Combination Treatment with Normobaric Hyperoxia and Cilostazol Protects Mice against Focal Cerebral Ischemia-Induced Neuronal Damage Better Than Each Treatment Alone

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ABSTRACT

Normobaric hyperoxia (NBO) and cilostazol (6-[4-((1-cyclohexy-1H-tetrazol-5-y)butoxyl]-3,4-dihydro-2-(1H)-quinolinone) (a selective inhibitor of phosphodiesterase 3) have each been reported to exert neuroprotective effects against acute brain injury after cerebral ischemia in rodents. Here, we evaluated the potential neuroprotective effects of combination treatment with NBO and cilostazol against acute and subacute brain injuries after simulated stroke. Mice subjected to 2-h filamental middle cerebral artery (MCA) occlusion were treated with NBO (95% O₂ during the ischemia) alone, with cilostazol (3 mg/kg i.p. after the ischemia) alone, with both of these treatments (combination), or with vehicle. The histological and neurobehavioral outcomes were assessed at acute (1 day) or subacute (7 days) stages after reperfusion. We measured regional cerebral blood flow (rCBF) during and after ischemia by laser-Doppler flowmetry and recovery (versus vehicle) in the combination therapy group just after reperfusion. Mean acute and subacute lesion volumes were significantly reduced in the combination group but not in the two monotherapy groups. The combination therapy increased endothelial nitric-oxide synthase (eNOS) activity in the lesion area after ischemia versus vehicle. Combination therapy with NBO plus cilostazol protected mice subjected to focal cerebral ischemia by improvement of rCBF after reperfusion, in part in association with eNOS activity.

Recent experiments in animals have shown that normobaric hyperoxia (NBO) exerts beneficial effects when administered during transient cerebral ischemia without increasing markers of oxidative stress (Singhal et al., 2002a,b). Shin et al. (2007) reported that administration of 100% O₂ prevented the expansion of the zone with a severe cerebral blood flow deficit occurring during distal middle cerebral artery (MCA) occlusion in mice. In practice, oxygen treatment has the advantage of minimal technique, and it can be delivered in an ambulance or even at the patient’s home after a stroke. A phase II study to assess the therapeutic potential of NBO given within 9 h of stroke onset is underway in the United States (Massachusetts General Hospital and National Institute of Neurological Disorders and Stroke).

Cilostazol, an antiplatelet drug used to treat intermittent claudication, increases the intracellular level of cAMP by inhibiting its hydrolysis by type III phosphodiesterase. Its principal reported actions include inhibition of platelet aggregation (Kimura et al., 1985; Kohda et al., 1999), anti-thrombosis in feline cerebral ischemia, and vasodilation via its anti-inflammatory and antiapoptotic effects mediated by scavenging hydroxyl radicals, decrease the formation of tumor necrosis factor-α, and inhibition of poly(ADP-ribose) polymerase activity (Lee et al., 2004, 2007, 2008). We further reported that cilostazol provided neuroprotection against the permanent, filamental MCA occlusion-mediated increases in maltothionein-1 and -2 (Wakida et al., 2006). In addition, increasing evidence has shown that cilostazol may also have...
endothelial protection mediated by inhibition of lipopolysaccharide-induced apoptosis (Kim et al., 2002) and induced nitric oxide (NO) production by endothelial NO synthase (eNOS) activation (Hashimoto et al., 2006). NO produced by eNOS may play an important role in improving cerebral blood flow (CBF) and a neuroprotective role after cerebral ischemia (Lo et al., 1996; Murphy, 2000; Hua et al., 2008).

If combining two low-dose therapies with different neuroprotective actions were found to increase the target effects and reduce the side effects (compared with single therapy at a regular dose), such combination therapy would be beneficial for stroke patients. For the present study, we chose NBO and cilostazol, both of which have proved to be safe and effective therapies against ischemia in animals and for which the experimental evidence indicates different mechanisms of action, and we evaluated the therapeutic effects of combination treatment. Therefore, the purpose of this study was to clarify whether this combination therapy has neuroprotection and to clarify the mechanisms in postischemic processes.

To clarify an effect of the combination therapy, we examined the potential neuroprotective effects of concurrent treatment with insufficient time of NBO and a suboptimal dose of cilostazol on the cerebral infarct size and the neurobehavioral deficits present after subjecting mice to a 2-h occlusion of MCA followed by a 22-h or 7-day reperfusion. Concomitantly, we performed terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining to identify apoptotic cells, evaluated eNOS activity in lesion area after ischemia using Western blot analysis and immunohistochemical analysis for a marker of oxidative damage by using 8-hydroxy-2’-deoxyguanosine (8-OHdG), and we measured regional CBF (rCBF) by a laser-Doppler flowmetry before and after the ischemia.

### Materials and Methods

#### Animals and Drugs

The experimental designs and all procedures were in accordance with the Animal Care Guidelines of the Animal Experimental Committee of Gifu Pharmaceutical University. Male ddY mice (body weight, 24–28 g, Japan SLC Ltd., Shizuoka, Japan) were housed at controlled room temperature (24.5–25.0°C), with a 12-/12-h light/dark cycle. Mouse food pellets and tap water were provided ad libitum.

Cilostazol was kindly gifted by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). Mice of the cilostazol and combination groups were treated with an intraperitoneal injection of cilostazol at 3 or 10 mg/kg body weight once, immediately after completion of MCA occlusion. Mice of the vehicle and NBO groups received an intraperitoneal injection of 8 ml/kg body weight 0.5% carboxymethyl cellulose sodium salt without cilostazol at the timing and volume similar to those used in the cilostazol and combination groups.

#### Experiment Protocol

This study consisted of two different experiments. Experiment 1 aimed to investigate the neuroprotective potential of each single therapy NBO or cilostazol, and experiment 2 aimed to investigate the neuroprotective effect of combination therapy with NBO and cilostazol.

**Experiment 1.** To investigate the effecting time of NBO exposing, mice were randomly assigned to three groups: normoxia, 2-h NBO, and 3-h NBO (Fig. 1A). Two- and 3-h NBO groups received NBO...
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During MCA occlusion. The 3-h NBO group received additional 1-h NBO treatments after reperfusion. Each group breathed room air after NBO treatment. The normoxia group breathed room air throughout this procedure.

To investigate the effecting dose of cilostazol, mice were randomly assigned to three groups, vehicle, cilostazol 3, and cilostazol 10, which were administered vehicle (carboxymethyl cellulose sodium salt), 3 mg/kg cilostazol, and 10 mg/kg cilostazol, respectively, just after the induction of ischemia (Fig. 1D).

**Experiment 2.** To investigate the effect of combination therapy with NBO and cilostazol, mice were randomly assigned to four groups, vehicle, 2-h NBO, cilostazol 3, and combination (Fig. 1C). The effects of these treatments were assessed by neurobehavioral and histopathological evaluation until the subacute phase (Fig. 1D).

**Transient Focal Cerebral Ischemia**

Mice were anesthetized with 1.0 to 1.5% isoflurane (Merck Hoei Co. Ltd., Osaka, Japan) in air (21% O₂) via a face mask (Soft Lander; Sin-ei Industry, Saitama, Japan). Focal cerebral ischemia was induced by introducing an 8-0 monofilament (Ethicon, Somerville, NJ) coated with silicone hardener mixture (Xantopren; Bayer Dental, Osaka, Japan) into the left common carotid artery and advancing it along the internal carotid artery until the tip occluded the proximal stem of the middle cerebral artery, as described by Hara et al. (1996). Two hours after ischemia, animals were briefly reanesthetized with isoflurane, and the filament was withdrawn to allow reperfusion through the common carotid artery. In all animals during surgery, the body temperature was maintained between 37.0 and 37.5°C with a heating lamp and heating pad. Any mice with an intracranial hemorrhage, pulmonary insufficiency, asphyxia, and/or without an ischemic brain infarct were excluded. Sham mice underwent the same operation but did not undergo ischemia and did not receive either NBO or an injection of cilostazol or vehicle.

**NBO**

The hyperoxia groups (2-h NBO, 3-h NBO, and combination groups) were exposed to 95% O₂ in the chamber [regulated by an oxygen controller (ETCIC, Somerville, NJ) coated with silicone hardener mixture (Xantopren; Bayer Dental, Osaka, Japan)] into the left common carotid artery and advancing it along the internal carotid artery until the tip occluded the proximal stem of the middle cerebral artery, as described by Hara et al. (1996). The normoxia group breathed room air (21% O₂) with keeping percentage of isoflurane for anesthetizing during 2-h ischemia.

The rCBF was determined by laser-Doppler flowmetry (Omegaflow flo-N1; Omegawave Inc., Tokyo, Japan) using a flexible 0.5-mm fiberoptic extension to the master probe, as described previously (Kotani et al., 2008). Steady-state baseline values were recorded just before MCA occlusion, and further rCBF values were obtained during MCA occlusion and 30 min after reperfusion and expressed as a percentage of the baseline value. The area under the rCBF curve was calculated for the period during ischemia and for the period after reperfusion. Mice that did not demonstrate either a significant reduction (to less than 40% baseline rCBF values) during MCA occlusion or a rapid restoration of the rCBF signal during reperfusion were excluded.

**Physiological and rCBF Monitoring**

Mice were anesthetized by the same way of MCA occlusion, the left femoral artery was cannulated, and blood pressure was measured during the preparation. In all animals during surgery and monitoring, the body temperature was maintained between 37.0 and 37.5°C with the aid of a heating lamp and heating pad. The temperatures were measured before, during (every 30 min), and after ischemia. Mean systemic arterial blood pressure (PowerLab; ADInstruments Ltd., Chalgrove, Oxfordshire, UK) was measured for three 3-min periods (starting at 30 min before and just after MCA occlusion and at 1 h after reperfusion). Arterial blood gases were sampled just after these vital measurements. Blood samples of 50 μl were taken 30 min before and just after the induction of ischemia and at 1 h after reperfusion and analyzed for pH, oxygen (PaO₂), and carbon dioxide (PaCO₂) (i-STAT 300F; i-STAT Corporation, West Windsor, NJ). The normoxia (sham, vehicle, and cilostazol 3) groups received air (21% O₂) via a face mask, and the hyperoxia (2-h NBO and combination) groups received 95% O₂ (0.75 l/min 100% O₂ kept at 95% O₂ in the center of the face mask), with keeping percentage of isoflurane for anesthetizing during 2-h ischemia.

Neurobehavioral Assessments

**Neurological Deficit Scoring.** Mice were tested for neurological deficits just before reperfusion, 22 h after reperfusion, and every day until 7 days after the ischemia (day 7), by an observer (Y.N.) who was blind to the animal groups. Scoring was done using the following scale: 0, no observable neurological deficits; 1, failure to extend the right forepaw; 2, circling to the contralateral side; and 3, loss of walking or righting reflex, as described by Hara et al. (1996).

**Behavioral Assessment.** To assess the effects of combination treatment with NBO and cilostazol on functional changes after ischemia in mice, behavioral assessment was performed. A Rotorod apparatus was used to assess motor coordination, strength, and balance (Jones and Roberts 1968). Each mouse received three trials on the day before surgery (day −1) and again on day 5. In each trial, we noted the number of mice that were able to walk on a rod rotating at 8 rpm for more than 600 s before falling, with any moribund mice being excluded. In addition, we assessed locomotor activity in a novel environment. To this end, mice were individually placed in a transparent acrylic cage (length, 175 × width, 245 × height, 125 mm) with sawdust bedding on the floor, and locomotion was measured every 5 min for 120 min using digital counters with infrared sensors (NS-ASS01; Neuroscience, Inc., Tokyo, Japan). This test was performed once per animal on day 7.

**Assessment of Acute Brain Injury**

**Infarct Volume Measurement.** At 22 h after reperfusion, mice were given an overdose of pentobarbital sodium (Dainippon Sumitomo Pharma, Osaka, Japan) and then decapitated. The brain was cut into 2-mm-thick coronal block slices. These slices were immersed for 30 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, St. Louis, MO) in normal saline at 37°C, then fixed in 10% phosphate-buffered formalin at 4°C. TTC reacts with intact mitochondrial respiratory enzymes to generate a bright-red color that contrasts with the pale color of the infarction. The caudal face of each slice was photographed (COOLPIX 4500; Nikon, Tokyo, Japan). The unstained areas of the total, cortical, and subcortical infarctions were measured using ImageJ (http://rsb.info.nih.gov/ij/download/), and the infarction volume was calculated as in a previous report (Hara et al., 1997).

**Histopathological Examination.** Mice under anesthesia were perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 mM phosphate buffer, pH 7.4, at 22 h after reperfusion. Then, the brains were postfixed overnight in the same fixative and immersed in 30% sucrose solution in the phosphate buffer. The brains were frozen and cut into 20-μm-thick coronal sections from 0.4 to 1.0 mm anterior to bregma (through the anterior commissure) using a cryostat (Leica, Wetzlar, Germany). To detect degenerated neurons, the sections were stained with TUNEL, as described previously (Kotani et al., 2008). The TUNEL assay was performed according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Immunohistochemical analysis for 8-OHdG was performed according as follows. Sections were rinsed three times.
in PBS, incubated in 3% H₂O₂ in methanol for 30 min, then placed in PBS and blocked with 1% mouse serum for 30 min. A monoclonal antibody against 8-OHdG was applied to sections overnight at 4°C. Secondary antibody (M.O.M. (Mouse on Mouse) biotinylated anti-mouse) was applied for 10 min. The avidin/biotinylated HRP complex (ABC Elite kit; Vector Laboratories, Peterborough, UK) was applied for 30 min, and the sections were allowed to develop Chromagen in a 3,3-diaminobenzidine + nickel solution (Sigma-Aldrich) for 2 min. To quantify the number of TUNEL- and 8-OHdG-positive cells in the ischemic boundary, zones within the cortex and subcortex were counted. The regions of interest for the micrographs were the periphery region between 100 and 500 µm from the lesion boundary. These were done in two high-power fields (×400) within each lesion by masked investigators (Y.N. and K.H.). Each count was expressed as number per millimeter squared (n = 5).

**Western Blot Analysis.** Mice in ischemic groups were deeply anesthetized and decapitated at 10 min after reperfusion. As control group, no-surgery and nontreated mice were used. The brains were quickly removed, and an 8-mm coronal section was cut from the left hemisphere (between 2 and 10 mm from the frontal end of the forebrain). Moreover, brain samples were divided into cortex and subcortex, and the cortex was homogenized in 10 ml/g tissue ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 50 mM EDTA, 1% Triton X-100, and protease/phosphatase inhibitor mixture) and centrifuged at 14,000g for 10 min at 4°C. An aliquot of 5 µg of protein was subjected to a 5 to 20% gradient SDS-polyacrylamide gel electrophoresis (SuperSep Ace; Wako Pure Chemicals, Osaka, Japan), with separated protein being transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA). For immunoblotting, the following primary antibodies were used: polyclonal antibody to nitric-oxide synthase (endothelial) (1:1000 dilution; Funakoshi, Tokyo, Japan), phosphor-ylated-eNOS (P-eNOS) (Ser1177) antibody (1:1000 dilution; Cell Signaling Technology Inc., Danvers, MA), and monoclonal anti-β-actin (1:1000 dilution; Sigma-Aldrich). The secondary antibody was anti-rabbit HRP-conjugated IgG (1:2000 dilution) and anti-mouse HRP-conjugated IgG (1:2000 dilution). The immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA). The band intensity was measured using a Lumino Imaging Analyzer (FAS-1000; Toyobo Engineering, Osaka, Japan) and Gel Pro Analyzer (Media Cybernetics, Inc., Bethesda, MD).

**Assessment of Subacute Brain Injury in Experiment 2**

Perfused and fixed brains were removed at 7 days after ischemia (day 7) and photographed using a digital camera (COOLPIX 4500; Nikon) to allow us to observe the changes on the brain surface. The brains were then cut into 2-mm-thick coronal slices. Coronal brain sections (20 µm/2 mm) were cut on a cryostat and stained with cresyl violet for histological measurements at a late time after ischemia, as previously described in rats (Iizuka et al., 1990). The various sections were used to detect the lesion area, and hypoxic or neutrophil infiltration area was judged by lesion area. In addition, whole-brain volume minus ventricles volume was measured to allow us to assess brain atrophy by means of an indirect method that assesses the difference between the remaining healthy ipsilateral hemisphere and the contralateral one (Grabowski et al., 1993).

**Statistical Analysis**

Data are presented as means ± S.E.M. Statistical comparisons were made using an analysis of variance followed by a Student’s t test or a Dunnett’s test or a Student-Newman-Keuls test or a Mann-Whitney U test with Bonferroni correction or a χ² test. P < 0.05 was considered to indicate statistical significance.

**Results**

**Physiological Parameters and Mortality**

In the two hyperoxia (2-h NBO and combination) groups, arterial PaO₂ values increased approximately 3.5-fold during the administration of 95% oxygen. The other physiological parameters were not significantly different among the four groups (Supplemental Table 1).

The mortality of mice was approximately 10% (range from 4.5–10.0%) 24 h after ischemia and 20% (range from 12.5–25.0%) 7 days after ischemia in each group of experiments 1 and 2, and there were no significant differences among groups (P > 0.05, χ² test; data not shown). The excluded mice, because of death or intracranial hemorrhage and so on, were approximately 15% (range from 13.6–20.0%) 24 h after ischemia, and there were no significant differences among groups (P > 0.05, χ² test; data not shown). All mice without death-assessed subacute brain injury in experiment 2 were evaluated.

**Experiment 1**

Figure 2, A and B, shows the comparison of exposing 2-h with 3-h NBO on the infarct area and volume. Expanding 3-h NBO, but not 2-h NBO, showed a significant suppression of infarct size compared with normoxia. Figure 2, C and D, shows the comparison of 3 with 10 mg/kg cilostazol on the infarct area and volume. Cilostazol, 10 but not 3 mg/kg, showed a significant infarct reduction of infarct size compared with vehicle.

**Experiment 2**

**Cerebral Blood Flow.** After the ischemic onset, rCBF dropped to approximately 30% of the preischemic baseline in all animals, and there was no significant difference either in the rCBF value at a given time point or in the average rCBF value among the four groups during the ischemia (Fig. 3, A and B). Almost immediately after reperfusion, the rCBF of the combination group recovered almost to the preischemic value, whereas the vehicle group exhibited incomplete recovery (to approximately 70% of baseline). These values indicated more or less successful reperfusion of previously ischemic tissue. However, there were significant differences in rCBF values at 2, 4, and 30 min after reperfusion between the vehicle and combination groups, with the average rCBF values obtained after reperfusion being 70.4 ± 7.1 and 96.8 ± 6.1% of baseline, respectively (P < 0.05, Fig. 3, A and C).

**Acute Brain Injury.** The ischemic mice showed apparent neurological deficits just before and at 22 h after ischemia. There was no significant difference in neurological deficit score among the four groups just before reperfusion (data not shown). In contrast, at 22 h after reperfusion, the combination group exhibited significantly reduced neurological deficit scores (P < 0.05, combination versus vehicle, Fig. 4D). These data included the mice that were assessed for subacute brain injury. The infarct tissue was visualized by TTC staining in the ischemic hemisphere (Fig. 4A), and combination treatment with 2-h NBO plus cilostazol 3 significantly reduced both the infarct area and infarct volume (versus vehicle) (Fig. 4, B and C). An ischemic lesion was consistently identified affecting the cortex and subcortex of the left cerebral hemisphere. A combination treatment-induced reduction of infarct volume...
could be seen both in the cortex and the subcortex (Fig. 4E). In the subcortex, cilostazol 3 alone significantly reduced the infarct volume, whereas in the cortex, 2-h NBO alone tended (nonsignificantly) to reduce the infarct volume (Fig. 4E).

A large number of cells showed strong TUNEL-positive staining on the ischemic side of the brain but not on the contralateral side. The numbers of TUNEL-positive degenerated cells in the ischemic boundary zones within the cerebral cortex (Fig. 4F) and subcortex (Fig. 4G) showed increases at 22 h after reperfusion. These numbers were significantly reduced by the combination treatment of the cortex and subcortex (Fig. 4, I and J, respectively). The numbers of TUNEL-positive cells were also significantly reduced by 2-h NBO treatment in the cerebral cortex (Fig. 4I) and by cilostazol 3 treatment in the cerebral subcortex (Fig. 4J).

P-eNOS and eNOS were almost 140 kDa. P-eNOS was...
Fig. 4. Acute brain injuries in experiment 2. A, TTC-stained coronal sections show infarct tissues (pale unstained region). Brain sections (from extreme left) were obtained from vehicle, 2-h NBO, cilostazol 3, and combination mice, respectively. B, total infarct area; C, total infarct volume; E, infarct volumes in cortex and subcortex. *, *P < 0.05; **, *P < 0.01 versus vehicle (Student-Newman-Keuls test, n = 11–17). Data are expressed as mean ± S.E.M. D, neurological deficit scores 22 h after reperfusion. *, *P < 0.05 versus vehicle (Mann-Whitney U test with Bonferroni correction, n = 27–36 adding the mice that were assessed subacute brain injury). F to J, TUNEL staining. F and G show representative photomicrographs of TUNEL staining in the ischemic boundary zones of the cerebral cortex (F, upper row) and subcortex (G, lower row). Bar, 50 μm. H, schematic drawing showing the brain regions at a level of 0.4 to 1.0 mm anterior to bregma (through the anterior commissure). 1, cortical; 2, subcortical ischemic boundary zones. I to J, quantitative representation of TUNEL-positive cells in ischemic brains from each group. *, *P < 0.05; **, *P < 0.01 versus vehicle; #, *P < 0.05; ##, *P < 0.01 between indicated groups (Student-Newman-Keuls test, n = 5). Data are expressed as mean ± S.E.M.
increased after MCAO (Fig. 5A). It was more prominent in all therapeutic groups than in the control. On the other hand, eNOS was not different among all groups. In quantitative analysis, P-eNOS was significantly greater in NBO, cilostazol, and combination groups than in the control group but not in the vehicle group (Fig. 5B). Moreover, P-eNOS was significantly greater in the combination group than in the vehicle group. However, eNOS was not different among all groups (Fig. 5C). There was a significant difference in the ratio of P-eNOS/eNOS between control and combination groups (Fig. 5D).

At 22 h after reperfusion, strong 8-OHdG immunoreactivity was detected in the cortex and subcortex on the ischemic side of the brain but not on the contralateral side in each group (Supplemental Fig. 1, A and B). Cilostazol group and combination group significantly reduced the number of 8-OHdG-positive cells compared with the vehicle group. It is important that the number of 8-OHdG-positive cells was significantly reduced by the combination treatment in the cortex (versus vehicle, 2-h NBO, and cilostazol groups, Supplemental Fig. 1C) and in the subcortex (versus vehicle group, Supplemental Fig. 1D).

Subacute Brain Injury

The neurological deficit score, which was maximal just before reperfusion, was reduced within 3 days and remained at its reduced level until 7 days after reperfusion (day 7). There was no significant difference in neurological deficit score between each group just before reperfusion. However, from day 1 onwards, the combination group displayed a significantly lower score than the vehicle group (P < 0.05, Fig. 6A).

**Rotorod Performance.** The Rotorod test has been used before to assess motor coordination, strength, and balance alterations after ischemic brain injury in rodents (Jones and Roberts, 1968; Rogers et al., 1997). At 1 day before surgery (day −1), no difference between the vehicle and combination groups was observed in Rotorod performance (data not shown). On day 5, the combination group contained a significantly greater proportion of mice [64.3% (nine of 14), P < 0.05] who could stay on the rod for more than 600 s [versus the vehicle group, 16.7% (three of 18)] (Fig. 6B).

**Locomotor Activity.** Locomotor activity data are shown in Fig. 6, C and D. The 2-h observation period was divided into 10-min intervals to display the time dependence of the behavior. Figure 6C shows that all of the mice moved less as time passed. In comparison with the sham group, the vehicle group showed a significantly lower total activity count over the first 1 h (0–60 min, Fig. 6D). Combination and 2-h NBO therapy prevented this decrease in novel environment activity after ischemia in the subacute phase. From the results of behavioral experiments, combination therapy prevented neurobehavioral function even until the subacute phase. These neurobehavioral findings are consistent with the observed morphological outcomes.

**Assessment of Brain Injury.** On day 7, obvious atrophy in the ischemic hemisphere could be seen in the gross photographs of whole brains (arrows in Fig. 7A) and in brain sections stained with cresyl violet (Fig. 7B). The shrinkage (percentage of the contralateral corresponding brain region) because of brain atrophy was significantly different between the vehicle and combination groups (P < 0.05, Fig. 7C). In the 4-, 6-, and 8-mm coronal slices, lesion area was significantly smaller in the combination group than in the vehicle group (P < 0.05 at each level, Fig. 7D). Lesion volume at 7 days after ischemia was significantly reduced by combination therapy (P < 0.01 versus vehicle, Fig. 7E).

**Discussion**

We found that 3-h NBO treatment reduced infarct volume more than the 2-h NBO treatment. Shinghal et al. (2002b) have reported similar results and demonstrated that NBO
treatment against brain ischemia did not increase oxidative stress.

Moreover, 10 but not 3 mg/kg cilostazol administered intraperitoneally just after the induction of ischemia showed significant suppression of infarct size, and a similar report has been published previously (Choi et al., 2002). Our study, for the first time to our knowledge, demonstrated that combination therapy with insufficient time of 2-h NBO and a suboptimal dose (3 mg/kg i.p.) of cilostazol had neuroprotective effects.

Furthermore, we examined another experiment whether optimal treatment with 3-h NBO plus cilostazol 10 could reduce infarct volume most at 22 h after 2-h ischemia. The infarct volume of combination therapy with 3-h NBO plus cilostazol 10 was 31.4 ± 7.2 mm³ (n = 4), significant (P < 0.05) difference compared with vehicle (64.8 ± 6.0 mm³, n = 17), and a little less (but not significant) than that with 2-h NBO plus cilostazol 3 (36.1 ± 4.3 mm³, n = 11). This result might be caused from the limitation of protection in our model of focal cerebral ischemia in mice.

In this study, monotherapy with 2-h NBO protected the cortex rather than the subcortex, as indicated by our assessment of TTC and TUNEL staining. Singhal et al. (2002a,b) showed previously in the rat that NBO significantly reduced cortical infarct volumes and, moreover, that the neuroprotective effects of NBO were most evident in the cerebral cortex itself. Shin et al. (2007) found that NBO improves CBF during ischemia of the cerebral cortex (by means of real-time, two-dimensional laser speckle flowmetry). Combination therapy in the present study demonstrated a more complete recovery of rCBF than the vehicle group immediately after reperfusion. Ischemia is particularly germane to CBF, and it is possible that improving rCBF after reperfusion may be a therapeutic potential to save salvageable ischemic brain.

eNOS is an enzyme that increases the activity just after ischemia (Zhang et al., 1993), and NO produced by eNOS plays an important role in improving CBF and a neuroprotective role after cerebral ischemia (Lo et al., 1996; Murphy, 2000; Hua et al., 2008). Huang et al. (1996) have reported that mice lacking expression of the eNOS developed larger infarcts after MCA occlusion. Cilostazol induces NO production by eNOS activation (Hashimoto et al., 2006), and it is possible that hyperoxia could augment eNOS activity in ischemic area (Shin et al., 2007). From these points of view, we have evaluated eNOS activity and rCBF before and after the ischemia. The eNOS is activated by phosphorylation at Ser1177 (Chen et al., 1999). The present study demonstrated that the amount of phospho-Ser1177 eNOS in ischemic area after reperfusion was significantly elevated in the combination therapy, and this result may correlate with the result that combination therapy was not only improving rCBF after reperfusion but also protecting the ischemic brain with functional and morphological outcome. eNOS activity may be, in part, the mechanism under combination therapy against neuroprotective effect.

Recent studies suggest that the benefits of NBO may exceed the risk of enhanced oxidative stress (Singhal et al., 2002a; Kim et al., 2005), which reportedly plays a major role in the pathogenesis of the cerebral infarction occurring as a reperfusion injury after focal ischemia (Yang et al., 1994). However, a theoretical concern remains about reperfusion injury being caused by an increase in oxygen free radicals.
because of NBO. A previous study indicated that free radicals increased in the blood of humans undergoing hyperbaric oxygen therapy (Narkowicz et al., 1993). However, the generation of superoxide and hydrogen peroxide can be suppressed by increased cAMP (Takei et al., 1998), and previous reports suggested that cilostazol suppresses superoxide production via an increase in cAMP (Choi et al., 2002; Park et al., 2006). We have reported that cilostazol provided neuroprotection against brain ischemia-mediated increases in metallothionein-1 and -2, having a potential of antioxidative stress (Wakida et al., 2006). Hence, it is possible that cilostazol suppresses any superoxide production that might be induced by NBO.

Both NBO and cilostazol therapy have been reported to have side effects. In humans, NBO may lead to lung damage (Jenkinson 1993), and prolonged high oxygen exposure is reported to induce cough and shortness of breath, decrease vital capacity, and increase alveolocapillary permeability (Capellier et al., 1999). For these reasons, prolonged exposure to high oxygen concentration should be avoidance. Clinically, despite cilostazol being generally well tolerated (Huang et al., 2008), untoward side effects (including headache and palpitation) limit its use, even though they are generally mild to moderate in intensity (Chapman and Goa 2003). From these points of view, it is desirable to gain enough effect by two or more therapies used even at an insufficient dose or time. This study demonstrated that concurrent treatment with insufficient time of NBO and a sub-optimal dose of cilostazol had neuroprotective effects no less than sufficient single therapies. This study may bring hope for the patients who cannot receive sufficient therapy because of their complication.

In conclusion, the present study demonstrated that combination therapy involving NBO and cilostazol: 1) protected against not only acute but also subacute cerebral injury in mice that had undergone focal cerebral ischemia and 2) inhibited apoptotic cell death. These effects may be, at least in part, due to improvement of rCBF just after reperfusion in association with eNOS activity.

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