Neuroprotection by a Central Nervous System–Type Prostacyclin Receptor Ligand Demonstrated in Monkeys Subjected to Middle Cerebral Artery Occlusion and Reperfusion
A Positron Emission Tomography Study

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Background and Purpose—Recently, we found that a novel subtype of prostacyclin (PGI2) receptor clearly distinct from the peripheral subtype in terms of ligand specificity is expressed in the central nervous system (CNS). (15R)-16-m-tolyl-17,18,19,20-tetranorisocarbacyclin (15R-TIC) was synthesized and demonstrated to be a specific ligand for this CNS-type PGI2 receptor. Previously, we demonstrated 15R-TIC to be neuroprotective in vivo during transient forebrain ischemia in gerbils and permanent middle cerebral artery occlusion (MCAO) in rats. Furthermore, this compound was shown to exert an anti-apoptotic effect on primary cultured hippocampal neurons, indicating its neuroprotective effect against ischemic insults occurs via direct action on CNS-type PGI2 receptor.

Methods—Local cerebral hemodynamics and oxygen metabolism were measured simultaneously by using positron emission tomography with the 15O steady-state method, before and up to 18 hours after 3-hour transient MCAO reperfusion in cynomolgus monkeys. Methyl ester of 15R-TIC (50 µg/kg, n=4) or its vehicle (10% Intralipos, n=4) was injected intravenously within 5 minutes after onset of MCAO and continuously infused for 5 hours (50 µg/kg per hour).

Results—Neuropathology showed that 15R-TIC significantly reduced cortical damage after 3-hour MCAO. Positron emission tomography results showed 15R-TIC significantly reduced the volume of “infarct” region of interest and attenuated the decrease in cerebral metabolic rate of oxygen and oxygen extraction fraction, and these protective effects were not attributable to improvement of cerebral circulation.

Conclusions—These results suggest that 15R-TIC has a potent neuroprotective effect against focal cerebral ischemia in a monkey MCAO via its direct action on CNS-type PGI2 receptors. (Stroke. 2006;37:2830-2836.)

Key Words: cerebral blood flow ■ cerebral metabolic rate of oxygen ■ middle cerebral artery occlusion ■ oxygen extraction fraction ■ prostacyclin receptor

Prostacyclin (PGI2) protects neurons against ischemic insults by dilating vascular smooth muscle and inhibiting platelet aggregation,1 and has been developed as a therapeutic agent for ischemic injury in the central nervous system (CNS).12 However, PGI2 often reduces cerebral blood flow (CBF) by reducing systemic blood pressure, which is a disadvantage for drug development.34 Using several PGI2 analogs and an in vitro autoradiographic technique, we found a novel subtype of PGI2 receptor having a clearly distinct ligand specificity to be expressed in the rostral region of the CNS.56 (15R)-16-m-tolyl-17,18,19,20-tetranorisocarbacyclin (15R-TIC) was synthesized and demonstrated to be a specific ligand for this CNS-type PGI2 receptor.57 This compound had a quite low affinity for cloned peripheral-type PGI2 receptors; therefore, little effect on circulatory parameters, such as blood pressure and heart rate,8 and on platelet aggregation.7 Recently, we demonstrated that 15R-TIC protects hippocampal CA1 pyramidal neurons against the ischemia-induced delayed neuronal death in gerbils9 and attenuates focal...
cerebral ischemia-induced neuronal damage in rats after permanent middle cerebral artery occlusion (MCAO). The protective effect of this compound was also observed in neurons in primary culture: 15R-TIC prevented neuronal death induced by high (50%) oxygen, xanthine plus xanthine oxidase, or serum deprivation. These observations suggest that 15R-TIC protects against ischemic insults by acting directly on CNS-type PGI2 receptors.

Concomitant measurement of local cerebral hemodynamics and oxygen metabolism by sequential positron emission tomography (PET) scanning by the 15O steady-state method has been well-established to evaluate the pathophysiological states of ischemic brain tissue. Moreover, the Stroke Therapy Academic Industry Roundtable recommended that neuroprotection from ischemia or stroke damage should be evaluated in larger species such as primates rather than in rodents, because primate neurologic functions and cerebrovascular anatomy are well-characterized and proven to be similar to those of humans. Thus, to evaluate nonvascular neuroprotective property of 15R-TIC against ischemic insult, we used PET to repeatedly and simultaneously measure local CBF and cerebral metabolic rate of oxygen (CMRO2) in cynomolgus monkeys, before and up to 18 hours after 3-hour transient MCAO reperfusion.

**Materials and Methods**

All experimental protocols were approved by the Ethics Committee on Animal Care and Use of the Central Research Laboratory of Hamamatsu Photonics, and were performed in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985).

**Animal Preparation**

Eight male cynomolgus monkeys (Macaca fascicularis) weighing 4.9 to 6.6 kg (Clea Japan Inc, Tokyo, Japan) were used. The experimental procedures were essentially the same as described in our previous studies. In summary, anesthesia was induced by intramuscular injection of ketamine (10 mg/kg). The monkeys were tracheotomized, immobilized by intramuscular injection of pancuronium (0.05 mg/kg) every 2 hours, and artificially ventilated. Anesthesia was maintained with a mixture of 0.8% isoflurane and nitrous oxide/oxygen gas (7:3) during the entire experiment. Under anesthesia the left femoral artery was catheterized for measurement of mean arterial blood pressure and heart rate and for arterial blood sampling. A thermocouple probe was inserted into the rectum for monitoring rectal temperature. During the experiment, body temperatures were maintained at 37°C with a heating blanket.

**Transient MCAO and Drug Administration**

Transient focal cerebral ischemia was produced by occlusion of the right middle cerebral artery (MCA) via the transorbital approach. After intramuscular injection of atropine (0.05 mg/kg), the right globe was removed. Then craniotomy was performed suprolateral to the optic nerve. After opening the dura, two microvascular clips were placed on the MCA to occlude circulation and carefully removed 3 hours later.

The methyl ester of 15R-TIC (15R-TIC-Me) was dissolved in saline with 10% Intralipos (Otsuka Pharmaceutical Factory, Inc) and administered intravenously. 15R-TIC-Me (50 µg/kg, n = 4) or its vehicle (10% Intralipos, n = 4) was injected intravenously within 5 minutes after onset of MCAO and continuously infused for 5 hours.

**PET Studies**

Five consecutive PET scans were performed in each monkey before and up to 18 hours after 3-hour transient MCAO (Figure 1). Serial PET scans were performed with an animal PET camera (SHR7700; Hamamatsu Photonics K.K.). After enucleation of the right globe, the head of each monkey was fixed in a plastic stereotaxic apparatus and then positioned in the PET scanner gantry by using a laser beam localization system, so that the head position could be reproduced. After positioning and fixation, a 30-minute transmission scan with a rotating 22Na pin source and the first PET scan were performed. After the first PET scan, the monkey was moved to an operating table and the MCAO was performed. After reposition of monkey in the PET scanner, a 30-minute transmission scan and further PET scanning were performed. After the fourth scan, the right eyelid was carefully sutured, and then the monkey was allowed to fully recover and was returned to its cage for the night. On the second day, the monkey was placed on the MCA to occlude circulation and carefully removed 3 hours later.

**Regions of Interest**

**Ischemic Regions of Interest Based on CBF Decrease at 2 Hours After Onset of MCAO**

Areas of 0% to 40%, 41% to 60%, 61% to 80%, and 81% to 100% of CBF before MCAO in the ipsilateral hemisphere were defined as...
ischemic regions of interest (ROI) and delineated by using an image analysis system (Alice; Hayden Imaging Processing Group, Boulder, Colo).

Infarct ROI Based on CMRO₂ Decrease at 18 Hours After Reperfusion of MCAO

In accordance with earlier reports and our previous study, a probable infarct region, so-called infarct, was delineated on the last PET image (18 hours after reperfusion [Rep/18 H], CMRO₂ < 60% of prelevel).

For each monkey, these ROIs were copied to all other PET images sets by using the Alice software. All related ROIs were stacked into the volume of interest, and then the mean values for the 3 metabolic/hemodynamic parameters were calculated for each volume of interest.

Histological Analysis

Twenty hours after reperfusion of MCAO (after the final PET scan), most of the monkeys (except for a vehicle-treated monkey, which was used in a long-term study) were anesthetized with pentobarbital (100 mg/kg, intraperitoneally) and perfused transcardially with 10% formalin neutral buffer (pH 7.4). Each brain section was embedded in paraffin, and 10-μm-thick coronal sections were cut and stained with hematoxylin and eosin. After correction for the extent of brain edema by the method of Swanson et al., the neuronal damage in each section was defined and measured with a computerized image analysis system. Neuronal damage was evaluated in blind fashion by an outsider researcher.

Data Analysis

All results were expressed as the mean ± SEM. One-way ANOVA with Dunnett’s multiple-comparison procedure was used to test for changes in physiological variables over time. Two-way ANOVA with Scheffe’s multiple-comparison procedure was used to test for differences in temporal changes in CBF, CMRO₂, and OEF and in physiological or biochemical parameters between 15R-TIC-Me–treated and vehicle-treated groups. Because of the small sample, we used both parametrical (unpaired t test) and nonparametric (Mann–Whitney U test) tests to analyze the statistical significance of differences in neuronal damage and volume changes of CBF, CMRO₂, and OEF in each ROI between 15R-TIC-Me–treated and vehicle-treated groups. For these statistical comparisons, we considered a difference as significant if the P < 0.05 level for both tests were reached.

Results

Physiological variables during the prolonged experiments remained within the normal range (Figure 2). No significant changes in physiological variables were noted during the long-term study, except for the plasma glucose level (P < 0.05, 1-way ANOVA, Dunnett multiple-comparison procedure). Two-way ANOVA with Scheffe’s multiple-comparison procedure revealed no significant differences in these parameters between the 15R-TIC-Me–treated and vehicle-treated groups.

PET Study

Before MCAO, there were no differences in CBF, CMRO₂, and OEF between hemispheres ipsilateral and contralateral to the site of MCAO or between the vehicle-treated and the 15R-TIC-Me–treated groups. During MCAO, CBF greatly decreased with a moderate reduction in CMRO₂ and a great increase in OEF in ipsilateral MCA territory of both groups, as described in our previous study.

During MCAO, the volume of each region, defined by percent changes in CBF, CMRO₂, and OEF (0% to 40%, 41% to 60%, 61% to 80%, 81% to 100%, 101% to 120%, 121% to 140%, 141% to 160%, and >160% of CBF, CMRO₂, and OEF in MCAO/2 H PET image set compared with the pre-MCAO PET set) are shown in Figure 3. No volume differences were noted between the vehicle-treated (n = 4) and 15R-TIC-Me–treated (n = 4) groups, except for the volume of the CMRO₂ region that was 41% to 60% of the pre-MCAO level. The volume of this CMRO₂ region was significantly reduced by 15R-TIC-Me treatment (P = 0.043 and 0.043 for unpaired t test and Mann–Whitney U test, respectively).

The temporal changes in CBF, CMRO₂, and OEF in the ischemic ROIs (based on the CBF decrease during MCAO) in both groups were similar (Figure 4). CBF in each ischemic ROI decreased variably in both groups at MCAO/2 H, reflecting the extent of ischemia in these ROIs. No significant differences in CBF temporal change in these ROIs were noted between the vehicle-treated and 15R-TIC-Me–treated groups. Corresponding with the ischemic extent, CMRO₂ decreased variably and OEF increased inversely in these ROIs during ischemia.

Figure 2. Physiological parameters. MABP, mean arterial blood pressure; PCO₂, partial pressure of carbon dioxide; PO₂, partial pressure of oxygen; #P < 0.05, 1-way ANOVA, Dunnett’s multiple-comparison procedure.
After reperfusion, OEF decreased and remained lower than the pre-MCAO level in the vehicle group, indicating "luxury perfusion." OEF in most ROIs of the treated group decreased to essentially the pre-MCAO level and remained there until the end of the experiment, indicating "matched perfusion." 15R-TIC-Me significantly attenuated the decrease in CMRO2 and OEF at several time points (P < 0.05, 2-way ANOVA, Scheffe’s multiple-comparison procedure). Eighteen hours after reperfusion, CMRO2 in severely ischemic ROIs (0% to 40% and 41% to 60% ROIs) of the vehicle group was less than 60% of the pre-MCAO level, a critical threshold of irreversible neuronal damage.12,16 However, CMRO2 in the 41% to 60% ROI of the 15R-TIC-Me–treated group remained higher than 60% of the pre-MCAO level, indicating a neuroprotective effect at that extent of ischemia.

The typical CMRO2 images from vehicle-treated and 15R-TIC-Me–treated monkeys 18 hours after reperfusion are given in Figure 5A and 5B. In accordance with previous reports,12,16 a probable infarction was delineated on these last PET images (Rep/18 H, CMRO2 < 60% of pre-level). Topographic studies showed the “infarct” ROI to be located mainly in the deep MCA territory and extended to the cortical MCA territory. The obvious extent of that was usually seen in the vehicle-treated monkeys. The volume of “infarct” ROI was significantly reduced by 15R-TIC-Me (vehicle, 24.3 ± 4.7% of hemisphere; 15R-TIC-Me, 3.3 ± 1.0% of hemisphere; P = 0.030 and 0.043 for unpaired t test and Mann–Whitney U test, respectively).

Neuropathologic Study
Consistent necrotic damage was seen throughout the cortex and basal ganglia 20 hours after 3-hour transient MCAO in the vehicle-treated group (n = 3, Figure 5C), as described in the previous study.15 15R-TIC-Me (n = 4) significantly reduced cortical damage by 89% (P = 0.033 and 0.034 for unpaired t test and Mann–Whitney U test, respectively), and reduced basal ganglia damage by 43% (P = 0.063 and 0.034 for unpaired t test and Mann–Whitney U test, respectively) (Figure 5D and 5E).

Discussion
Presently we demonstrated that 15R-TIC, a CNS-type PGI2 receptor-specific ligand, was potently neuroprotective (nonvascular but parenchymal) against focal cerebral ischemia in a monkey MCAO model: (1) it “neuroprotected” the cerebral cortex preferentially; (2) it significantly reduced the volume of “infarct” ROI; and (3) it greatly attenuated the decrease in CMRO2 and OEF in most ROIs, although the temporal changes in CBF in these ROIs were not different from those in the vehicle group. Sequential PET studies in a MCAO-reperfusion primate model have demonstrated that CMRO2 is the best predictor of reversible or irreversible brain damage and that the critical metabolic threshold appears to be a CMRO2 that is < 60% of the contralateral tissue.12 In the present study, we defined the probable infarct region in PET image at 18 hours after reperfusion in monkeys subjected to 3-hour transient MCAO (CMRO2 < 60%; see Materials and Methods) and demonstrated that 15R-TIC-Me treatment significantly reduced the volume of the “infarct” ROI. Furthermore, 15R-TIC-Me prevented the CMRO2 from falling under the critical metabolic threshold in the severely ischemic ROI (41% to 60% decrease in CBF ROI). These results demonstrate the neuroprotective effect of 15R-TIC-Me against focal cerebral ischemia in the monkey MCAO model.

The main finding of the present study is that the neuroprotective effect of 15R-TIC was not caused by improvement in cerebral circulation. 15R-TIC was synthesized and demonstrated to be a specific ligand for CNS-type PGI2 receptor. This receptor has a ligand specificity that is clearly distinct from that of the peripheral subtype and is extensively expressed in the rostral region of the CNS, eg, cerebral cortex, hippocampus, thalamus, and striatum.54 The neuron specificity of 15R-TIC is indicated by our hemi-lesion experiments with kainate in the rat striatum, which revealed that the
CNS-type PGI\textsubscript{2} receptor is expressed mainly in neurons, but not in glial cells.\textsuperscript{5} 15R-TIC did not affect the blood pressure or heart rate in rats\textsuperscript{8} and in rhesus monkeys under physiological conditions (unpublished data) and did not inhibit platelet aggregation.\textsuperscript{7} In the present study, 15R-TIC-Me affected neither the volume of ischemic severity during MCAO (Figure 3) nor the temporal changes in CBF (Figure 4). Previous studies also suggest the nonvascular neuroprotective effects of 15R-TIC.\textsuperscript{8–10} In hippocampal neurons in primary culture, synthetic ligands including 15R-TIC prevented neuronal death induced by high (50\%) oxygen, xanthine plus xanthine oxidase, or serum deprivation.\textsuperscript{10} In the in vivo

Figure 4. Temporal changes in CBF, CMRO\textsubscript{2}, and OEF in the ROI are based on CBF reduction at 2 hours after MCAO. The ranges of CBF changes were 0\% to 40\%, 41\% to 60\%, 61\% to 80\%, 81\% to 100\% of the pre-MCAO PET set. *\textsubscript{P}<0.05, 2-way ANOVA, Scheffe's multiple-comparison procedure.
ischemic models, these compounds protected hippocampal CA1 pyramidal neurons against ischemia-induced delayed neuronal death in gerbils and attenuated focal cerebral ischemia-induced neuronal damage in rats subjected to permanent MCAO. In contrast, ligands specific for peripheral type PG\textsubscript{I}\textsubscript{2} receptors did not show such neuroprotective effects. Taken together, these observations suggest that 15R-TIC exerted neuroprotection by acting directly on the receptor expressed in neurons.

The temporal change in OEF (measured by sequential PET with \textsuperscript{15}O steady-state inhalation) is an ideal tool for evaluating residual tissue function after cerebral ischemic insults. OEF increases in the early stages of an infarction and falls (even if the regional CBF does not fall) depending mainly on the depth and duration of ischemia, and when mitochondrial function becomes impaired. This decline in oxygen uptake, not dictated by a further reduction in oxygen delivery, is an indicator of impending infarction. In the present study, the OEF in most ROIs of the 15R-TIC-Me--treated group was restored to its pre-MCAO level with essentially normal CBF after reperfusion (Figure 4). These results also support the neuroprotective effect of 15R-TIC. To our knowledge, the present study is the first to evaluate the effectiveness of such an agent in a primate MCAO model by observing temporal changes in oxygen metabolism combined with local hemodynamics.

In conclusion, 15R-TIC prevented neuronal damage after 3-hour transient MCAO in primates, as evaluated by neuro-pathological and PET studies. The simultaneous observation of local hemodynamics and oxygen metabolism showed that neuroprotection was not caused by improvement in cerebral circulation. These results indicate the possible therapeutic usefulness of PG\textsubscript{I} or its analogs acting directly on the CNS-type PG\textsubscript{I} receptor. The mechanism of neuronal protection is unclear, though it is known that 15R-TIC has anti-apoptotic effects in vitro. At least, 15R-TIC did not attenuate production of reactive oxygen species and did not affect intracellular Ca\textsuperscript{2+}, cAMP, IP\textsubscript{3} or cytochrome C levels (data not shown). The intracellular mechanism of neuroprotection by CNS-type PG\textsubscript{I}\textsubscript{2} ligands is currently undergoing study in our laboratory.

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