Evaluation of Carbamazepine Pharmacokinetic Profiles in Mice with Kainic Acid-Induced Acute Seizures

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The purpose of the present study was to evaluate the effect of kainic acid (KA)-induced acute seizures on the pharmacokinetic profiles of antiepileptic drug, carbamazepine (CBZ) in mice. Experimental acute seizure in mice was induced by intraperitoneal injection of KA (30 mg/kg), and mice were provided for experiments after 48 h of KA treatment. The portal plasma concentrations of CBZ and its metabolite carbamazepine-10,11-epoxide (CBZ-epo) had trends to decrease as compared to the control mice, whereas the brain CBZ and CBZ-epo concentrations was actually lower in KA treated mice. On the other hand, the exosorption of CBZ from blood to the intestinal lumen via P-glycoprotein (P-gp) in KA treated-mice was significantly increased in parallel with that of Rhodamine-123 (Rho123), a P-gp substrate. Western blotting analysis for intestinal and cerebral P-gp showed that the P-gp expression was induced in the KA-treated mice. The apparent brain-to-plasma concentration ratio (Kp) of CBZ in the KA-treated mice showed significant decrease but that of CBZ-epo did not. Moreover, in the KA-treated mice, the percentage of protein binding was significantly increased, and found to be an inverse proportion in the relationship between the Kp and protein binding of CBZ. In conclusion, the mechanism responsible for a decreased brain CBZ concentration in the KA-induced seizure mice is based on the up-regulation of P-gp function in tissues and plasma protein binding of CBZ.

Key words carbamazepine; kainic acid-induced seizure; P-glycoprotein; antiepileptic drug; brain distribution

Approximately 30% of epileptic patients do not respond to the usual antiepileptic drugs (AEDs), representing a major problem associated with increased morbidity and mortality. 1) The underlying mechanisms are not completely understood; it has been believed that mechanisms leading to AEDs resistance are most likely complex network phenomena including development of tolerance to antiepileptic drugs’ action or alterations in drug targets based on pharmacogenomic factor. 2) Among various transporters, P-glycoprotein (P-gp) has been identified as being important regulators of bioavailability of drugs, which is located in the endothelial cells of the blood–brain barrier and/or the small intestine, and its outwardly directed active efflux mechanisms from the blood to intestinal lumen or cerebral cell to the blood appear to act as a second line defense mechanism. P-gp, gene product of the multidrug resistance 1 (MDR1 gene), limiting brain accumulation and intestinal absorption of many lipophilic drugs. 3)–6)

Because of lipophilic properties, some AEDs have been established as a substrate of P-gp, and P-gp participates in the regulation of their extracellular brain concentrations. 7) In case of carbamazepine (one of major AEDs), however, there are contradictions among the papers on the relationship between P-gp function and CBZ disposition. One reports concluded that CBZ is not a substrate for P-gp using mdr1a/b(−/−) knockout mice or P-gp transfected cell lines, 8)9) and the other reports concluded that CBZ is appropriate substrate for P-gp using P-gp transfected cell lines or normal rats with a microdialysis method. 2)7) In addition, study on pharmacoresistance in patients with epilepsy using a radio-labeled P-gp substrate (verapamil) and positron-emission tomography (PET) method showed that regionally enhanced P-gp activity in brain might contribute to drug resistance in some patients with temporal lobe epilepsy. 10) These reports indicate that there is complexity to explain the relationship between CBZ therapy and drug resistance through the P-gp function, and it is not known to what extent the P-gp is involved in the transport of CBZ in a living body in epileptic patients. Therefore, it is important that confirmation of P-gp function during seizure in the living organism should be conducted to relief contradictions among those literatures.

MATERIALS AND METHODS

Materials Carbamazepine (CBZ), carbamazepine-10,11-epoxide (CBZ-epo), midazolam (MDZ) and Rhodamine-123 (Rho123) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Kainic acid (KA) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). N-Methyl-2-pyrrolidone (Pharmasolve®) and Polyoxyethylene (40) hydrogenated castor oil (HCO-40) were kindly supplied by International Specialy Products Technologies Inc. (Wayne NJ, U.S.A.) and Nikko Chemical Co., Ltd. (Tokyo, Japan). As a primary antibody for P-glycoprotein (P-gp), C219 antibody was purchased from Calbiochemi, Co. (CA, U.S.A.). As a secondary antibody, anti-mouse IgG, immune-star HRP chemiluminescent (ECL) kit was purchased from Bio-Rad Laboratory, Inc. (CA, U.S.A.). All other chemicals were of the highest grade available.

Animals All animal experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto Pharmaceutical University. Male ddY mice (6 weeks old, 28—35 g) were purchased from Japan SLC Inc. (Hamatsu, Japan), and housed in a temperature and humidity-controlled room with free access to water and standard mouse chow. Experimental acute seizure was induced by an intraperitoneal injection of KA (30 mg/kg). Saline-treated mice served as controls. Mice with seizure in the stage 4—5,
which go into convulsions at least within 90 min after the administration of KA, were selected for the experiments, and then were provided for each experiment at 48 h after the treatment by KA.

Preparation of Standard and Test Solutions The standard stock solutions for calibration curves of drugs were prepared by dissolving them in methanol at various concentrations, and were stored at 4 °C in the dark. Calibration curve samples were prepared by adding known amounts of these standard stock solutions to plasma or dialysate in a volume ratio of 1:100. The test solution of CBZ or MDZ for intravenous administration were prepared by dissolving CBZ or MDZ in a vehicle composed of 5% ethanol, 5% HCO-40 and 5% Pharmasolve® in 0.9% saline at a final concentration of 0.4 mg/ml or 0.5 mg/ml, respectively. The test solution of Rho123 for intravenous administration were prepared by dissolving Rho123 in a vehicle composed of 5% Pharmasolve® in 0.9% saline at a final concentration of 0.2 mg/ml. For absorption studies of in-situ loop method, the test solutions of CBZ was prepared by suspending them in 1% carmellose sodium (CMCNa) at final concentrations at 0.5 mg/ml. All test solutions were prepared just before experiments.

In-Situ Intraloop Administration Method Mice were fasted for at least 12 h with free access to water, and were divided into 4 groups. Then mice were anaesthetized with an intraperitoneal injection of sodium pentobarbital (32 mg/kg). The mice were placed in a spine position on a heating pad under a surgical lamp to maintain constant normal body temperature. A midline longitudinal abdominal incision was made, and the inlet or outlet silicon tube (2.1 mm i.d.) was placed at ileum to make 10-cm loop. Then the loop was flushed 3 times with prewarmed (37 °C) phosphate buffered saline (PBS) containing 25 mM glucose (G-PBS, pH 7.4). The test solution of CBZ (0.5 mg/ml/kg) was administered to the intestinal loop. After intraloop administration of CBZ, the blood samples were collected at 0.5, 1.0, 2.0, 3.0 h through the portal vein. Simultaneously, the brain was removed by sacrificing mice at each correspondence time. Plasma was separated (9000×g, 5 min, 4 °C), and brain was flushed by 0.9% saline and homogenized to make 10% (w/v) tissue sample with 0.9% saline. Plasma or brain tissue samples were stored at −80 °C until the day of assay.

In-Situ Single Perfusion Method Mice were fasted for at least 12 h with free access to water and were anaesthetized with an intraperitoneal injection of sodium pentobarbital (32 mg/kg). The mice were placed in a spine position on a heating pad under a surgical lamp to maintain constant normal body temperature. A midline longitudinal abdominal incision was made, and the proximal end of jejunum was catheterized with an inlet silicon tube (2.1 mm i.d.), which was connected to a perfusion pump. The distal end of the ileum was also cannulated with an outlet silicon tube to collect the intestinal effluent. Then the 20 cm lumen of the whole intestine was flushed 3 times with prewarmed G-PBS. Subsequently, G-PBS was perfused through the intestine at a flow rate of 0.2 ml/min. After 10 min of perfusion for stabilization, the test solution of CBZ (0.4 mg/ml/kg) or Rho123 (0.2 mg/ml/kg) was intravenously injected via the jugular vein. Simultaneously, intestinal effluent was collected at designated intervals for 120 min. Samples were kept at −80 °C until the day of assay.

Everted Sac Method According to the method of Barr and Reigelman, an intestinal everted sac was prepared from drug free mice ileum to investigate whether KA has direct effect on the Rho123 uptake via P-gp. The 10 cm length of everted sac was preincubated with KA (1.0 mg/ml) at 37 °C for 30 min in 50-ml glass test-tubes containing 10 ml of Krebs–Henselit buffer (pH 6.0) with supplying 95% O2–5% CO2. Then, Rho123 or CBZ was added in the mucosal side at a final concentration of 50 μg/ml. After the incubation, mucosal fluid was sampled, and the uptake amounts of Rho123 were calculated.

Protein Binding and Apparent Coefficient of Brain Distribution Mice were fasted for at least 12 h with free access to water and were anaesthetized with an intraperitoneal injection of sodium pentobarbital (32 mg/kg). Mice received oral CBZ (0.5 mg/ml/kg), then blood from jugular vein and brain were obtained at 30 min after the oral administration. Using 0.5 ml of plasma, plasma water was obtained by an ultracentrifugation by means of Centrifree® (Millipore, MA, U.S.A.). Brain was homogenated as described above. Samples were kept at −80 °C until the day of assay.

Western Blot Analysis Crude protein fractions were prepared from the intestinal mucosa or brain of control or KA-treated mice, according with manufacturer’s instructions with some modifications. Briefly, after the luminal contents of small intestine were isolated and flushed with phosphate-buffered saline, the intestine was opened and laid on a chilled glass plate. Intestinal membrane tissue was collected by scraping with a slide glass and homogenized by adding a T-PER (Pierce, Rockford, IL, U.S.A.) containing 1 mM PMSF at 20 ml/g tissue. For the cerebral crude protein, 1 wet-gram of brain was homogenized in 20 ml of T-PER containing 1 mM PMSF. These homogenates were centrifuged at 5400×g for 10 min (4 °C), and then the resulting supernatant was centrifuged at 14000×g for 30 min (4 °C). The pellets were resuspended in buffer containing 300 mM mannitol and 40 μg/ml PMSF (pH 7.5) with a syringe needle. Protein concentrations in those crude suspensions were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as the standard. The amount of protein in the brain or intestinal samples was adjusted to 180 μg/lane or 40 μg/lane, respectively, and then was loaded onto the 7.5% SDS-gel. The electrophoresis procedure using primary (C219) and secondary antibodies was described in our previous reports using ECL system. The final optimal density was measured by means of Dolphin-Chemi (Kurabo, Osaka, Japan).

Drug Assay The determination of Rho123 concentrations in the buffer solution was performed immediately after the experiment. An equal volume of methanol was added to 100 μl aliquots of samples. The mixture was vortexed for 30 s, and centrifuged at 9000×g for 10 min at 4 °C. One hundred microliter aliquot of the supernatant was then added to the 96-well microplate, and Rho123 concentrations in the samples were analyzed by a Fluoroscan Acent CF (Dainippon-Pharm. Co., Tokyo, Japan) with fluorimetric detection, with an excitation wavelength of 485 nm and an emission wavelength of 527 nm. Concentrations of CBZ and CBZ-epo in plasma, plasma water, and 10% brain homogenate and intestinal perfusates, and MDZ in plasma were measured by a liquid chromatography-mass spectrometry (LC/MS). For ex-
traction procedure of plasma and 10% brain homogenate samples, to 100 µl aliquot of samples 10 µl of an internal standard solution (indinavir, 10 µg/ml in methanol) was added and vortexed. Then 100 µl of 2% (w/v) ZnSO₄ in 50% methanol solution was added and vortexed to precipitate proteins. After centrifuging at 9000 × g for 10 min (4 °C), clear supernatant was transferred to a clean centrifuging tube and then 1 ml of diethylether was added to extract analytes. The diethylether phase was decanted and evaporated at 40 °C under reduced pressure. For the intestinal perfusate and plasma water, adding the internal standard the analytes were directly extracted from 100 µl samples by ether without 2% (w/v) ZnSO₄ in 50% methanol. The LC/MS system (Shimadzu, Kyoto, Japan) consisted of the following components: a SIL-10A system controller, a LC-10Advp pump, a SPD-10A UV detector, a SIL-10ADvp automatic injector, a CTO-10A column oven and LCMS-QP8000 mass spectrometer equipped with a CLASS-8000 work station. The analytical column for the analytes was a Cadenza CD-C₁₈ (2.1 mm i.d.×100 mm, 5 µm size, Intakt, Kyoto, Japan), and the column temperature was maintained at 65 °C for all separations. Elution was carriedout isocratically at a flow-rate of 0.2 ml/min with 90% acetonitrile containing 1% acetic acid. The mobile phase was degassed before use. Mass spectrometry was performed utilizing atmospheric pressure chemical ionization (APCI) at a positive mode. The voltages of APCI probe, curved desolvation line (CDL) and deflectors were set at 5 kV, −30 V and +55 V, respectively, and the flow rate of nebulizing gas (N₂) was set at 2.5 l/min. The temperatures of APCI probe and CDL were set at 400 °C and 230 °C, respectively. The final extraction residues from samples were reconstituted with 70 µl of the mobilephase and 20 µl of sample was injected into the LC/MS system. CBZ, CBZ-epo, MDZ, it's hydroxyl body (MDZ-OH) and indinavir were detected by molecular mass of 273 m/z, 253 m/z, 327 m/z, 343 m/z and 615 m/z, respectively.

Data Analysis  The apparent distribution constant (Kp) of CBZ and CBZ-epo to brain was calculated by dividing brain concentration by plasma concentration in jugular vein. All values are expressed as the mean±S.D. Statistical differences of the means were assumed to be significant when p<0.05 by one-way ANOVA followed by Tukey’s multiple range tests.

RESULTS

Cerebral and Portal Kinetics of CBZ after Intraloop Administration of CBZ  Time courses of cerebral andportal concentration of CBZ and its metabolite, CBZ-epo following intraloop administration at a dose of 0.5 mg/kg CBZ to KA-treated and control mice are shown in Fig. 1. The portal plasma concentrations of CBZ in the KA-treated mice was significantly decreased (Fig. 1b), and CBZ-epo had trends to decrease as compared to the control mice (Fig. 1d). While in the brain, CBZ and CBZ-epo concentrations after intraloop administration of CBZ were lower in the KA treated mice, indicating that the transferability of CBZ or CBZ-epo to the brain in KA-treated mice was decreased (Figs. 1a, c).

Effects of KA Treatment on CBZ and Rho123 Uptake to the Everted Sac  To confirm the effects of KA treatment on the transport via P-gp, the uptake studies of Rho123 (an established substrate of P-gp) and CBZ, were conducted using a everted sac method. As shown in Fig. 2, there was no
change in the Rho123 or CBZ uptake to the everted sac between with and without KA (1.0 mg/ml), suggesting that KA has no potential to affect the function of P-gp.

Intestinal Exsorption of CBZ and Rho123 in Mice
The cumulative amounts of CBZ and Rho123 in the intestinal perfusate following intravenous administration are shown in Fig. 3. The cumulative amounts of CBZ in the intestinal perfusate at 120 min after the intravenous administration in the KA-treated- and control mice were 25.1 ± 3.1 μg and 20.1 ± 1.8 μg, respectively, and those of Rho123 were 1.4 ± 0.3 μg and 0.8 ± 0.3 μg, respectively. The intestinal exsorption of CBZ and Rho123 in the KA treated-mice were significantly increased as compared with those of respective control mice.

Western Blotting Analysis of Intestinal and Cerebral P-gp
To further characterize the specific interaction of CBZ with P-gp in the KA-treated mice, we investigated the expression of P-gp in the intestine and brain using C219 antibody. As shown in Fig. 4, highly immunoreactive band at 170 kDa is present in the brain and intestine in the KA-treated mice as compared to the control. The optical intensity of P-gp band for the intestine and brain showed significant 2.2-fold and 2.5-fold increases, respectively (Fig. 4c). These observations indicate that the P-gp expression was induced in the KA-treated mice.

Brain-to-Plasma Concentration Ratio (Kp) and Protein Binding of CBZ and CBZ-Epo in KA-Treated Mice
The concentrations of CBZ and CBZ-epo in jugular plasma and brain, the Kp values and the percentages of protein binding after oral administration of CBZ (0.5 mg/kg) are listed in Table 1. There were no significant changes in plasma–drug concentrations in the KA-treated and control mice. However the brain concentrations of CBZ and CBZ-epo were significantly decreased as compared to the controls. The Kp value of CBZ in the KA-treated mice showed significant decrease. The Kp value of CBZ-epo in the KA-treated mice also had trend to decrease, but was found to be no significant. In the in KA-treated mice, the percentage of protein binding was significantly increased, and found to be an inverse proportion in the relationship between the Kp and protein binding of CBZ in the in KA-treated mice.

Intravenous Administration of MDZ as a Specific Substrate for Cytochrome P450 3A (CYP3A)
To confirm the effects of KA treatment on the liver metabolism via CYP3A, MDZ (0.5 mg/kg) was intravenously administered to the KA-treated and non-treated mice. However, no significant changes in the plasma concentrations of MDZ or its hydroxyl metabolites (MDZ-OH) were observed (Fig. 5).

DISCUSSION
About 30 percentage of epileptic patients develop intractable seizures, and it has been believed that those epileptic patients have drug-resist epilepsies. However, there has been no clear answer to explain the mechanism in the relationship between invagination of epileptic fit and CBZ efficacy. On the other hand, P-gp, which locates on the inside cells of capillary vessel of blood–brain barrier (BBB), regulates the transfer of drug from blood circulation to the central nerve system to avoid neurotoxicity. In addition, P-gp,
which locates on the brush-border membrane in gut epithelial cell of the intestine, plays a role of barrier to regulate drug absorption or metabolism. Therefore, alteration in the amount of P-gp expression should lead to alteration in the pharmacokinetics of drugs. Using a cerebral microdialysis method, Potschka et al. reported that CBZ, phenytoin and valproate were substrates of P-gp in the BBB, and the pharmacokinetics and pharmacodynamics of these antiepileptic drugs were regulated by the function of P-gp. Moreover, Kwan et al. reported that phenytoin, gabapentin and CBZ are good substrates for P-gp in mdr1a knock-out mice. For carbamazepine (CBZ), however, there are paradoxical observations in the literatures. Baltes et al. demonstrated that CBZ was not transported by any type of P-gp from human or mouse and did not inhibit the transport of cyclosporine using P-gp-transfected kidney cell lines. However these experiments were conducted in specific conditions based on gene manipulation. Therefore, the confirmation of P-gp function during epilepsy in the living organism without gene manipulation should be required to relief contradictions among those literatures.

In this study, we used kainic acid (KA) to prepare an experimental model of acute seizures in mice, and KA-induced seizures model has been established to cause specified and continuous limbic-system convulsion based on the degeneration of nerve cells around cerebral cortex or hippocampus. After intraloop administration, the brain CBZ and its active metabolite CBZ-epo concentrations in KA-treated mice were significantly retarded as compared to the control mice, and no significant decrease was found in plasma CBZ and CBZ-epo in the portal vein (Fig. 1). These observations indicate that KA-induced seizures decreases a transfer of CBZ or CBZ-epo from the blood circulation to the brain. In preliminary study using an ultracentrifugation method, we confirmed that KA did not have any influence on the protein binding of CBZ or CBZ-epo in 10% homogenate solution of rat brain. Therefore, the possibility that the KA treatment in-
hibits the bindings of CBZ and CBZ-epo to the brain tissue is denied. From the evidences that the hepatic extraction ratio of CBZ is reported to be 0.04\(^{13}\) and there were no changes in the activity of hepatic CYP3A (Fig. 5), it was considered that the portal CBZ reflects relative levels of peripheral CBZ concentration. In addition, Langer et al. investigated the pharmacoresistance in patients with epilepsy using a P-gp substrate (radio-labeled verapamil) and positron-emission tomography (PET).\(^{10}\) After administration of radio-labeled verapamil, they evaluated P-gp activity in epileptogenic and nonepileptogenic brain regions of patients by measuring an ipsilateral influx or efflux rate constants of verapamil. Even though they failed to detect statistically significant differences in the results, they reported that regionally enhanced P-gp activity in brain might contribute to drug resistance in some patients with temporal lobe epilepsy. That is, their report greatly supports our consideration that repeated events of seizures itself increase the drug resistance through the P-gp functions.

In the in-situ perfusion study, cumulative amounts of Rho123 and CBZ after intravenous administration in the KA-treated mice showed significant increases in the intestinal perfuse (Fig. 3). Since Rho123 is a substrate of P-gp, a significant increase in the intestinal exsorption of Rho123 indicates that the P-gp function in the gut cells was enhanced by seizures induced by KA, where KA has no direct effect on the gut P-gp function (Fig. 2). In addition, this in-situ study also indicates that CBZ is a substrate of P-gp. Hence, these observations suggest that seizures induced by KA enhances the P-gp function, namely, these P-gp substrates (Rho123 and CBZ) in the basal-membrane side were more actively transported to the apical-membrane side in the presence of seizures.

From these in-situ studies, it was speculated that P-gp function in the intestine and blood-brain-barrier is enhanced by KA-induced seizures. To further clarify the participation of enhanced P-gp function during seizures to the disposition of CBZ, we conducted western-blot analysis. The optical densities obtained from the blotting gel for the intestinal and cerebral P-gp were significantly increased in the KA-treated mice (Fig. 4). In addition, these in-situ studies also indicate that CBZ is a substrate of P-gp. Hence, these observations suggest that seizures induced by KA enhances the P-gp function, namely, these P-gp substrates (Rho123 and CBZ) in the basal-membrane side were more actively transported to the apical-membrane side in the presence of seizures.

In mice, CBZ is metabolized by CYP 3A11 or 3A13,\(^{21}\) therefore, it is considered that an alteration in the metabolism via CYP3A also important factor to change the disposition and efficacy of CBZ. As cerebral transfer of CBZ-epo (an active metabolite of CBZ) was also retarded in the presence of seizure in this study, there was a possibility that the activity of CYP3A also increased in KA-induced mice. However, no significant changes in the elimination phase of MDZ and its hydroxyl metabolites (MDZ-OH) were observed (Fig. 5), when both mice groups received intravenous MDZ, a specific substrate of CYP3A. In the KA-treated mice, therefore, the activity of CYP3A was considered to be unchanged. As another factor to alter the disposition of CBZ, it is considered that protein binding of CBZ is altered in the KA-treated mice. Contin et al. reported that CBZ and CBZ-epo have higher affinity for \(\alpha_1\)-acid glycoprotein because these are basic drugs.\(^{22}\) Therefore, there is a possibility that changes in the \(\alpha_1\)-acid glycoprotein levels in plasma lead to the alteration in tissue clearance of CBZ. In the protein binding study, we found that the percentage of CBZ protein binding in the KA-treated mice was significantly increased, whereas the Kp value was significantly decreased, but not in CBZ-epo (Table 1). These observations clearly demonstrate that the tissue transfer of CBZ was decreased in the KA-treated mice because of decreasing in the free fraction of CBZ in plasma. An increase in the plasma \(\alpha_1\)-acid glycoprotein levels was found in several inflammable disease such as burn, Crohn’s disease, myocardial infarction, neuralgia, infectious disease, rheumatism or gastric ulcer, however, it is unclear whether KA-induced seizure has an increasing effect on \(\alpha_1\)-acid glycoprotein levels in plasma at present.

In summary, it was found that KA-induced acute seizures provide the up-regulation of intestinal and cerebral P-gp in mice. An increasing in the percentage of protein binding of CBZ is another important factor in concerned with the CBZ transfer to the brain. These observations may provide a new insight into the altered clinical efficacy of CBZ in patients with invagination of epileptic fit.

REFERENCES

