Effect of a Novel Thromboxane A2 Receptor Antagonist, S-1452, on Postischemic Brain Injury in Rats

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Background and Purpose: Arachidonic metabolites have been implicated in the development of cerebral injury after ischemia. Particular importance has been placed on the balance of thromboxane A2 and prostaglandin I2 because of its regulatory activity on platelet functions and arterial tone. The purpose of the present study was to shed light on the role of thromboxane B2 in postischemic brain injury.

Methods: We evaluated the effects of S-1452, a novel thromboxane A2 receptor antagonist, on brain edema, infarct areas, and survival rate in rats with middle cerebral artery occlusion. A transient middle cerebral artery occlusion model was produced by inserting a piece of silicon-coated nylon thread into the internal carotid artery.

Results: The ratio of plasma thromboxane B2 to 6-keto-prostaglandin F1a significantly rose at 0 hour (P<.05), 1 hour (P<.01), 3 hours (P<.05), and 12 hours (P<.05) and then nearly returned to the normal level at 24 hours after reperfusion following 1-hour occlusion. Pretreatment with S-1452 (5, 10, or 50 mg/kg PO) significantly attenuated the increase in postischemic water content in the cerebral cortex perfused by the anterior cerebral artery and the cerebral cortex perfused by the middle cerebral artery in a dose-dependent manner but slightly attenuated it in the caudate putamen 24 hours after reperfusion following 1-hour occlusion. Pretreatment with S-1452 (10 mg/kg PO) also significantly decreased the areas of infarction in the front parts of the cerebrum. The survival rate of animals after 2 hours of occlusion tended to be improved by treatment with S-1452 (10 mg · kg⁻¹ · d⁻¹ PO), although there was no statistical significance.

Conclusions: Our results suggest that thromboxane A2 is closely related to postischemic brain injury in the early phase of recirculation and that S-1452 may have a protective effect on postischemic brain injury. (Stroke. 1993;24:2059-2065.)

Key Words • cerebral ischemia • thromboxane antagonists • rats

Many different mechanisms have been implicated in the development of cerebral injury after ischemia.1-5 Vasconstrictor prostanoids liberated under ischemia-reperfusion conditions have been considered mediators in many cerebrovascular disorders.6-10 Particular importance has been attached to the role of thromboxane A2 (TXA2) in causing platelet aggregation and vasconstriction leading to the reduction of microcirculatory blood flow in various organs.11 Under cerebral ischemia-reperfusion conditions, a massive amount of arachidonates are liberated, and they are converted to prostaglandins (PGs), leukotrienes (LTs), and other arachidonate metabolites.12-19 The increase in TXB2 levels was found to correlate with an increase in dysfunction in spinal cord ischemia.20 Other investigators have described the beneficial effects of thromboxane synthase inhibition in animal models of brain ischemia.21-24 (+)-(SZ)-7-[3-Endo-[phenylsulfonyl] amino]bicyclo-[2.2.1]hept-2-exo-y]heptanoic acid (S-1452) is a stable calcium salt of the (+)-isomer of S-145 that has been shown to be a potent and selective TXA2 receptor antagonist. S-145 has been found to be an effective blocker of TXA2-induced platelet aggregation and vasconstriction and to protect against collagen-induced ECG changes and thrombopenia and experimental bronchial asthma in rodents.25-30 Permanent middle cerebral artery (MCA) occlusion models in the rat have generally been used to assess the protective effects of various drugs against ischemic brain damage.31,32 However, in human ischemic stroke, there is a possibility that recirculation occurs frequently after focal ischemia, particularly in the case of cerebral embolism. Thus, a recirculation model after MCA occlusion may be useful for simulating focal ischemia in humans. In the present study, we used a transient MCA occlusion model in the rat that is produced by inserting a silicon-coated nylon thread from the external carotid artery (ECA) into the internal carotid artery (ICA).

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This MCA occlusion model is relatively simple and easy to reperfuse.

The aim of the present study was to shed light on the role of TXA$_2$ in postischemic brain injury. Thus, we evaluated the effects of S-1452 on brain edema, infarct areas, and survival rate in the rats with a transient right MCA occlusion.

Materials and Methods

We induced transient focal ischemia by right MCA occlusion in male Wistar rats by the modified method of Longa et al. adult male Wistar rats (270 to 320 g) that were specific pathogen free were anesthetized with a gas mixture of 70% N$_2$O–30% O$_2$–2% halothane. After a median incision of the neck skin, the right ECA was carefully dissected, and an 18-mm-long 4-0 nylon thread (Nitcho Kogyo Co, Ltd) precoated with silicon (Xantpren, Bayer Dental) mixed with a hardener (Optisol Activator, Bayer Dental) to increase the thickness of the distal half was inserted from the ECA lumen into the right ICA lumen to occlude the origin of the right MCA. The surgery was performed within 15 minutes with no bleeding. Body temperature was kept at 37°C with a heating pad.

After surgery, anesthesia was discontinued, and the rats were allowed free access to food and water until the next procedure was performed. Neurological deficits characterized by severe left-sided hemiparesis and right Horner’s syndrome were used as criteria for ischemic insult. Ischemic animals exhibited severe hemiparesis in the upper extremities with counterclockwise circling and rolling to the left side. Rats with convulsions, sustained consciousness disturbance, or no neurological deficits were excluded from further study. Most cases of convulsion and sustained unconsciousness were due to artificial subarachnoid hemorrhage (SAH) by rupture of intracranial ICA, and most cases of no neurological deficits were caused by unsuccessful MCA occlusion. Approximately 30% of the experimental animals were excluded by the above reasons, and the rest (approximately 70%) of the experimental animals were used as ischemic animals in further study.

After 1 or 2 hours of MCA occlusion, the thread was removed to allow complete reperfusion of the ischemic area via the right common carotid artery (CCA).

Rats were decapitated at 0 hour (just after reperfusion), 1 hour, 3 hours, 6 hours, 12 hours, and 24 hours after reperfusion following 1-hour transient MCA occlusion. For measurement of brain water content, brain samples of both hemispheres were taken from the cerebral cortex perfused by the anterior cerebral artery (ACA area), the cerebral cortex perfused by the MCA (MCA area), and the caudate putamen. The water content of these samples was calculated as follows: 100×(weight−dry weight)/weight (%). MCA and ACA areas were defined as described by Nagasawa and Kogure. Before the experiment, the extent of the ischemic area was clarified by transcardiac perfusion of three ischemic animals with carbon black. The colorless area was considered to correspond to the territory supplied by the occluded MCA. The cerebral cortex corresponding to the colorless area was taken as MCA area, and the remainder of the cortex was taken as ACA area from both hemispheres.

Before decapitation, blood was drawn from the right jugular vein to measure plasma TXB$_2$ and 6-keto-PGF$_{1\alpha}$. The blood in a tube containing ethylenediaminetetra-acetic acid (EDTA) and indomethacin was centrifuged at 2000g for 10 minutes at 4°C. A mixture of 0.95 mL of an EDTA solution (2% disodium EDTA and 0.8% NaCl adjusted to pH 7.4 with NaOH) with 0.05 mL of a 0.04 mol/L indomethacin solution dissolved in absolute ethanol was used for the treatment of 10 mL of blood. The plasma samples were frozen with dry ice powder immediately after separation and then stored at -80°C until the measurement of TXB$_2$ and 6-keto-PGF$_{1\alpha}$. Brain water content and plasma TXB$_2$ and 6-keto-PGF$_{1\alpha}$ in complete sham-operated rats also were measured (represented as normal in results and figures).

Radioimmunoassay of TXB$_2$ and 6-keto-PGF$_{1\alpha}$ was conducted after extraction of the plasma. The plasma was acidified to pH 3.0 with 1N HCl and loaded on an Amnprep C2 100-mg column (Amersham, UK), which was washed with 2 mL of 100% methanol and 2 mL of water before use. Next, the column was washed successively with 5 mL of water, 5 mL of 10% ethanol, and 5 mL of hexane. Prostaglandins were finally eluted with 5 mL of methylformate. The eluant was evaporated under a stream of nitrogen gas at room temperature, and the residue was dissolved in assay buffer solution that consisted of 0.1% gelatin and 0.01% thimerosal in 0.05 mol/L phosphate buffer (pH 7.3). TXB$_2$ and 6-keto-PGF$_{1\alpha}$ were analyzed by radioimmunoassay using kits from Amersham: TXB$_2$ (no. RPA 516; threshold of detection, 20 pg/mL) and 6-keto-PGF$_{1\alpha}$ (no. RPA 515; threshold of detection, 20 pg/mL).

To assess the contribution of TXA$_2$ to postischemic brain edemas and infarct areas, rats were administered S-1452 (5, 10, and 50 mg/kg PO) 15 minutes before 1-hour transient MCA occlusion. Brain water contents 24 hours after reperfusion were measured by the dry-weight method described above. To measure infarct areas, rats were perfused with heparinized physiological saline 24 hours after reperfusion. The brains were removed from skulls, cut into 1-mm coronal sections, and immersed in 2% triphenyltetrazolium chloride (TTC) solution at 37°C for 30 minutes. The areas colorless to TTC staining, which reflect mitochondrial damage, were quantified as infarct areas by an imaging analysis system (Kontron M14, Zeiss, West Germany).

The survival of animals treated with S-1452 or vehicle (0.5% calboxymethyl cellulose) alone was compared at the time points of 3, 6, 12, 24, 48, and 168 hours after reperfusion following 2-hour transient MCA occlusion. The mortality of animals was approximately 60% at 168 hours after reperfusion following 2-hour occlusion, but 1-hour occlusion was a nonlethal ischemia. Then, we used 2-hour MCA occlusion to evaluate the effect of S-1452 on the survival rate after transient cerebral ischemia. Rats were administered S-1452 (10 mg/kg PO) 15 minutes before 2-hour transient MCA occlusion and every 24 hours after reperfusion.

Each experiment group consisted of more than five animals. All values are presented as mean±SEM. For statistical analyses, Dunnett’s multiple-range test, unpaired t test, and Fisher’s exact probability test were used.
Results

After a 1-hour unilateral MCA occlusion, brain water contents continued to rise significantly until 24 hours after reperfusion in the MCA areas and caudate putamen. The percent water of the MCA areas increased from 79.31±0.09% (nonoccluded side) to 83.39±0.66% (occluded side, P<.01) and that of the caudate putamen increased from 75.59±0.22% (nonoccluded side) to 81.60±0.50% (occluded side, P<.01) at 24 hours after reperfusion (Fig 1). However, increase of water content in the ACA areas was also significant but minimal (nonoccluded side, 79.09±0.13%; occluded side, 80.33±0.26%; P<.01 at 24 hours after reperfusion; Fig 1).

The level of plasma TXB2, a stable metabolite of TXA2, significantly increased from 92.8±20.4 pg/mL (normal) to a maximum of 369.8±67.2 pg/mL (P<.01) at 0 hour (just after reperfusion), 219.4±38.7 pg/mL (P<.05) at 1 hour, and 212.7±30.0 pg/mL (P<.05) at 12 hours and nearly returned to the normal level by 24 hours after reperfusion. The level of plasma 6-keto-PGF1α, a stable metabolite of PG12, was almost unchanged but slightly increased from 54.1±4.6 pg/mL (normal) to 81.6±8.9 pg/mL (P<.05) at 0 hour and 76.2±6.7 pg/mL (P<.05) at 6 hours after reperfusion (Fig 2).

In addition, the ratio of plasma TXB2 to 6-keto-PGF1α significantly rose from 1.73±0.40 (normal) to 4.73±0.84 (P<.05) at 0 hour, 4.67±0.68 (P<.01) at 1 hour, 3.76±0.64 (P<.05) at 3 hours, and 3.13±0.41 (P<.05) at 12 hours and nearly returned to the normal level at 24 hours after reperfusion (Fig 2). The elevation of the ratio until 3 hours after reperfusion was noteworthy.

The effect of S-1452 on brain edema after transient focal ischemia is shown in Fig 3. Animals administered S-1452 (5, 10, or 50 mg/kg PO) 15 minutes before 1 hour of MCA occlusion showed significant attenuation of the increase in postischemic water contents in the ACA areas (vehicle: 81.53±0.61%; S-1452: 5 mg/kg, 81.31±0.44%, NS; 10 mg/kg, 79.64±0.09%, P<.01; 50 mg/kg, 79.72±0.20%, P<.01) and the MCA areas (vehicle: 85.23±0.63%; S-1452: 5 mg/kg, 84.77±0.81%, NS; 10 mg/kg, 82.75±0.51%, P<.05; 50 mg/kg, 82.46±0.78%, P<.05) in a dose-dependent manner but only slight attenuation in the caudate putamen (vehicle: 83.31±0.56%; S-1452: 5 mg/kg, 84.18±0.36%, NS; 10 mg/kg, 81.56±0.61%, P<.05; 50 mg/kg, 81.82±0.58%, NS) at 24 hours after reperfusion.

Pretreatment with S-1452 (10 mg/kg PO) also significantly decreased the areas of infarction in the front parts of the cerebrum (bregma 1.7 mm: vehicle, 16.8±1.8%; S-1452, 4.9±1.5%, P<.01; bregma 0.7 mm: vehicle, 22.5±2.2%; S-1452, 17.2±0.6%, P<.05; bregma −0.3 mm: vehicle, 21.3±0.6%; S-1452, 17.1±1.0%, P<.01). There was no change in the hind parts of the cerebrum (Fig 4).

The effect of S-1452 (10 mg·kg−1·d−1 PO) on the survival rate of animals after 2 hours of MCA occlusion is shown in Fig 5. At 3, 6, 12, and 24 hours and 7 days...
after reperfusion, the survival rate of the vehicle group was 88.9%, 72.2%, 55.6%, 38.9%, and 38.9%, respectively. A time-dependent decrease in the survival rate was observed after reperfusion. On the other hand, the survival rate of the S-1452 group was 100%, 94.4%, 77.8%, 66.7%, and 66.7% at 3, 6, 12, and 24 hours and 7 days after reperfusion, respectively. Although there was no statistical significance by Fisher’s exact probability test, the survival rate tended to be improved in S-1452–treated animals.

### Discussion

Recirculation affects cerebral ischemia and modifies postischemic events in various ways. There is a possibility that recirculation occurs frequently after spontaneous thrombolysis and break-up of cerebral emboli in a common clinical event. Models of focal ischemia in the rat have normally been produced by occlusion of the MCA. Direct microsurgical techniques for occluding the MCA through a craniectomy have been described. These techniques are invasive, and it is difficult to achieve reperfusion. The MCA occlusion models that were used in our present study can be developed by relatively simple extracranial microsurgical dissection and are easy to reperfuse. These models are good for investigating the influence of reperfusion on focal ischemic brain injury.

Our results indicate that reperfusion after focal ischemia elevates the ratio of plasma TXB2 to 6-keto-PGF1α until 3 hours after reperfusion; then, this ratio declines to nearly the normal level by 24 hours after reperfusion, and pretreatment with S-1452, a TXA2 receptor antagonist, significantly attenuates the increase of brain water contents and infarct areas after focal ischemia. Moreover, the survival rate tended to be improved in S-1452–treated animals. Although postinjury studies would be more therapeutically meaningful, the present study has been done on the effect of pretreatment with S-1452. However, our results still suggest that S-1452 has the potential to safeguard the brain against ischemic stroke. In human ischemic stroke, there is a possibility that the brain alternates between ischemia and recirculation,
particularly in the case of cerebral embolism. We suggest that preventive administration of S-1452 to stroke patients can attenuate reperfusion injury after spontaneous thrombolysis and break-up of cerebral emboli.

The physiological effects of arachidonate metabolites often have opposing actions. For example, PGI₂ and PGF₂α are potent vasodilators, whereas TXA₂ and PGF₂α are vasoconstrictors. Under ischemic reperfusion conditions, a large quantity of arachidonates are liberated and are converted to PGs, LTs, and other arachidonate metabolites by the cyclooxygenase and lipoxygenase enzyme systems. Arachidonate metabolites have been implicated in the development of cerebral injury after ischemia. Particular importance has been assigned to the balance of TXA₂ and PGI₂ because of its regulative activity on platelet functions and arterial tone. The increase of TXB₂ levels has been reported to correlate well with an increase in neurological dysfunction in spinal cord ischemia. On the other hand, PGI₂ has been suspected to improve the regional blood flow after cerebral ischemia.

Recently, stable PGI₂ analogues were synthesized, and clinical trials on stroke have been reported for their therapeutic potency. TXA₂ analogues (U46619, SQ-26655) and PGF₂α are vasoconstrictors, and TXA₂ synthetase inhibition is considered to cause beneficial effects in the ischemic brain. Our present data show marked increase of the plasma TXB₂ level and slight increase of the plasma 6-keto-PGF₁α. We observed marked elevation of the ratio of plasma TXB₂ to 6-keto-PGF₁α due to breakdown of the balance between the TXA₂ and PGI₂ levels after ischemia. Furthermore, our data show that S-1452, a TXA₂ receptor antagonist, ameliorates brain edema and cerebral infarct areas. These results strongly indicate that the elevation of plasma TXA₂ level after reperfusion following brain ischemia closely participates in brain injury.

In our studies, S-1452 significantly suppresses the elevation of water content in the cerebral cortex after reperfusion following transient ischemia. However, suppression of the water content elevation by S-1452 is slight in the caudate putamen. Moreover, improvement of the infarct areas by S-1452 after focal ischemia is restricted in only the front parts of the cerebrum. The caudate putamen and the hind parts of the cerebrum are areas that become the ischemic core by MCA occlusion. On the other hand, we consider that the cerebral cortex and the front parts of the cerebrum are the ischemic penumbra areas or the areas that are easily perfused by collateral blood flow such as from the ACA. Pettigrew et al. have reported that TXA₂ synthase inhibition suppressed the elevation of the ratio of cerebral TXB₂ to 6-keto-PGF₁α and enhanced the cerebral blood flow after reperfusion following cerebral ischemia. In our ischemia models, S-1452 may retard the vasoconstriction that would contribute to depression of the cerebral blood flow during ischemia and reperfusion. Alternatively, S-1452 may prevent microvascular occlusion by inhibiting secondary aggregation of platelets stimulated by toxic products released during ischemia. We suppose that TXA₂ synthetase inhibitors and TXA₂ receptor antagonists such as S-1452 can alleviate brain injury after transient ischemia not by direct effects on the neurons but rather by the increase of collateral blood flow through its antplatelet and vasodilating activities, which are particular to the ischemic penumbra.

Other investigators also described the beneficial effects of thromboxane system inhibition in animal models of brain ischemia. Sadoshima et al. performed bilateral CCA occlusion on spontaneously hypertensive rats given the TXA₂ synthetase inhibitor OKY-046 and found significant increases in the cerebral blood flow and improvements of cerebral glucose metabolism during ischemia. Roy et al. reported that reduced TXB₂ levels due to TXA₂ synthetase inhibition correlated with increased regional cerebral blood flow in the ischemic penumbra in cats with a permanent MCA occlusion. Their findings suggest that thromboxane synthase inhibition may be important for the preservation of neurons threatened but not destroyed by hypoperfusion.

On the other hand, platelets may adhere to the damaged endothelium and subendothelium due to ischemia and release vasoactive substances that promote platelet aggregation and vasoconstriction. There is increasing evidence that platelets play an important role in the development of injury after SAH. We consider similar mechanisms to be involved in the development of cerebral injury after reperfusion following ischemia.

In conclusion, we have demonstrated that ratios of rat plasma TXB₂ to 6-keto-PGF₁α rose until 3 hours and then returned to control levels at later stages of reperfusion after transient MCA occlusion and that S-1452, a novel TXA₂ receptor antagonist, could reduce the postischemic brain injury. These results suggest that TXA₂ closely participates in postischemic brain injury in the early phase of recirculation. TXA₂ receptor antagonists such as S-1452 may have a protective effect on postischemic brain injury.

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References
The role of the microcirculation in cerebral ischemia is just beginning to attract widespread attention. There has been some research in such issues as reperfusion injury and the effects of leukocytes, but they have mostly been considered laboratory curiosities with little clinical relevance. Evidence is now accumulating that damage at the microcirculatory level can be therapeutically altered. Especially for embolic strokes, there is considerable reason to suspect that spontaneous thrombolysis occurs frequently. Furthermore, if thrombolytic therapy is proven to be effective, the problems associated with reperfusion will assume increased importance.

**Editorial Comment**

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