischemia. Yamashita et al. (1996) reported that TTC-909, given intravenously 10 min after transient forebrain ischemia, protected against delayed neuronal death in the CA1 pyramidal cell layer of the hippocampus in stroke-prone SHR. TEI-7165, infused into the lateral ventricle of gerbils after 3 min forebrain ischemia, protected against delayed neuronal death in the CA1 pyramidal cell layer of the hippocampus (Matsuda et al., 1997). Clinprost and TTC-909 seem to pass through the blood–brain barrier and are hydrolyzed into TEI-7165 by esterase. A novel prostacyclin receptor showing high affinity for TEI-7165 (isocarbacyclin) has been detected in a variety of brain regions, such as the thalamus, lateral septal nucleus, hippocampus, cerebral cortex, striatum and dorsal cochlear nucleus (Takechi et al., 1996). This novel subtype has been shown to exist on neuronal cells. Although the function of this subtype is not clear, these findings suggest that TTC-909 has protective effects against ischemic damage to CA1 pyramidal neurons as a result of a direct action of TEI-7165 after it enters the brain through the blood–brain barrier.

Ozagrel, a thromboxane A_2 synthetase inhibitor, infused after thrombus formation reduced the infarct volume significantly 24 h after photochemically induced thrombotic occlusion of the middle cerebral artery in stroke-prone SHR. It has been reported that ozagrel has a beneficial effect in the decrease in local cerebral blood flow after bilateral carotid artery occlusion in stroke-prone SHR (Ishikawa et al., 1991). Ozagrel inhibits the platelet aggregation induced by arachidonic acid and collagen in rabbit platelets (Naito et al., 1983). However, it has a very low blood–brain barrier permeability (Nishiyama et al., 1986) whereas TTC-909 and clinprost can pass through into the brain (Kohno et al., 1995; Minagawa et al., 1996). In addition, ozagrel had no protective effect against neuronal damage in the CA1 pyramidal cell layer of the stroke-prone SHR hippocampus following transient bilateral carotid artery occlusion (Yamashita et al., 1996). Thus, ozagrel might not have a direct protective effect against ischemic neuronal damage. These findings regarding to ozagrel suggest that the inhibition of cerebral infarction by ozagrel is mediated by the improvement in cerebral blood flow and in part by an antiplatelet effect, but not by a neuroprotective effect.

In conclusion, the inhibition of cerebral infarction by TTC-909 could be mediated by its vasodilator and antiplatelet activity, and possibly by a neuroprotective effect, in photochemically induced thrombotic occlusion of the middle cerebral artery in stroke-prone SHR.

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