In conclusion, CsA-induced hyperpermeability and P-gp dysfunction of MBEC4 cells were markedly aggravated in co-cultures with brain pericytes. This aggravation appears to occur due to CsA-induced inhibition of TGF-β1 expression in brain pericytes. These findings suggest that an inhibition of brain pericyte-derived TGF-β1 contributes to the occurrence of CsA-induced dysfunction of the BBB, thereby triggering neurotoxicity.

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Rapid Communication

An Inhibitory Role of Nitric Oxide in the Dynamic Regulation of the Blood-Brain Barrier Function

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SUMMARY

1. The present study aimed at elucidating the effects of nitric oxide (NO) on blood-brain barrier (BBB) function with mouse brain capillary endothelial (MBEC4) cells.

2. Histamine (20–100 μM) evoked NO production (1.6–7 μM) in MBEC4 cells in a dose-dependent manner.

3. The permeability coefficient of sodium fluorescein for MBEC4 cells and the cellular accumulation of rhodamine 123 in MBEC4 cells were increased dose-dependently by addition of NO solutions (14 and 28 μM) every 10 min during a 30-min period.

4. The present study demonstrated that NO increased the permeability and inhibited the P-glycoprotein efflux pump of brain capillary endothelial cells, suggesting that NO plays an inhibitory role in the dynamic regulation of the BBB function.

KEY WORDS: nitric oxide; blood-brain barrier (BBB); permeability; P-glycoprotein; mouse brain endothelial cells .

INTRODUCTION

The blood-brain barrier (BBB) contributes to brain homeostasis and fulfills a protective function by regulating the access of solutes and toxic substances to the central nervous system. The BBB is formed by brain capillary endothelial cells, which are closely sealed by tight junctions (Pardridge, 1999). The tight junctions in the

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BBB prevent significant passive movement of small hydrophilic molecules from the blood to the brain, but specialized transport systems mediate the entry of essential substances such as glucose, amino acids, choline, monocarboxylic acids, amines, thyroid hormones, purine bases, and nucleosides (Tsuji and Tamai, 1999; Kusuhara and Sugiyama, 2001). The efflux transporter P-glycoprotein (P-gp) is a key element of the molecular machinery that confers special permeability properties on the BBB. P-gp, which was initially recognized for its ability to excrete anticancer drugs from multidrug-resistant cancer cells, is strongly expressed in brain capillaries. Its expression in the BBB limits the accumulation of many hydrophobic molecules and potentially toxic substances in the brain.

Nitric oxide (NO) is a transient product of inflammatory processes, generated from L-arginine by the enzyme NO synthase (NOS). NO appears to be involved in numerous vital cellular functions including neurotransmission, blood-pressure control, and the regulation of vascular tone. The basal production of NO appears to be required for biological regulation, and yet an excess of this same molecule can be cytotoxic to organism. But the molecular mechanisms mediating NO-induced tissue injury and breakdown of the BBB are not completely understood.

In the present study, to clarify the role of NO in the dynamic regulation of the BBB, we examined effects of NO on the function of tight junctions and P-gp in mouse brain capillary endothelial (MBEC4) cells. MBEC4 cells show the highly specialized characteristics of brain microvascular endothelial cells including P-gp expression (Tatsuta *et al.*, 1992, 1994).

MATERIALS AND METHODS

A saturated NO solution (typically containing approximately 1.4 mM NO) was prepared according to Ikesue *et al.* (2000). Deionized water (2 mL) was bubbled with argon for 20 min to remove oxygen. Then, the solution was bubbled with pure NO gas for 20 min and kept in a glass flask with a rubber septum under a NO atmosphere prior to use.

MBEC4 cells, which were isolated from BALB/c mouse brain cortices and immortalized by SV40-transformation (Tatsuta *et al.*, 1992), were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. They were grown in 2.5-cm^2 dish, 12-well Transwells (Costar, MA) and 24-well plates in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Direct and continuous electrochemical measurement of NO was performed with a three-electrode potentiostatic EMS-100 system (BIO-LOGIC, Grenoble, France), as previously described (Ikesue *et al.*, 2000; Trevin *et al.*, 1998). In brief, confluent MBEC4 cells in a 2.5-cm^2 dish were washed three times with Krebs–Ringer solution (143.0 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, and 11.0 mM D-glucose, pH 7.4). The dish was placed on the stage of an inverted microscope (ECLIPSE TE300, Nikon, Tokyo, Japan) mounted with an NO monitoring system. The NO-biosensor (ASTEC, Fukuoka, Japan) was positioned about 10 μm above the cell surface. Ten minutes after treatment with 1-mM L-arginine (Sigma, St. Louis, MO), histamine (Wako, Osaka, Japan) in a volume of 10 μL was added to the cells in 1 mL of Krebs–Ringer solution with a transient mixing step to give the final concentration indicated. The level of production of NO in MBEC4 cells was monitored for a 15-min period after the addition of histamine.

MBEC4 cells (42,000 cells/cm^2) were cultured on the collagen-coated polycarbonate membrane of the Transwell insert (3.0-μm pore size, 12-well type). After 3 days, they were washed three times with serum-free medium. Cells were exposed to 5 or 10 μL of NO solution (final concentration, 14 and 28 μM, respectively) injected into the inside of the insert (luminal side) every 10 min during a 30-min period. To initiate the transport experiments, the medium was removed, and cells were washed three times with Krebs–Ringer buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-glucose, pH 7.4). Krebs–Ringer buffer (1.5 mL) was added to the outside of the insert (abluminal side). Krebs–Ringer buffer (0.5 mL) containing 100 μg/mL of sodium fluorescein (Na-F; Sigma) was loaded on the luminal side of the insert. Samples (0.5 mL) were removed from the abluminal chamber at 10, 20, 30, and 60 min and were immediately replaced with fresh Krebs–Ringer buffer. Aliquots (5 μL) from the abluminal chamber samples were mixed with 200 μL of Krebs–Ringer buffer, and then the concentration of Na-F was determined using a multiwell fluorometer ($E_x(\lambda)$ 485 nm; $E_m(\lambda)$ 530 nm; CytoFluor Series 4000, PerSeptive Biosystems, Framingham, MA). The permeability coefficient and clearance were calculated according to the method described by Dehouck *et al.* (1992). Clearance was expressed as microliters of tracer diffusing from the luminal to abluminal chamber and was calculated from the initial concentration of tracer in the luminal chamber and final concentration in the abluminal chamber: Clearance (μL) $= [C]_A \times V_A/[C]_L$ where $[C]_L$ is the initial luminal tracer concentration, $[C]_A$ is the abluminal tracer concentration, and V_A is the volume of the abluminal chamber. During a 60-min period of the experiment, the clearance volume increased linearly with time. The average volume cleared was plotted versus time, and the slope was estimated by linear regression analysis. The slope of clearance curves for the MBEC4 monolayer was denoted by PS_{app}, where PS is the permeability-surface area product (in microliters per minute). The slope of the clearance curve with a control membrane was denoted by $PS_{membrane}$. The real PS value for the MBEC4 monolayer (PS_{trans}) was calculated from $1/PS_{app} = 1/PS_{membrane} + 1/PS_{trans}$. The PS_{trans} values were divided by the surface area of the Transwell inserts to generate the permeability coefficient (P_{trans}, in centimeters per minute).

The functional activity of P-gp was determined by measuring the cellular accumulation of rhodamine 123 (Sigma) according to the method of Fontaine *et al.* (1996). MBEC4 cells (21,000 cells/cm^2) were cultured on collagen-coated 24-well plates. Three days after seeding, they were washed three times with serum-free medium and then exposed to 14–28 μM of NO solution every 10 min during a 30-min period. The medium was removed, and the cells were washed three times with assay buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES, and 11 mM D-glucose, pH 7.4). The cells were incubated with 0.5 mL of assay buffer containing 5 μM of rhodamine 123 for 60 min. Then, the solution was removed, and the cells were washed three times with ice-cold phosphate-buffered saline and solubilized in 1 M NaOH (0.2 mL). The solution was neutralized with 1-M HCl (0.2 mL), and the rhodamine 123 content was determined using a multiwell fluorometer ($E_x(\lambda)$ 485 nm; $E_m(\lambda)$ 530 nm). The cellular protein was measured by the method of Bradford (Bradford, 1976).

The effect of the NO solution on cell viability was assessed using a WST-8 assay (Cell Counting Kit, DOJINDO, Kumamoto, Japan). A highly water-soluble formazan dye, reduced by mitochondrial dehydrogenase, was measured by determining the absorbance of each sample with a 450-nm test wavelength and a 700-nm reference wavelength.

The values are expressed as means ± SEM. Statistical analysis was performed using Student's *t*-test. One-way analysis of variance (ANOVA) followed by the Dunnett test was applied to multiple comparisons. The differences between means were considered to be significant when *P* values were less than 0.05.

RESULTS

Figure 1A shows a representative current-time curve obtained with the NO biosensor in MBEC4 cells. When L-arginine (1 mM) was added to cells 10 min before the addition of histamine, the signal became stable within 1–2 min. The NO-biosensor signal increased rapidly to reach a peak within 1 min after the injection of histamine. Following this period, the signal decreased slowly to the baseline about 5–7 min post-injection. Histamine at concentrations of 20–100 μM dose-dependently increased NO production (1.55 ± 0.65–6.94 ± 1.43 μM) in MBEC4 cells (Fig. 1B).

To evaluate effect of NO on the BBB function, the concentration and the exposure time of NO were determined on the basis of NO production evoked by histamine. When the NO solution at the final concentrations of 14 and 28 μM was added every 10 min during a 30-min period, the permeability coefficients of Na-F for MBEC4 cells increased dose-dependently to 113.6 ± 12.9 and 123.7 ± 2.8%, respectively (Fig. 2A). The exposure to NO solution had no effect on cell viability assessed with the WST-8 assay (14 μM: 93.7 ± 3.3%, 28 μM: 101.3 ± 3.2% of control).

As shown in Fig. 2B, the accumulation of rhodamine 123 in MBEC4 cells increased dose-dependently to 108.3 ± 4.8 and 164.5 ± 14.6% of the control value after a 30-min exposure to the NO solution at concentrations of 14 and 28 μM, respectively.

DISCUSSION

As shown in Fig. 1, direct and continuous electrochemical monitoring revealed that histamine stimulated MBEC4 cells to release NO over a short period (about 5 min). This phenomenon appears to be due to an activation of the constitutive endothelial form of NO synthase (eNOS) through a Ca^{2+}/calmodulin-dependent pathway in response to H_1 receptor-mediated increases in intracellular Ca^{2+} (Daum

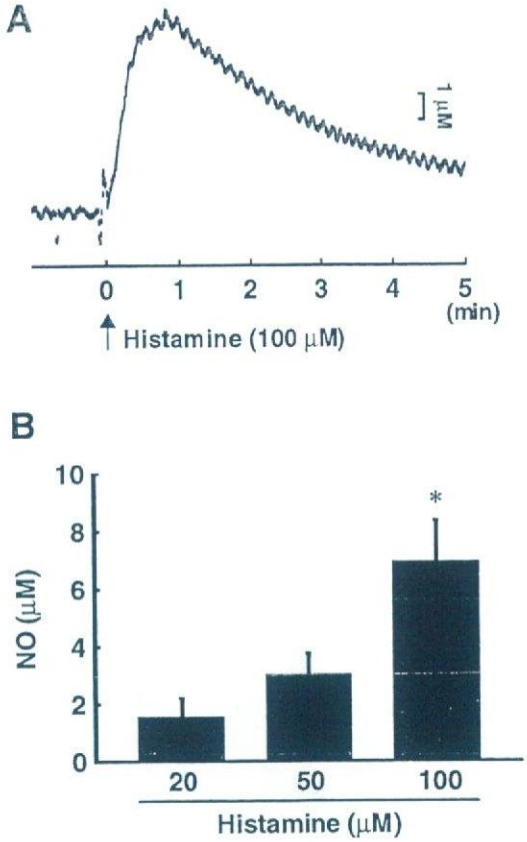

Fig. 1. Histamine-induced NO production in MBEC cells. (A) A representative differential pulse amperogram obtained using a NO biosensor shows the level of NO production evoked by histamine (100 μM). (B) Concentration-response relationship of histamine-induced NO production using direct electrochemical monitoring in MBEC cells. Values are means ± SEM ($n = 3$). $^*p < 0.05$, significant difference from 20 μM histamine.

et al., 1983; Kishi *et al.*, 1996). Various endogenous substances including histamine stimulate NO production in the microvascular endothelium and/or glial and neuronal cells in the brain (Mayhan, 1996), probably contributing to dynamic regulation of the BBB function.

The permeability of MBEC4 cells to Na-F was apparently increased by a brief exposure to NO solutions at 2- to 4-fold the concentrations induced by histamine (Fig. 2A). The NO solutions employed here are more prominent than convenient NO donors such as sodium nitroprusside with respect to biological properties including distribution and degradation. The cell viability was not influenced by NO at the concentrations employed here (14–28 μM), suggesting that the increased paracellular permeability of MBEC4 cells was not due to the cytotoxicity of NO. Therefore, NO is highly likely to lower the functions of the tight junctions at the

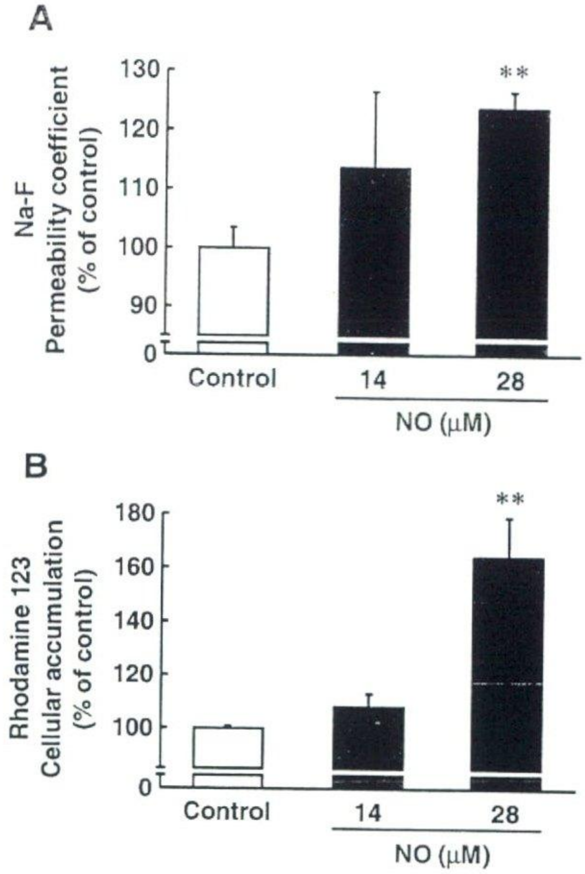

Fig. 2. Changes in the permeability coefficient of Na-F (A) and the cellular accmulation of rhodamine 123 (B) in MBEC4 cell monolayers after addition of NO solutions every 10 min during a 30-min period. Data are expressed as a percentage of the corresponding control value (A: 1.47 ± 0.21 × 10^{-4} cm/min, B: 0.95 ± 0.07 nmol/mg protein). Values are shown as means ± SEM (n = 3–18). $^{**}P < 0.01$, significant difference from control.

BBB, supporting early reports (Hurst and Fritz, 1996; Mayhan, 2000: Shukula *et al.*, 1996). The mechanisms by which NO donors increased vascular endothelial permeability involved an increase in the level of cyclic guanosine monophosphate (cGMP) A1 (Gimeno *et al.*, 1998) or the formation of peroxynitrite (Menconi *et al.*, 1998). These substances conceivably influence the intrinsic tight junction proteins and the associated actin cytoskeleton through a direct or second signaling pathway. Further studies are required to clarify this mechanism.

In the present study, the most important finding was that the accumulation of rhodamine 123, a substrate of P-gp (Fontaine *et al.*, 1996), in MBEC4 cells was increased by treatment with NO solutions (Fig. 2B). P-gp is an energy-dependent efflux pump mediating the transportation of substances from the basement membrane to the luminal surface. NO induced a depletion of endothelial ATP by inhibiting glyceraldehyde-3-phosphate dehydrogenase (glycolytic enzyme) activity (Hurst *et al.*, 2001). This inhibition of energy metabolism is probably associated with the NO-triggered inhibition of P-gp function.

In the present study, NO induced hyperpermeability in MBEC4 cells and inhibition of the P-gp efflux pump. These findings suggest that NO in the brain plays an inhibitory role in the dynamic regulation of the BBB function.

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Abstract:

Chelating compound, chrysoidine, is more effective in both anti-prion activity and brain endothelial permeability than quinacrine

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Running title: Aromatic azo chemicals with anti-prion activity

SUMMARY

1. As an extension of our previous study of quinacrine and its derivatives, chelating chemicals were screened to obtain more effective, better brain-permeable anti-prion compounds using either prion-infected neuroblastoma cells or brain capillary endothelial cells.
2. Eleven chemicals were found to have anti-prion activity. Most of them shared a common structure consisting of benzene or naphthalene at either end of an azo bond. Structure-activity data suggest that chelating activity is not necessary but might contribute to the anti-prion action.
3. Chrysoidine, a representative compound found here, was about 27 times more effective in the anti-prion activity and five times more efficiently permeable through the brain capillary endothelial cells than quinacrine was.
4. These chemicals might be useful as compounds for development of therapeutics for prion diseases.

KEY WORDS: prion; chrysoidine; blood-brain barrier; aromatic azo compounds; therapy; chelating agents; brain endothelial cells; prion-infected neuroblastoma cells

INTRODUCTION

Transmissible spongiform encephalopathies or prion diseases are fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease and Gerstmann–Sträussler–Scheincker syndrome in humans, and scrapie, bovine spongiform encephalopathy and chronic wasting disease in animals. These disorders are characterized by accumulation in the brain of an abnormal isoform of prion protein (PrP), which is putatively a main component of pathogens or the pathogen itself, and which is rich in beta-sheet structure and resistant to digestion with proteinase K (Prusiner, 1991). Recent outbreaks of variant Creutzfeldt-Jakob disease and iatrogenic Creutzfeldt-Jakob disease through use of cadaveric growth hormone or dural grafts in younger people have necessitated the development of suitable therapies.

We previously found quinacrine and its derivatives to have potent anti-prion activity in prion-infected cells (Doh-Ura *et al.*, 2000; Murakami-Kubo *et al.*, 2004). The common structure of these chemicals, a quinoline ring with a side chain containing a nitrogen atom located at a particular distance from another nitrogen atom in the ring indicates that the chemicals have chelating activity, but the involvement of chelating metals in their anti-prion activity has never been confirmed. Quinacrine has been used recently for clinical trials of patients with prion diseases in several countries. Orally administered quinacrine is reportedly effective in transiently improving cognitive functions of patients (Nakajima *et al.*, 2004), but it frequently causes such adverse effects as liver dysfunction. For that reason, either improving its penetration into the brain (the target organ of prion diseases) or reducing its uptake into the liver is suggested for producing more beneficial results (Dohgu *et al.*, 2004).

Here, to obtain more effective anti-prion compounds with better brain permeability than quinacrine, we screened chelating chemicals in prion-infected neuroblastoma cells. We investigated the brain permeability of a representative chemical using an *in-vitro* model for the blood-brain barrier.

MATERIALS AND METHODS

Chemicals and cells Chemicals used in the study were purchased from Sigma-Aldrich Corp. (St. Louis, MO), Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), and Wako Pure Chemical Industries Ltd. (Osaka, Japan). All chemicals, except for chrysoidine, were dissolved in 100% dimethyl sulfoxide (DMSO), although chrysoidine was dissolved in distilled water.

Acetylated Yellow AB was obtained as follows. Yellow AB was dissolved in dichloromethane and mixed with excess glacial acetic acid. After its complete acetylation was observed by thin layer chromatography, the acetylated product was purified using silica gel column chromatography (dichloromethane/ethyl acetic acid: 9/1 (v/v)). The residual solid was lyophilized and identified as acetylated Yellow AB by both fast atom bombardment mass spectrometry and elemental analysis.

Murine neuroblastoma cells that had been persistently infected with the scrapie prion strain RML (ScNB cells) (Race *et al.,* 1988) were used for the assay of anti-prion activity and grown in Opti-MEM (Invitrogen Corp., CA) containing 10% fetal bovine serum. For the assay of brain endothelial permeability, immortalized endothelial cells from the murine brain capillary (MBEC4 cells) (Tatsuta *et al.,* 1992) were used and grown in DMEM (Invitrogen Corp., CA) containing 10% fetal bovine serum, 100 µg/mL streptomycin and 100 units/mL penicillin.

Anti-prion activity assay Anti-prion activity of a chemical was assayed by measuring its 50% inhibition dose (IC_{50}) for abnormal PrP formation in ScNB cells, as described previously (Doh-Ura *et al.,* 2000; Ishikawa *et al.,* 2004). Each chemical was added at designated concentrations when cells were passed at 10% confluency. Final concentration of DMSO in the medium was maintained as less than 0.5%. The cells were allowed to grow to confluence and were lysed with a lysis buffer (0.5% sodium deoxycholate, 0.5% Nonidet P-40, PBS). The lysates were digested with 10 µg/mL proteinase K for 30 min and centrifuged at 100,000 × *g* for 30 min at 4°C. The pellets were resuspended in the sample loading buffer and boiled. The samples were separated using electrophoresis on a 15% Tris-glycine-SDS-polyacrylamide gel and electroblotted. Detection of PrP was done using an antibody PrP-2B, followed by an alkaline phosphatase-conjugated secondary antibody. Immunoreactive signals were visualized with CDP-Star detection reagent (GE Healthcare Bio-Science, NJ) and were analyzed densitometrically. Three independent assays were performed in each experiment.

Surface plasmon resonance assay Binding assay of a chemical with recombinant PrP was performed using an optical biosensor (Biacore AB, Uppsala, Sweden), as described previously (Kawatake *et al.*, 2006). Briefly, recombinant mouse PrP (amino acids 121-231; PrP121-231) was immobilized on a biosensor chip at a density of *ca.* 3,000 resonance units (RU) using amine coupling. Test chemicals were diluted to 50 μM with the running buffer (3% DMSO in PBS, pH 7.4) and were injected over both the PrP flow cell and the reference for 60 s at a flow rate of 20 μL/min. The dissociation phase was monitored for 60 s with injection of the running buffer at a flow rate of 20 μL/min. The flow cell was washed with 10 mM NaOH for 30 s between sample injections. Buffer blanks for double referencing were injected before sample analyses.

Brain endothelial permeability assay Permeability assay was performed as described previously (Dohgu *et al.*, 2004). Briefly, MBEC4 cells were cultured on the collagen-coated polycarbonate membrane of a Transwell insert (Corning Coster Corp., MA). Before assay, the cells were washed with Krebs-Ringer buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11 mM D-glucose, pH 7.4). Then, the buffer (1.5 mL) was added outside of the insert (abluminal side), and the buffer (0.5 mL) containing 100 μM of a chemical was loaded on the luminal side of the insert. Samples (0.5 mL) were recovered from the abluminal chamber at 10, 20, 30, and 60 min and replaced immediately with fresh Krebs-Ringer buffer. Sodium fluorescein (Na-F, MW 376; Sigma-Aldrich Corp., MO) was used as a paracellular transport marker, and chrysoidine (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) as a test chemical, in addition to quinacrine as a control. The chemical concentration was measured by either determining the fluorescent intensity of Na-F (Ex(λ) 485 nm; Em(λ) 530 nm) and quinacrine (Ex(λ) 450 nm; Em(λ) 530 nm) or determining the absorbance of chrysoidine at 450 nm. The permeability coefficient was calculated using the slope of clearance curve for each chemical obtained during the 60-min period according to the method described by Dehouck et al. (Dehouck *et al.*, 1992). Statistical analysis was performed using one-way analysis of variance followed by Tukey-Kramer method for multiple comparisons.

RESULTS

Anti-prion screening *in vitro* To evaluate functional groups of anti-prion chelating chemicals, various chelating chemicals were examined for whether they inhibited abnormal PrP formation in prion-infected ScNB cells. Thirty-five chelating chemicals were analyzed; 11 of them were effective in inhibiting abnormal PrP formation for doses at which cell toxicity was not observed (Tables 1 and 2). Nine of the 11 effective chemicals had a common structure, which consisted of aromatic rings (terminals 1 and 2 in Table 2) in both ends of an azo bond. Although both 4-methyl-2-(2-thiazolylazo)phenol and 4-(2-pyridylazo)resorcinol were not effective, they also exhibited this structure, with a thiazole ring and a pyridine ring in the terminal 1 portion, respectively. Their lack of effectiveness might be attributable to cell toxicity, which occurred at lower doses than for chemicals carrying a benzene ring in the terminal 1 portion. On the other hand, all chemicals carrying either a benzene ring or a naphthalene ring in the terminal 2 portion were effective. Therefore, the data suggest that a structure with such an aromatic ring as benzene or naphthalene in either end of an azo bond might be responsible for inhibiting abnormal PrP formation in ScNB cells.

Mechanism of anti-prion action We tested whether the effective chemicals cause any alteration of the cellular PrP level in the treated cells because reduction in the cellular PrP level engenders reduction in abnormal PrP formation. The results revealed no reduction in the cellular PrP level of the cells (data not shown). Furthermore, either to examine whether the chemicals directly destabilize or denature the abnormal PrP structure or to exclude the possibility of interference with preparation and immunodetection of the abnormal PrP, the cell lysate either alone or mixed with the chemicals was incubated at 37°C for one hour prior to proteinase K digestion; it was then processed ordinarily to obtain the abnormal PrP. The results indicated that the chemicals did not affect the abnormal PrP signals (data not shown).

Because it was predicted that the chemicals might exert their anti-prion action through a certain mechanism involving chelating metals, the most effective chemical found here, chrysoidine, was pre-incubated before addition to the ScNB culture medium with an equivalent dose or lower doses of various metal ions, including copper, zinc, cobalt, and aluminum ions. The results revealed no change in the inhibition activity of the chemical (Fig. 1). Furthermore, to examine whether chelating activity is necessary for anti-prion action, we modified Yellow AB in such a manner that its amino base was acetylated to remove its chelating activity. The acetylated Yellow AB was tested in ScNB cells, and it was one-eighth as effective in inhibiting abnormal PrP formation as Yellow AB (Fig. 2A). Finally, as a chemical bearing the effective structure but lacking chelating activity, the chemical azobenzene, which is most similar in the structure to the chemical chrysoidine, was tested. It was about 30 times less effective than chrysoidine (Fig. 2B). These findings suggest that chelating activity is not essential for the anti-prion action but might influence it.

Interaction with recombinant PrP We previously reported that more potent anti-prion agents have higher affinity to recombinant PrP121-231 in surface plasmon resonance (SPR) analysis (Kawatake *et al.*, 2006). Therefore, we examined whether this is also demonstrated in the effective chelating chemicals found here. Six of the chemicals (each at 50 μM) were tested. The SPR sensorgrams of the chemicals except 4-(2-pyridylazo)resorcinol showed weak signal responses of less than 100 RU (Fig. 3). However, neither 4-(2-thiazolylazo)resorcinol nor Yellow AB reached the equilibrium state at the association phase; neither 4-(2-thiazolylazo)resorcinol nor 2-phenylazo-4-methylphenol returned to the baseline at the dissociation phase. In contrast, 4-(2-pyridylazo)resorcinol showed the strongest response of more than 200 RU and neither reached the equilibrium state at the association phase nor returned to the base line at the dissociation phase. The binding response value from the sensorgram (equilibrium or maximum response value divided by molecular weight), which is an index for estimating the interaction of a chemical with the molecules sited on a biosensor chip (Frostell-Karlsson *et al.*, 2000), showed no apparent relationship with the IC_{50} value of anti-prion activity (data not shown), suggesting that the chemicals found here might exert their anti-prion action in a manner that differs from those of previously reported anti-prion chemicals such as antimalarias and amyloid binding dyes.

Brain endothelial permeability The brain is the main organ that is affected in prion diseases. Therefore, therapeutic compounds must penetrate into the brain. To examine the permeability of a chemical through the blood-brain barrier, we used a simple analytical model consisting of brain capillary endothelial MBEC4 cells. As a representative of the effective chemicals found in the study, chrysoidine was examined in this model and compared with a paracellular marker, Na-F, as well as a control, quinacrine, which has been used for clinical trials of patients with prion diseases. The results showed that the respective permeability coefficients of Na-F, quinacrine and chrysoidine were 2.17×10^{-3}, 0.96×10^{-3} and 4.63×10^{-3} cm/min. Therefore, chrysoidine penetrated the brain capillary endothelial cells about five times more efficiently than quinacrine.

DISCUSSION

Here we revealed that chelating chemicals, especially aromatic azo compounds, have anti-prion activity. Mechanisms of their anti-prion action apparently include neither alteration of cellular PrP level nor direct modification of abnormal PrP. Taken together with previous findings related to the interaction of PrP with metals (review in, Brown, 2004), the data obtained through the present study suggest that the chelating activity might influence the anti-prion action but is not essential for it. This inference is consistent with our previous results from quinacrine derivatives carrying chelating activities (Murakami-Kubo *et al.,* 2004).

Chrysoidine, a representative chemical found in this study, is far superior to quinacrine in both the anti-prion activity and the brain endothelial permeability. The respective anti-prion activities of chrysoidine and quinacrine in ScNB cells were 15 nM and 400 nM in IC_{50}, indicating that chrysoidine is about 27 times more effective than quinacrine. Furthermore, chrysoidine penetrated brain capillary endothelial cells about five times more efficiently than quinacrine. In addition, chrysoidine is much less toxic than quinacrine because a maximal dose at which the ScNB cell growth to confluence is still tolerant was more than 100 μM in chrysoidine or 2 μM in quinacrine (Table 2). These findings suggest that chrysoidine might be more beneficial *in vivo* than quinacrine, but the *in-vivo* efficacy of chrysoidine remains to be evaluated.

Results from the SPR analysis obtained here were not consistent with those of our previous study (Kawatake *et al.,* 2006), where the SPR binding response correlates with the inhibition activity of abnormal PrP formation in ScNB cells. Chrysoidine, the most effective chemical in the study, has a similar structure to either half of a symmetrical compound, Congo red, whose anti-prion activity (IC_{50}=14 nM) is as prominent as that of chrysoidine (IC_{50} = 15 nM) (Table 2). Interaction with recombinant PrP121-231, however, differs greatly between chrysoidine and Congo red. Congo red has very high affinity (K_D = 1.6 μM) and strong binding response (1.7 RU/Da at 10 μM using a *ca.* 3,000 RU PrP-bound biosensor chip) to the PrP121-231 (Kawatake *et al.,* 2006), whereas chrysoidine shows a sensorgram pattern of low affinity compounds and has very low binding response (0.1 RU/Da at 50 μM using a