3-[2-[4-(3-Chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1H-indazole Dihydrochloride 3.5 Hydrate (DY-9760e), a Novel Calmodulin Antagonist, Reduces Brain Edema through the Inhibition of Enhanced Blood-Brain Barrier Permeability after Transient Focal Ischemia

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Received October 2, 2002; accepted November 20, 2002

ABSTRACT
An alteration of the blood-brain barrier (BBB) permeability contributes to the development of brain edema after stroke. In this study, we evaluated the effects of 3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1H-indazole dihydrochloride 3.5 hydrate (DY-9760e), a novel calmodulin antagonist, on brain edema formation and BBB integrity in rats subjected to transient focal ischemia. DY-9760e (1 mg/kg/h) was intravenously infused for 6 h, starting immediately after reperfusion of a 1-h middle cerebral artery occlusion. Treatment with DY-9760e significantly suppressed the increase in water content and the extravasation of Evans blue dye after transient focal ischemia. Analysis of a magnetic resonance imaging method revealed that DY-9760e significantly prevented the development of brain edema in the cortical region of the ipsilateral hemisphere. Tri-fluoperazine, a calmodulin antagonist that is structurally different from DY-9760e, also attenuated brain edema elicited by transient focal ischemia. Furthermore, DY-9760e and trifluoperazine reduced tumor necrosis factor-α-induced hyperpermeability of inulin through a cultured brain microvascular endothelial cell monolayer, suggesting an involvement of calmodulin in the regulation of brain microvascular barrier function. The present results demonstrate that DY-9760e ameliorates brain edema formation and suggest that this effect may be mediated in part by the inhibition of enhanced BBB permeability after ischemic insults. Thus, DY-9760e is expected to be a therapeutic drug for treatment of acute stroke patients.

In acute-stage cerebral ischemia, the development of infarction is accompanied by the formation of severe edema. Brain edema produces increased intracranial pressure, leading to a compression of the microvasculature, which causes further cerebrocircular disorder followed by secondary expansion of the infarct volume and often leads to a fatal condition (Klatzo et al., 1986). Therefore, alleviation of brain edema may not only improve clinical symptoms by restricting subsequent infarction but also reduce mortality.

The blood-brain barrier (BBB) functions to eliminate the free passage of hormones, drug, and other neuroactive and neurotoxic substances into the central nervous system. Ischemia and reperfusion injury causes BBB disruption, which accelerates the development of abnormal vascular permeability and exacerbates postischemic edema (Cole et al., 1991; Yang and Betz, 1994). Although the precise cellular mechanisms underlying changes in BBB permeability after cerebral ischemia are unclear, there are several lines of evidence that calcium and cytokines may play an important role in altering BBB permeability (Tschugguel et al., 1995; Merrill and Murphy, 1997; Abbruscato and Davis, 1999; Brown and Davis, 2002). Tumor necrosis factor-α (TNFα) is an inflammatory cytokine that elicits enhanced vascular permeability (Deli et al., 1995; Mark and Miller, 1999). Increases in TNFα mRNA and protein expression occur in the rat and mouse brain after ischemic insults (Buttini et al., 1996; Uno et al., 1997). Furthermore, it has been shown that TNFα exacerbates ischemic brain injury, whereas blockade of TNFα exerts neuroprotection (Barone et al., 1997). Thus, TNFα may

ABBRVIATIONS: BBB, blood-brain barrier; TNFα, tumor necrosis factor-α; MCA, middle cerebral artery; MRI, magnetic resonance imaging; HBMEC, human brain microvascular endothelial cell; ANOVA, analysis of variance; PLSD, protected least significant difference; NOS, nitric-oxide synthase; A23187, 5-(methylamino)-2-[(2R,3R,6S,8S,9R,11R)-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-(1H-pyrrol-2-yl)-ethyl]-1,7-dioxaspiro[5,5]undec-2-yl]methyl]-4-benzoxazolecarboxylic acid.
be a mediator in altering BBB permeability and producing brain injury after cerebral ischemia.

We have developed a competitive calmodulin antagonist, 3-[2-(4-(3-chloro-2-methylphenyl)-1-piperazinyl)ethyl]-5,6-dimethoxy-1-(4-imidazoylethyl)-1H-indazole dihydrochloride 3.5 hydrate (DY-9760e), that exerts cytoprotective action (Sugimura et al., 1997) and reduces the cerebral infarct volume after transient and permanent focal ischemia in rats (Sato et al., 1999; Takagi et al., 2001). The purpose of this study was 1) to evaluate the effects of DY-9760e on brain edema formation and BBB integrity in rats subjected to transient focal ischemia; 2) to determine whether DY-9760e directly affects changes in the vascular barrier function of brain endothelial cells treated with TNFα; and 3) to clarify the involvement of calmodulin in brain edema formation by using trifluoperazine, a calmodulin antagonist structurally unrelated to DY-9760e.

Materials and Methods

**Animals.** Male Wistar rats (Clea Japan, Inc., Tokyo, Japan), weighing 240 to 280 g, were used. Animals were housed in cages in a regulated environment (23 ± 2°C, 55 ± 15% relative humidity) under a 12-h light/dark cycle (on from 8:00 AM to 8:00 PM), and given food (F-2; Funabashi Farm, Chiba, Japan) and tap water ad libitum. All experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan).

**Transient Focal Ischemia Model.** Animals were anesthetized by inhalation of 2% halothane in 70% nitrous oxide and 30% oxygen, and the brains, excluding the cerebellum, were quickly removed. The contralateral and the ipsilateral hemispheres were weighed separately. The dry weight of the brain hemisphere was measured after it was dried at 120°C for 48 h. The water content in the hemisphere was calculated as follows: water content (%) = (weight − dry weight)/weight × 100.

**Measurement of Brain Water Content.** The rats were killed by decapitation under anesthesia with 2% halothane in 70% nitrous oxide and 30% oxygen, and the brains, excluding the cerebellum, were quickly removed. The contralateral and the ipsilateral hemispheres were weighed separately. The dry weight of the brain hemisphere was measured after it was dried at 120°C for 48 h. The water content in the hemisphere was calculated as follows: water content (%) = (weight − dry weight)/weight × 100.

**Examination of BBB Permeability.** Fourteen rats each were treated with vehicle or DY-9760e. The BBB permeability was assessed by the Evans blue extravasation method (Uyama et al., 1988; Belayev et al., 1996); Evans blue (2% in saline, 4 ml/kg) was intravenously administered via the tail vein at 4 h after the onset of MCA reperfusion. At the completion of test compound infusion (6 h after reperfusion), the thorax was opened under anesthesia with pentobarbital, and a needle was inserted into the left heart ventricle. Rat brains were perfused with saline containing 10 units/ml heparin at a flow rate of 16 ml/min for 20 min. The dry weight of the brain hemisphere was calculated in the vascular system. While still under anesthesia, the rats were decapitated, brains were quickly removed, and both the contralateral and the ipsilateral cortices were dissected. Brain samples were weighed and homogenized in 50% trichloroacetic acid solution (1.5 ml/g tissue). After centrifugation at 10,000g for 10 min, the supernatant was diluted with ethanol, and its fluorescence was determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrometer (SpectraMax Gemini; Molecular Devices Corp., Sunnyvale, CA). The changes of the BBB permeability after cerebral ischemia were expressed as the ratio of fluorescence intensity in the ipsilateral cortex to that in the contralateral cortex (Evans blue extravasation index).

**Macromolecular Permeability across the Brain Endothelial Cell Monolayer.** Human brain microvascular endothelial cells (HBMECs; ACBRI 376) were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). HBMECs at passage 4 to 8 were plated onto the inner membrane of a Cell Culture Insert (pore size 0.45 μm) for 24-well plates (Falcon, Nippon Becton Dickinson Co., Ltd., Tokyo, Japan). Culture medium (EGM-2; Sanko Junyaku Co., Ltd., Tokyo, Japan) was added to the upper compartment (i.e., into the insert cup, 200 μl/well) and the lower compartment (i.e., out of the insert cup, 800 μl/well), and cells were maintained in humidified 95% air and 5% CO2 at 37°C in a CO2 incubator (MCO-17AI; Sanyo Electric Co., Ltd., Moriguchi, Osaka, Japan). The culture medium was exchanged on a 4.7 T BIOSPEC 47/40 (Bruker, Ettingen, Germany) equipped with a 12-mm inner diameter gradient coil and a 72-mm inner diameter probehead of cylindrical design. Median sagittal MRI was performed to ensure correct head position with a gradient echo sequence [optimized parameters: repetition time, 100 ms; echo time, 12.1 ms; field of view, 80 mm; matrix size, 256 × 256; and slice thickness, 2.0 mm]. Subsequently, 10 T2-weighted coronal images were acquired by a multislice fast spin echo sequence [repetition time, 6000 ms; echo time, 20 ms; field of view, 50 mm; matrix size, 256 × 256; number of segmented k-spaces, 8; slice thickness, 2.0 mm; and slice separation, 0.2 mm].

**Magnetic Resonance Imaging (MRI).** The total number of animals used was 12 (vehicle, n = 6; DY-9760e, n = 6). At 24 h after ischemic induction, the extent of brain edema was determined by the MRI technique under anesthesia with 1.5% halothane in air (flow rate 1.5 l/min). The MRI was carried out on a 4.7 T BIOSPEC 47/40 (Bruker, Ettingen, Germany) equipped with a 12-mm inner diameter gradient coil and a 72-mm inner diameter probehead of cylindrical design. Median sagittal MRI was performed to ensure correct head position with a gradient echo sequence [optimized parameters: repetition time, 100 ms; echo time, 12.1 ms; field of view, 80 mm; matrix size, 256 × 256; and slice thickness, 2.0 mm]. Subsequently, 10 T2-weighted coronal images were acquired by a multislice fast spin echo sequence [repetition time, 6000 ms; echo time, 20 ms; field of view, 50 mm; matrix size, 256 × 256; number of segmented k-spaces, 8; slice thickness, 2.0 mm; and slice separation, 0.2 mm]. The lesion area on the image of each slice was quantified by an image analyzer (Quantimet-600; Leica, Wetzlar, Germany). The lesion volume was calculated for each animal from hyperintense brain areas on nine images multiplied by the slice thickness.
every 2 to 4 days until the cultures became confluent. Treatment of HBMECs with test compounds was carried out by adding 0.3 ng/ml TNFα and DY-9760e (0.1–3 μM) or trifluoperazine (0.1–3 μM) to both compartments for 24 h. In the naïve group, neither TNFα nor test compound was applied to the cells. The barrier function of the HB-MEC monolayer was assessed by using inulin, a macromolecule that does not permeate across the cell membrane (Wolburg et al., 1994). [3H]Inulin (0.4 μCi/well) was added to the upper compartment, and culture plates were maintained in a CO2 incubator for 30 min. The amount of radioactivity diffused into the lower compartment was determined by a liquid scintillation counter (LSC-6000; Aloka Co., Ltd., Mitaka, Tokyo, Japan). Inulin permeability in each of the control and the test compound-treated groups was expressed as a percentage of that in the naïve group.

Statistical Analysis. All data were expressed as the mean ± S.E.M. The statistical analysis was evaluated by Exsas version 5.00 based on SAS release 6.12 (Arm Corp., Osaka, Japan and SAS Institute Japan Ltd., Tokyo, Japan). Brain water content was analyzed by two-way ANOVA with Fischer’s PLSD test. Evans blue extravasation index and lesion volume measured with MRI were analyzed by the unpaired Student’s t test. Inulin permeability of the test compound-treated groups was compared with that of the control group (TNFα alone) by one-way ANOVA with Dunnett’s post hoc analysis. Differences with a value of p < 0.05 were considered statistically significant.

Results

T₂-Weighted Images. Brain edema was manifested as regions with increased signal intensities on T₂-weighted images taken 24 h after transient MCA occlusion (Fig. 1). All animals of the vehicle-treated group had increased signal intensities not only in cortical regions but also in subcortical regions of the ipsilateral hemisphere, suggesting pronounced brain edema (Fig. 1A). When DY-9760e was intravenously infused at a rate of 1 mg/kg/h for 6 h beginning from 1 h after MCA occlusion, high signal intensity was only detected in the ipsilateral dorsolateral portion of the striatum (Fig. 1B). The lesion volumes as measured by MRI are shown in Fig. 1C. DY-9760e significantly (p < 0.05) reduced the lesion volume in the ipsilateral hemisphere by 67%, and its reduction was evident in the cortex.

Effect of DY-9760e on Brain Edema. Immediately after the measurements by MRI, animals were decapitated and brain water content was determined. In the vehicle-treated group, water content progressively increased from 78.8 ± 0.1% in the contralateral hemisphere to 82.4 ± 0.4% in the ipsilateral hemisphere (Fig. 2), causing the development of brain edema. In the DY-9760e-treated group, water content of the ipsilateral hemisphere was significantly reduced to 80.3 ± 0.5% compared with the content in the vehicle-treated group (Fig. 2). In contrast, DY-9760e had no effect on water content in the contralateral hemisphere (Fig. 2). The fact that DY-9760e affected only the damaged regions but not normal regions may be due to its neuroprotective action, because hyperosmolar agents such as glycerol and mannitol reduce brain water content in both regions (Aoki et al., 2001).

Effect of DY-9760e on BBB Disruption. The effect of DY-9760e on BBB permeability after transient MCA occlusion is shown in Fig. 3. In the vehicle-treated group, transient focal ischemia induced a 5-fold increase in the Evans blue extravasation index, which was expressed as the ratio of the Evans blue content in the ipsilateral cortex to that in the contralateral cortex, implying enhanced BBB permeability.
To understand the mechanism by which calmodulin antagonists attenuate brain edema caused by transient focal ischemia, we next examined whether DY-9760e and trifluoperazine directly affect changes in the barrier function of brain endothelial cells. TNFα, a proinflammatory cytokine, has been shown to increase vascular permeability in brain endothelial cells (Deli et al., 1995). DY-9760e had no effect on the binding of TNFα to TNFα receptors at concentrations at least 100-fold higher (100 μM) than those of calmodulin inhibition (data not shown). We therefore assessed the effects of DY-9760e and trifluoperazine on vascular permeability by using HBMECs treated with TNFα. Vascular barrier function was determined by the permeability of [3H]inulin, a membrane-impermeable molecule, across the HBMEC monolayer. Exposure of cells to 0.3 ng/ml TNFα for 24 h induced a 1.5- to 1.9-fold increase in monolayer permeability to inulin, suggesting the vascular barrier dysfunction of brain endothelial cells. Addition of DY-9760e (0.3–3 μM) simultaneously with TNFα to the culture medium suppressed the TNFα-induced hyperpermeability of inulin in a concentration-dependent manner (Fig. 5A). Similarly, trifluoperazine (1–3 μM) also inhibited an increase in permeability (Fig. 5B). These results suggest that enhanced permeability by TNFα may be dependent on calmodulin.

**Discussion**

The BBB is present at the level of the brain capillaries and is critical for the maintenance of homeostasis in the central nervous system. An alteration of BBB permeability after cerebral ischemia and reperfusion may be an important factor in the development of ischemic brain damage, especially in brain edema formation (Albayrak et al., 1997). It has been reported that MCA occlusion followed by reperfusion results in BBB disruption and subsequently brain edema formation (Schell et al., 1992). Thus, compounds that regulate the permeability of the BBB could be new therapeutic drugs for the treatment of stroke and brain trauma that are accompanied by brain edema. In this article, we provide evidence that DY-9760e, a potent and novel calmodulin antagonist, ameliorates brain edema formation after transient focal ischemia in rats and this effect may be mediated in part by direct protection against BBB dysfunction.

It has been widely accepted that an excessive elevation of the intracellular Ca2⁺ during and after ischemia is a trigger to neuronal damage (Choi, 1988, 1995; Meldrum and Garthwaite, 1990; Mitani et al., 1993). Calmodulin is a major Ca2⁺ binding protein in the brain, where it plays an important role in the neuronal response to changes in the intracellular Ca2⁺ concentration (Zhou et al., 1985; James et al., 1995). In contrast, an excessive elevation of the intracellular Ca2⁺ concentration after ischemic insults may induce aberrant activation of the Ca2⁺/calmodulin signaling systems, resulting in neuronal damage. Indeed, it has been shown that Ca2⁺/calmodulin signaling systems are implicated in neuronal damage as follows: 1) increase of Ca2⁺-bound calmodulin during/after the ischemic insult (Picone et al., 1989); 2) significant up-regulation of the calmodulin gene expression in the CA1 pyramidal cell layer after cerebral ischemia (Palfi et al., 2001); 3) protection by calmodulin antagonists against hypoxic/hypoglycemia in organotypic hippocampal cultures (Sun et al., 1997); and 4) reduction in transient focal is-

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**Fig. 3.** Effect of DY-9760e on BBB disruption in transient MCA-occluded rats. Rats were treated with vehicle or DY-9760e at 1 mg/kg/h for 6 h, beginning 1 h after MCA occlusion. Evans blue dye was extracted from the cerebral cortex with 50% trichloroacetic acid, and the fluorescence of the extract was determined. The Evans blue extravasation index of the DR-9760e-treated group was significantly lower than that of the vehicle-treated group. This result suggests that DY-9760e may protect against BBB dysfunction after transient focal ischemia.

**Effect of Trifluoperazine on Water Content.** Because DY-9760e has an antagonistic effect against calmodulin (Sugimura et al., 1997), we then evaluated the effect of trifluoperazine, which is a calmodulin antagonist structurally unrelated to DY-9760e, on brain edema elicited by transient focal ischemia. Trifluoperazine (30 mg/kg), given intraperitoneally twice, 5 min before MCA occlusion and 4 h after MCA reperfusion, significantly suppressed the increase in water content compared with that in the vehicle-treated group (Fig. 4), suggesting that calmodulin antagonists prevent the development of brain edema.

**Macromolecule Permeability across the Endothelial Cell Monolayer.** To understand the mechanism by which calmodulin antagonists attenuate brain edema caused by transient focal ischemia, we next examined whether DY-9760e and trifluoperazine directly affect changes in the barrier function of brain endothelial cells. TNFα, a proinflammatory cytokine, has been shown to increase vascular permeability in brain endothelial cells (Deli et al., 1995). DY-9760e had no effect on the binding of TNFα to TNFα receptors at concentrations at least 100-fold higher (100 μM) than those of calmodulin inhibition (data not shown). We therefore assessed the effects of DY-9760e and trifluoperazine on vascular permeability by using HBMECs treated with TNFα. Vascular barrier function was determined by the permeability of [3H]inulin, a membrane-impermeable molecule, across the HBMEC monolayer. Exposure of cells to 0.3 ng/ml TNFα for 24 h induced a 1.5- to 1.9-fold increase in monolayer permeability to inulin, suggesting the vascular barrier dysfunction of brain endothelial cells. Addition of DY-9760e (0.3–3 μM) simultaneously with TNFα to the culture medium suppressed the TNFα-induced hyperpermeability of inulin in a concentration-dependent manner (Fig. 5A). Similarly, trifluoperazine (1–3 μM) also inhibited an increase in permeability (Fig. 5B). These results suggest that enhanced permeability by TNFα may be dependent on calmodulin.

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**Fig. 4.** Effect of trifluoperazine on brain edema in transient MCA-occluded rats. Vehicle (saline solution, 1 ml/kg, n = 8) or trifluoperazine (n = 11) at a dose of 30 mg/kg was intraperitoneally administered twice, 5 min before MCA occlusion and 4 h after MCA reperfusion. At 24 h after ischemic induction, brain water content was measured. Each value represents the mean ± S.E.M. (n = 14, in each group). ***, p < 0.001, significantly different from the vehicle-treated group (unpaired Student’s t test).
enhanced permeability of endothelial cells (Tschugguel et al., 1995; Merrill and Murphy, 1997; Abbissutto and Davis, 1999). These reports suggest that intracellular calcium may regulate vascular barrier function. In this study, transient focal ischemia caused a significant increase in Evans blue dye extravasation (Fig. 3), which accounts for the increased permeability of large molecules, including albumin, accelerating brain edema formation. We found that DY-9760e suppresses the increase in Evans blue dye extravasation, suggesting a protective action against BBB dysfunction. However, because calmodulin antagonists such as DY-9760e and trifluoperazine also reduce parenchymal cell injury (Kuroda et al., 1997; Sato et al., 1999; Takagi et al., 2001), it remains unclear whether they have a primary or secondary effect on the BBB.

We then assessed the direct effect of calmodulin antagonists on vascular barrier function in HBMECs. TNFα is a proinflammatory cytokine that increases in the brain after ischemic insult (Yang et al., 1999) and produces increased vascular permeability in brain endothelial cells (Deli et al., 1995). Barone et al. (1997) show that intraventricular injection of TNFα exacerbates brain edema after MCA occlusion in rats, whereas the injection of antibodies against TNFα or soluble TNF receptor I reduces brain injury. Taken together, these reports indicate that TNFα may be a major factor that exacerbates brain edema resulting from BBB disruption. Interestingly, we found that DY-9760e and trifluoperazine are capable of blocking the TNFα-induced increase in permeability of endothelial cells (Fig. 5). Because DY-9760e has no effect on the binding of TNFα to TNFα receptors (data not shown), the protective effects on the abnormal permeability are ascribed to the action against TNFα-induced intracellular events. Ishizu et al. (1995) demonstrate that the calmodulin antagonist W-7 blocks an interleukin-6-mediated increase in permeability of cultured rat endothelial cells. Borbiev et al. (2001) show that calmodulin kinase II activation is involved in endothelial barrier dysfunction. Together, these results suggest that calmodulin-dependent cascades may play a crucial role in the regulation of vascular barrier function. Furthermore, the maximum plasma level of DY-9760e in rats is approximately 1000 ng/ml (2 μM) when continuously infused at a rate of 1 mg/kg/h (Y. Fujimaki, unpublished data), a level similar to that yielding protection against vascular barrier dysfunction in vitro. The results imply that the effective concentration of DY-9760e on vascular barrier dysfunction in vivo corresponds to the plasma level at which DY-9760e suppresses brain edema. Fukunaga et al. (2000) recently demonstrate that DY-9760e inhibits neuronal and endothelial nitric-oxide synthases (NOSs), which are calmodulin-dependent enzymes, and it blocks the nitric oxide production elicited by A23187, a Ca2+ ionophore. Furthermore, TNFα has been shown to induce endothelial NOS activation (Bove et al., 2001). Our preliminary study revealed that the NOS inhibitors such as Nω-nitro-L-arginine methyl ester and Nω-methyl-L-arginine acetate suppress the TNFα-induced increase in inulin permeability across the HBMEC monolayer, implicating nitric oxide as the regulator of barrier function (data not shown). Further studies are needed to assess which Ca2+/calmodulin signaling cascades contribute to the regulation of BBB permeability.

In summary, DY-9760e and trifluoperazine ameliorate brain edema after transient focal ischemia, and these effects...
may be mediated in part by protection against BBB disruption. Thus, drugs that inhibit aberrant activation of calmodulin-dependent pathways may have a therapeutic effect in the acute phase of cerebral ischemic damage.

Acknowledgments

We thank K. Hirot a and T. Aoyagi for technical assistance with the MRI study, E. Oht a for the BBB permeability assay, and N. Ed o for technical assistance with endothelial cell cultures.

References


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