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Effect of TTC-909 on Cerebral Infarction Following Permanent Occlusion of the Middle Cerebral Artery in Stroke Prone Spontaneously Hypertensive Rats

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Abstract. We investigated the effect of TTC-909, a drug 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 cerebral infarction 7 days after permanent occlusion of the middle cerebral artery (MCA) in stroke prone spontaneously hypertensive rats (SHRSP). Under the anesthesia, the MCA was permanently occluded above the rhinal fissure. In schedule 1, vehicle or TTC-909 was injected i.v. once daily over 7 days starting immediately after MCA occlusion. In schedule 2, vehicle or TTC-909 was infused for 3 h starting immediately after MCA occlusion. In schedule 3, vehicle or TTC-909 was infused for 3 h starting immediately after MCA occlusion followed by bolus injection once daily over 6 days. Seven days later, the infarct volume was estimated following hematoxylin and eosin staining. Cerebral infarction produced by permanent occlusion of MCA was limited to the cerebral cortex. While this volume was reduced significantly in case of schedule 3, the infarct volume was not reduced significantly in schedules 1 and 2. Ozagrel, a thromboxane A_2 synthetase inhibitor, had no effect on the infarct volume in schedule 3. These results suggest that cerebral infarction can be developed progressively not only during the first few hours but also after a permanent occlusion of MCA in SHRSP. TTC-909 inhibited cerebral infarction, maybe by improving cerebral blood flow and by protecting against neuronal damage.

Keywords: stroke, focal cerebral ischemia, middle cerebral artery occlusion, stroke-prone spontaneously hypertensive rats, prostacyclin

Introduction

Reproducible animal models of stroke or focal ischemic infarction are crucial for studying the pathophysiology of ischemic brain injury and for evaluating different types of therapy. Several animal models of middle cerebral artery (MCA) occlusion have been developed and widely used as stroke models. In some models using normotensive rats, not only MCA but also common carotid artery were occluded to produce cerebral infarction (1, 2). Tamura et al. (3) reported a procedure for occluding proximal MCA in normotensive rats. However, this procedure is technically difficult and invasive.

In hypertensive rats, MCA occlusion gives rise to much larger infarcts than seen in normotensive strains. Coyle and Jokelainen (4) used a less invasive surgical approach with MCA occlusion above the rhinal fissure. While cerebral infarction in normotensive rats did not

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occur, a reproducible focal cerebral infarction did develop after the same occlusion in stroke-prone spontaneously hypertensive rats (SHRSP) (4, 5). Our previous study showed that cerebral infarction was produced in the ipsilateral cerebral cortex 7 days after the permanent occlusion of MCA above the rhinal fissure with a micro-bipolar coagulation in SHRSP (6). Thus, it seems that the MCA occlusion model in SHRSP is a unique and convenient model for studying cerebral infarction and/or stroke.

Generation of prostacyclin (prostaglandin I_2, PGI_2) from endothelial cells is crucial for the maintenance of homeostasis of cerebral blood flow, as PGI_2 counteracts the thromboxane generated from platelets (7). Because of its highly potent vasodilating and antiplatelet activity, PGI_2 has been used to treat various types of thrombotic disorders such as ischemic cerebrovascular diseases (8). The clinical assessment of PGI_2 might be useful in open trials (9), but not in double-blind trials (10, 11). The inconsistency of PGI_2 efficiency may be accounted for by the instability of PGI_2 properties, and the half-life of PGI_2 under in vivo conditions is about 3 min (12). In addition, the hypotensive effect of PGI_2 might reduce collateral blood flow in the ischemic area and offset any direct beneficial effects (13).

Isocarbacyclin methylester (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) and its active metabolite, isocarbacyclin (TEI-7165), are chemically stable PGI_2 analogues. TTC-909 is a drug preparation of clinprost incorporated into lipid microspheres (LM). The hypothetical sequence of events for TTC-909 to exert pharmacological effects is as follows: the LM would deliver clinprost to most tissues including the blood and the brain, clinprost would be released gradually from the LM, and then the clinprost would be hydrolyzed to TEI-7165 by esterase action to exert pharmacological activity. Both clinprost and TEI-7165 inhibit platelet aggregation and platelet adhesion in vitro and suppress prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contraction of isolated canine arteries (14). TTC-909 also has vasodilative and anti-platelet activity in vivo, similar to PGI_2 (15). It was shown that TTC-909 improved changes in microcirculation and glucose utilization following permanent occlusion of MCA in SHRSP (16). TTC-909 improved the increase of permeability in blood-brain barrier and prevented ischemic brain edema in this permanent occlusion model of MCA. On the other hand, ozagrel, a highly selective inhibitor of thromboxane (TX) A_2 synthase, is prescribed for thromboembolic disorders, cerebrovascular diseases, ischemic heart diseases, and asthma. Ozagrel inhibits both spasm of the basilar artery and the decreases regional cerebral blood flow by reducing TXA_2 production and increasing PGI_2 production (17).

In the present study, effects of TTC-909 and ozagrel on cerebral infarction induced by permanent occlusion of MCA in SHRSP were investigated.

Materials and Methods

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

Materials

TTC-909 was from Taisho Pharmaceutical Co., Ltd. (Tokyo) (Fig. 1). Intralipid 10%® (vehicle) was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo). TTC-909 was diluted with intralipid 10% as the vehicle. The schedules of treatment with TTC-909 were as follows: In schedule 1, vehicle or TTC-909 was injected i.v. once daily over 7 days starting immediately after MCA occlusion. In schedule 2, vehicle or TTC-909 was infused for 3 h starting immediately after MCA occlusion. In schedule 3, vehicle or TTC-909 was infused for 3 h starting immediately after MCA occlusion followed by bolus injection once daily over 6 days.

Ozagrel (Xambone injection®) was purchased from Kissei Pharmaceutical Co., Ltd. (Matsumoto) and was dissolved in saline as the vehicle. Ozagrel was infused for 3 h immediately after MCA occlusion followed by bolus injection once daily over 6 days. TTC-909, ozagrel, or each vehicle was infused or injected through the tail vein in a volume of 1 mL/kg.

Animals

Male SHRSP (n = 209), 12 – 20 weeks of age (Taisho Pharmaceutical Co., Ltd.), were housed in an air condi-

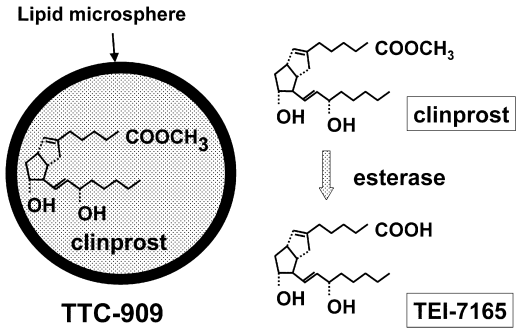

Fig. 1. Chemical structures of clinprost and isocarbacyclin (TEI-7165) and a model of lipid microsphere formation (TTC-909). TTC-909 is a drug preparation of clinprost incorporated into lipid microspheres with a diameter of 0.2 μm. Clinprost is hydrolyzed to TEI-7165 by esterase.

tioned room at 22°C with 12-h light-dark schedule (lights on at 7:00 a.m.). The animals were provided OA-2 diet (Clea Co., Ltd., Tokyo) with free intake of tap water. There were no signs of spontaneous strokes in these SHRSP. The systolic blood pressure in each conscious SHRSP was measured using a rat-tail sphygmomanometer system (KN-210, Riken Kaihatsu, Inc., Tokyo). All the SHRSP we used had a systolic blood pressure over 180 mmHg.

Preparation of middle cerebral artery occlusion model

Rats were anesthetized with 2% halothane and anesthesia was maintained with 1% halothane in room air, and the body temperature was kept at 37 ± 1°C using a heat pad. Under an operating microscope (MD-II; Nagashima, Inc., Tokyo), the left MCA was exposed through a burr-hole craniectomy of 2-3 mm in diameter performed by a transtemporal route, without damage to the zygomatic bone, and then the artery was dissected free of the meninges. The MCA above the rhinal fissure was occluded with a microbipolar coagulation (MICRO-3D; Mizuho Ika Kogyo, Inc., Tokyo), using a low power setting and was cut to ensure completeness of the vascular occlusion. The MCA was observed through the dura matter. The soft tissuees were put back into place and the skin was sutured. The rats were weaned from the respirator for anesthesia and set up in bolemann cages for the infusion of drugs.

Infarct size

At 7 days after permanent occlusion of MCA, the rats were anesthetized with ether; 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 & eosin (H.E.). 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, Inc., St-Catharines, Canada) by an investigator who was unaware of 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)

Physiological parameters

In the schedule 3, physiological parameters before the infusion of TTC-909, at the end of the infusion of TTC-909, 1 and 6 days after the MCA occlusion (15 min after each bolus injection of TTC-909) were measured in MCA occluded animals separate from those used for histopathological examination. Blood pressure was measured via an arterial cannula inserted in to the carotid artery, using a pressure transducer (AP-621G; Nihon Kohden, Co., Ltd., Tokyo). Haematological parameters, hematocrit (%), pH, PO_2 (mmHg), and PCO_2 (mmHg), were measured using hematological analyzer i-STAT (I-STAT Co., East Windsor, NJ, USA).

Statistical analyses

To evaluate the effects of TTC-909 and ozagrel on infarct volume induced by permanent occlusion of MCA, 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 to be statistically significant.

Results

Effects of TTC-900 and ozagrel on cerebral infarction

Effects of TTC-909 on cerebral infarction 7 days after permanent occlusion of MCA in SHRSP are shown in Fig. 2. In schedule 1 (Fig. 2a), cerebral infarction was induced in the cerebral cortex and the infarct volume was 77.5 ± 9.7 mm^3 (mean ± S.E.M.) in the vehicle treated group. When TTC-909 in doses of 30, 100, and 300 ng/kg per day was intravenously injected over 7 days, the infarct volume was not significantly reduced. In schedule 2 (Fig. 2b), the infarct volume was 92.2 ± 7.6 mm^3 in the vehicle-treated group. When TTC-909 in doses of 30, 100, 300, and 900 ng/kg per hour was intravenously infused for 3 h starting immediately after MCA occlusion, the infarct volume was not significantly reduced. In schedule 3 (Fig. 2: c and d), the infarct volume was 94.7 ± 6.2 mm^3 in the vehicle-treated group. When TTC-909 was infused for 3 h starting immediately after MCA occlusion in doses of 30, 100, 300, and 900 ng/kg per hour followed by bolus injection once daily over 6 days in a dose of 300 ng/kg per day, the infarct volume was dose-dependently reduced. The effect of TTC-909 was statistically significant with doses of 300 ng/kg per hour + 300 ng/kg per day ($P<0.05$) and 900 ng/kg per hour + 300 ng/kg per day ($P<0.01$), for 3 h-infusion + for daily injection, respectively.

Figure 3 shows a representative of the serial coronal sections of the whole brain in vehicle-treated animals (a) and TTC-909 (900 ng/kg per hour + 300 ng/kg per day)-treated animals (b) in schedule 3. In the TTC-909 treated rat, cerebral infarction was reduced.

Effect of ozagrel on cerebral infarction 7 days after

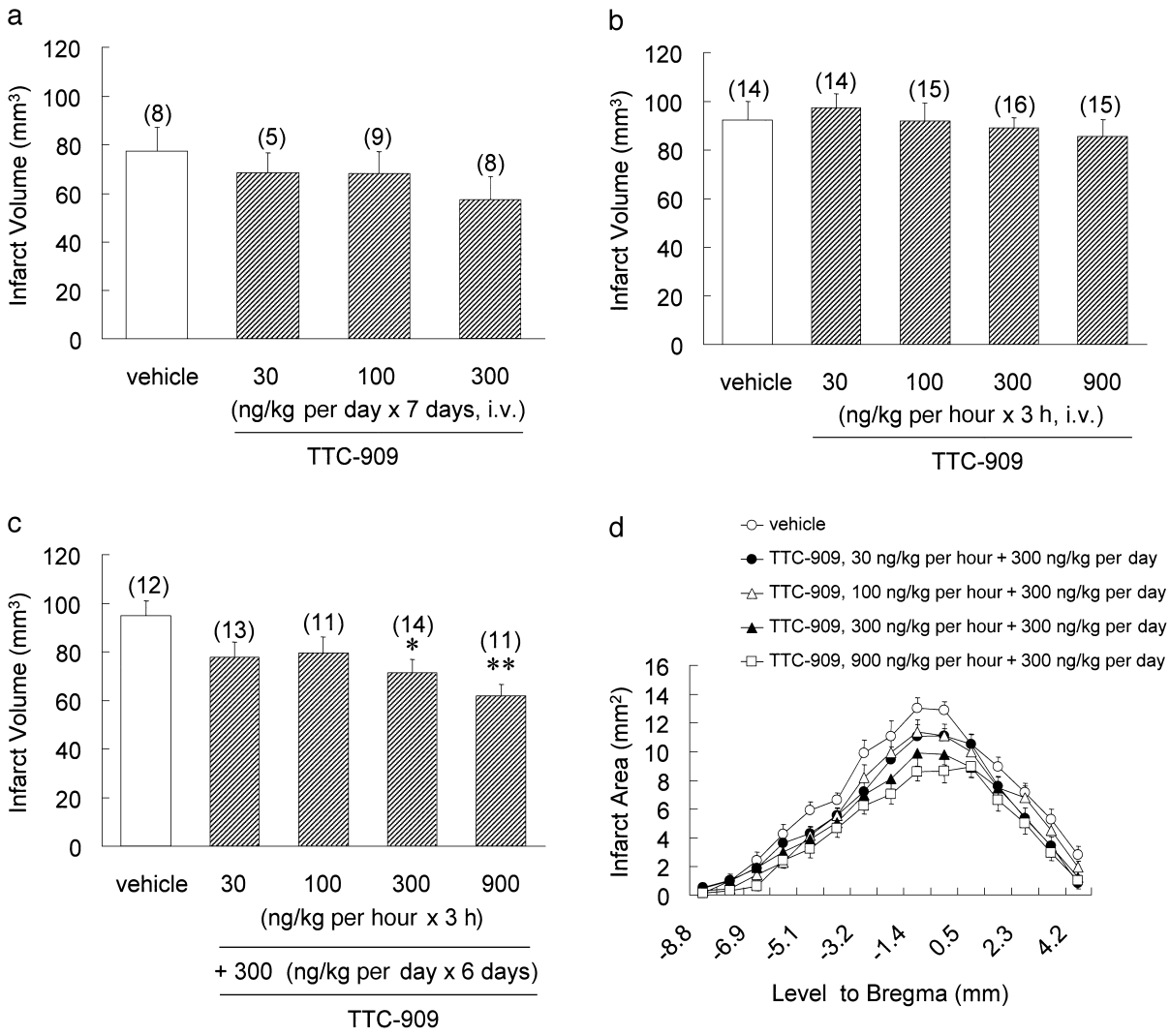

Fig. 2. Effects of TTC-909 on cerebral infarction 7 days after permanent occlusion of MCA in SHRSP. a: schedule 1, b: schedule 2, c and d: schedule 3. Panels a, b, and c show the infarct volume (mm^3) and panel d shows the infarct area (mm^2) of each section. Each point represents the mean ± S.E.M. (n = 5 – 16). **P*<0.05, ***P*<0.01, significantly different from the vehicle-treated group (Dunnett's test).

permanent occlusion of MCA is shown in Fig. 4. Ozagrel or vehicle was given in schedule 3. In the vehicle-treated group, the infarct volume was 88.7 ± 5.9 mm^3. When ozagrel was infused for 3 h starting immediately after MCA occlusion in doses of 1, 3, and 9 mg/kg per hour followed by bolus injection once daily over 6 days in a dose of 10 mg/kg per day, the infarct volume was not significantly reduced.

Physiological parameters

In schedule 3, physiological parameters were measured before the infusion of TTC-909, at the end of the infusion of TTC-909, 1 and 6 days after the MCA occlusion (15 min after each bolus injection of TTC-909) in MCA occluded animals. All parameters in the TTC-909-treated group were not significantly different from those in the vehicle-treated group, before and after the infusion of TTC-909 (Table 1).

Discussion

TTC-909 significantly reduced the infarct volume 7 days after the permanent occlusion of MCA, when it was infused for 3 h starting immediately after MCA occlusion followed by bolus injection once daily over 6 days (schedule 3). TTC-909 is considered to be effective because the infarct volume was reduced even at 7 days after permanent occlusion of MCA.

In the present study, TTC-909 did not reduce the infarct volume 7 days after the permanent occlusion

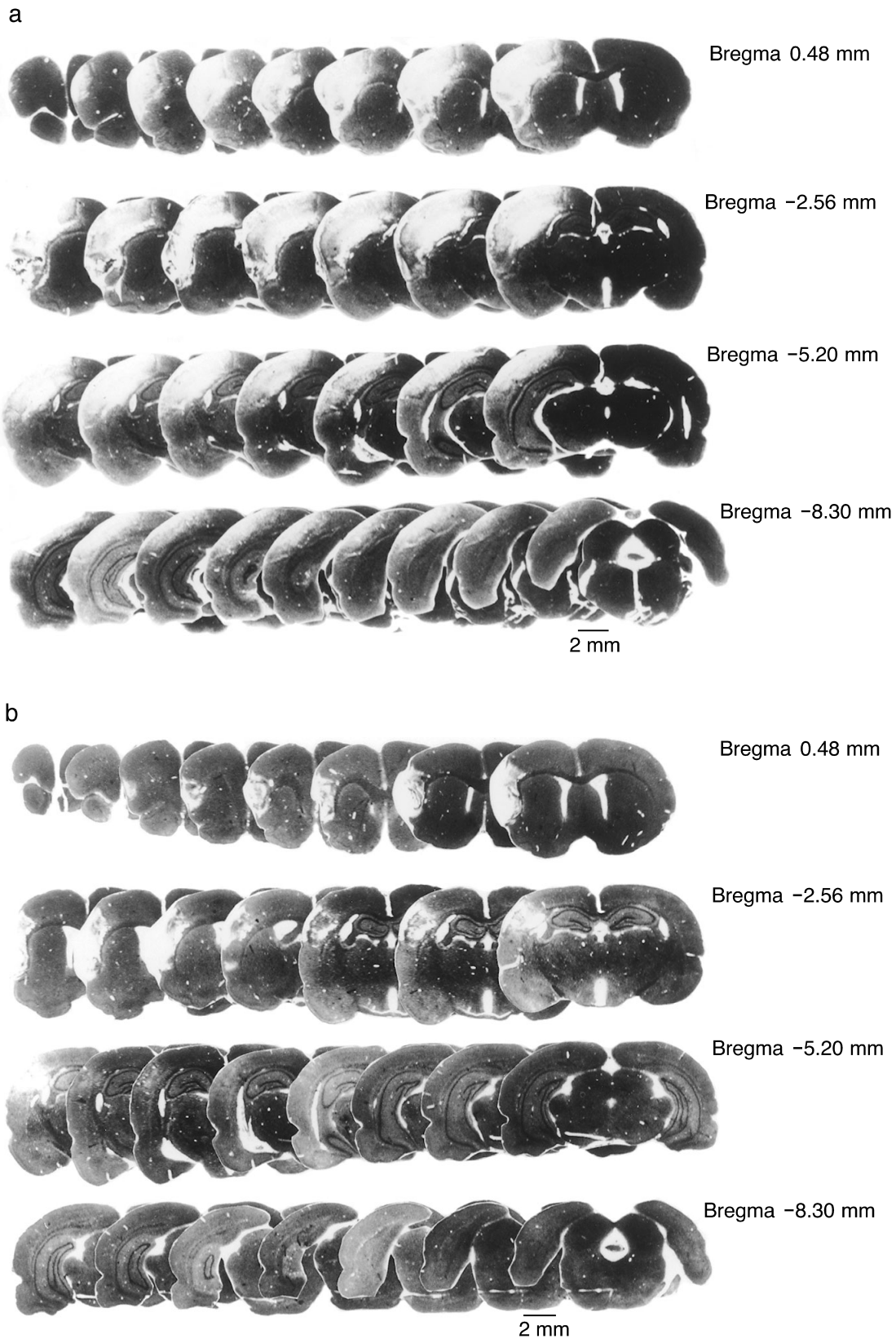

Fig. 3. Representative of serial coronal sections of the whole brain in vehicle-treated animals (a) and TTC-909 (900 ng/kg per hour for 3 h + 300 ng/kg per day × 6 days)-treated animals (b) in the schedule 3. The decreased intensity of the H.E.-stained region is the infarction. The numbers show the stereotaxic level relative to the Bregma (mm), according to the atlas of Paxinos and Watson (33).

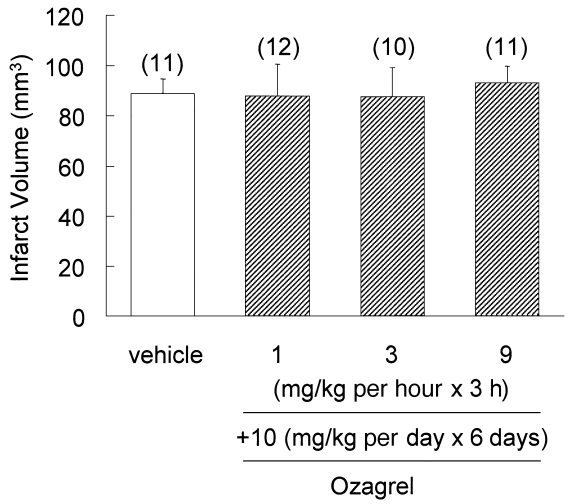

Fig. 4. Effect of ozagrel in schedule 3 of treatment on the infarct volume (mm^2) 7 days after permanent occlusion of MCA in SHRSP. Vehicle or ozagrel (1, 3, and 9 mg/kg per hour for 3 h + 10 mg/kg per day × 6 days) was infused for 3 h starting immediately after MCA occlusion occlusion followed by bolus injection once daily over 6 days. Each point represents the mean ± S.E.M. (n = 10 – 12). There were no significant differences between the vehicle-treated and ozagrel-treated groups.

of MCA, neither in schedule 1 nor in schedule 2. Takagaki et al. (18) showed that infarction in the striatum developed during the early stage (within 6 h) after MCA occlusion and that development of cortical infarction was slower than that seen in the striatum after permanent occlusion of MCA according to the method of Tamura et al. (3). On the other hand, it was reported that the ischemic region in the MRI analysis increased progressively over a few days after the occlusion of MCA in rats (19, 20). Furthermore, neuroprotective effects have been noted with the delayed treatment of some drugs, such as the Na^+/Ca^{2+} channel blocker NS-7 (21) and the low molecular weight heparin enoxaparin (22), in rats subjected to MCA occlusion. Thus, some cell responses to ischemic insult and changes in hematoleology still may continue after the few hours following MCA occlusion. The results in the present study suggest that cerebral infarction progressively develops both during the first few hours and beyond and that the daily bolus injection of TTC-909 since the next day after MCA occlusion prevented the development of neuronal damage. In addition, since the concentration of clinprost

Table 1. Physiological variables before infusion, at the end of the infusion of vehicle or TTC-909 (900 ng/kg per hour), and 1 and 6 days after the MCA occlusion (15 min after each bolus injection of vehicle or TTC-909, 300 ng/kg per day) in SHRSP subjected to permanent occlusion of MCA in schedule 3

	Before the infusion (before MCA occlusion)		Post ischemia at the end of the infusion	
	vehicle (n = 6)	TTC-909 (n = 6)	vehicle (n = 6)	TTC-909 (n = 6)
HR (beats/min)	380.5 ± 11.8	406.3 ± 11.8	378.3 ± 22.2	396.0 ± 13.6
MAP (mmHg)	166.3 ± 3.2	165.0 ± 8.7	139.0 ± 14.3	154.7 ± 8.9
RT (°C)	37.63 ± 0.11	37.42 ± 0.28	37.72 ± 0.16	37.33 ± 0.33
glucose (mg/dL)	109.7 ± 9.7	115.2 ± 10.6	148.5 ± 17.6	143.8 ± 15.6
Hct (%PCV)	47.7 ± 1.0	47.2 ± 1.1	44.0 ± 1.8	45.2 ± 2.5
Hb (g/dL)	16.3 ± 0.3	15.8 ± 0.4	14.8 ± 0.7	15.2 ± 0.9
pH	7.45 ± 0.02	7.46 ± 0.02	7.44 ± 0.01	7.45 ± 0.02
pCO_2 (mmHg)	37.5 ± 2.0	35.7 ± 4.0	35.8 ± 2.2	34.6 ± 2.6
pO_2 (mmHg)	94.0 ± 4.5	97.0 ± 8.2	101.0 ± 4.1	102.3 ± 3.8

	Post ischemia			
	1 day		6 day	
	vehicle (n = 6)	TTC-909 (n = 7)	vehicle (n = 7)	TTC-909 (n = 9)
HR (beats/min)	380.1 ± 13.3	400.7 ± 13.6	418.9 ± 8.9	415.0 ± 9.6
MAP (mmHg)	153.4 ± 5.8	155.0 ± 4.0	171.6 ± 5.9	173.8 ± 2.1
RT (°C)	37.6 ± 0.22	37.7 ± 0.22	37.9 ± 0.29	38.2 ± 0.17
glucose (mg/dL)	121.1 ± 14.7	109.7 ± 11.7	173.4 ± 25.7	145.8 ± 10.7
Hct (%PCV)	45.4 ± 1.3	46.6 ± 1.3	45.4 ± 1.5	46.3 ± 1.0
Hb (g/dL)	15.6 ± 0.4	15.9 ± 0.5	15.7 ± 0.5	15.7 ± 0.3
pH	7.43 ± 0.01	7.43 ± 0.07	7.43 ± 0.01	7.45 ± 0.01
pCO_2 (mmHg)	39.4 ± 0.9	37.3 ± 1.1	36.7 ± 1.5	36.7 ± 1.1
pO_2 (mmHg)	96.8 ± 2.3	96.3 ± 2.2	99.3 ± 1.3	100.8 ± 2.4

Each value represents the mean ± S.E.M. HR: heart rate, MBP: mean arterial pressure, RT: rectal temperature, Hct: hematocrit, Hb: hemogrobin.

or TEI-7165 will rapidly decrease because of the short half life (23), the maintenance of plasma concentration of TTC-909 above the constant level for a few hours after the MCA occlusion may be important to prevent formation of cerebral infarction.

TTC-909 has a vasorelaxant effect on the contraction induced by $PGF_{2\alpha}$ and U-46619, a TXA_2-receptor agonist, in basilar, coronary, renal, mesenteric, and femoral arteries in vitro (14). In the previous study, we have investigated the effect of TTC-909 on cerebral blood flow in the same stroke model as the present study. The changes in microcirculation were improved in post-ischemic rim and the surrounding area 7 days after permanent occlusion of MCA, when TTC-909 was injected in a dose of 100 ng/kg per day for 7 days (16). From these findings, the inhibiting effect of TTC-909 on cerebral infarction following permanent occlusion of MCA could be due to improvement in cerebral blood flow in ischemic penumbra.

In addition, TTC-909 also has direct protective effects on neuronal damage induced by cerebral ischemia. We have reported that TTC-909, given intravenously 10 min after the transient forebrain ischemia, protected against delayed neuronal death in the CA1 pyramidal cell layer of the hippocampus in SHRSP (24). In fact, radioactivity was distributed rapidly in most tissues including the brain after intravenous administration of [^{3}H]TTC-909 in rats (23). Minagawa et al. (25) reported that lipid microsphere (LM) containing clinprost was transported through the blood-brain-barrier by endocytosis of simple diffusion of clinprost released from LM and transport of TEI-7165 generated by hydrolysis of clinprost in normal and ischemic rats. Furthermore, radiolabeling was densely detected on pyramidal and granular neurons in the hippocampal region as well as endothelial cells in the brains of gerbils treated with [^{3}H]TTC-909 after ischemic insult, while radiolabeling was detected only in endothelial cells in the brains of sham-operated gerbils (26). We have shown that the active metabolite of clinprost, TEI-7165, protected against delayed neuronal death in the CA1 pyramidal cell layer of the hippocampus when infused into the lateral ventricle of gerbils subjected to 3-min forebrain ischemia (27). So, it is assumed that systemically administered TTC-909 arrives at the ischemic penumbra via improving or increasing cerebral blood flow and then exerts protective effects against neuronal damages after penetration through the blood-brain-barrier.

The precise mechanisms of the neuroprotective effects of TTC-909 are not clear. A novel prostacyclin receptor showing high affinity to TEI-7165 has been detected in a variety of brain regions, such as thalamus, lateral septal nucleus, hippocampus cerebral cortex, striatum and dorsal cochlear nucleus (28). This novel subtype was shown to exist on neuronal cells. We (29) reported that TEI-7165 and clinprost inhibited the elevation of intracellular Ca^{2+} levels induced by BAY K8644, an L-type voltage-sensitive Ca^{2+} channel activator, in rat striatal slices. Furthermore, TEI-7165 and clinprost protected against BAY K8644-induced striatal dysfunction, under conditions of transient ischemia (29). These findings do suggest that TTC-909 has protective effects on ischemic neuronal as a result of a direct action of TEI-7165, possibly by inhibiting the elevation of intracellular Ca^{2+}.

In contrast to TTC-909, ozagrel, a TXA_2 synthetase inhibitor, had no inhibiting effect on infarct volume 7 days after permanent occlusion of MCA, when infused for 3 h starting immediately after MCA occlusion followed by bolus injection once daily over 6 days (schedule 3). In the other treatment schedules of ozagrel, 1 and 2, ozagrel also had no inhibitory effect on infarct volume (data not shown). It has been reported that ozagrel has an improving effect on the decrease in local cerebral blood flow after bilateral carotid artery occlusion in SHRSP (30). Ozagrel inhibits the platelet aggregation induced by arachidonic acid and collagen in rabbit platelets (31) as does TTC-909, clinprost, and TEI-7165. However, it has been reported that ozagrel has very low blood-brain barrier permeability (32). In addition, Ozagrel had no protective effect on neuronal damage in the CA1 pyramidal cell layer of the SHRSP hippocampus following transient bilateral carotid artery occlusion in contrast with TTC-909 (24). Thus, the mechanism of action of TTC-909 must be different from that of ozagrel. Further investigations is are needed to clarify the difference of mechanism between TTC-909 and ozagrel.

In conclusion, the inhibition of cerebral infarction by TTC-909 could be mediated by improvement in cerebral blood flow and passively by a neuroprotective effect in the permanent MCA occlusion model in SHRSP.

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