Full Paper

Effects of First- and Second-Generation Histamine-H₁-Receptor Antagonists on the Pentobarbital-Induced Loss of the Righting Reflex in Streptozotocin-Induced Diabetic Mice

Junzo Kamei[1,*], Shoko Hirano[1], Shigeo Miyata[1], Akiyoshi Saitoh[1], and Kenji Onodera[2]

[1]*Department of Pathophysiology and Therapeutics, School of Pharmacy and Pharmaceutical Sciences, Hoshi University, Tokyo 142-8501, Japan*
[2]*Department of Dental Pharmacology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8525, Japan*

Abstract. The second-generation histamine-H₁-receptor antagonists, such as epinastine and cetirizine, are used as non-sedating antihistamines for treating allergic symptoms due to their poor ability to penetrate blood-brain barrier. Because it has been reported that the blood-brain barrier system is disturbed in diabetes, it is possible that second-generation histamine-H₁-receptor antagonists may easily penetrate the blood-brain barrier and cause potent sedation in diabetics. In the present study, we investigated the effects of first-generation (diphenhydramine) and second-generation (epinastine and cetirizine) histamine-H₁-receptor antagonists on the duration of pentobarbital-induced loss of the righting reflex (LORR) in non-diabetic and diabetic mice. Systemic treatment with diphenhydramine (3 – 30 mg/kg, s.c.), and intracerebroventricular treatment with epinastine (0.03 – 0.3 μg/mouse) and cetirizine (0.03 – 0.3 μg/mouse) dose-dependently and significantly increased the duration of pentobarbital-induced LORR in both non-diabetic and diabetic mice. Although systemic treatment with epinastine (3 – 30 mg/kg, s.c.) and cetirizine (3 – 30 mg/kg, s.c.) did not affect the duration of pentobarbital-induced LORR in non-diabetic mice, these treatments significantly prolonged it in diabetic mice. Our results suggest that the systemic administration of second-generation histamine-H₁-receptor antagonists may produce a central nervous system depressant effect in diabetes.

Keywords: loss of righting reflex, histamine-H₁-receptor antagonist, diabetes, pentobarbital, blood-brain barrier

Introduction

Histamine-H₁-receptor antagonists are the mainstays of treatment for several allergic disorders, particularly rhinitis, conjunctivitis, dermatitis, urticaria, and asthma (1). Two generations of histamine-H₁-receptor antagonists have been developed so far. First-generation histamine-H₁-receptor antagonists, such as diphenhydramine, chlorpheniramine, triprolidine, and hydroxyzine, readily penetrate the blood-brain barrier (BBB), and produce histamine blockade at histamine-H₁-receptors in the central nervous system (CNS). Therefore, CNS side effects, resulting mainly from the blockade of central histamine-H₁-receptors, are a major drawback of the classical antihistamine-based therapy (2 – 4). In contrast, second-generation histamine-H₁-receptor antagonists, such as epinastine, cetirizine, and fexofenadine, are associated with a reduced incidence of sedation due to their poor ability to penetrate the BBB (5).

The low CNS-penetration of second-generation antihistamines has previously been attributed to the physicochemical properties of the compounds (6) and to serum protein binding (7). However, diabetes alters the cerebral microvascular structure in various ways (8). Most of the changes are the result of cerebrovascular accidents (9). Other less dramatic changes, such as reduced density of cortical capillaries and increased capillary

*Corresponding author. FAX: +81-3-5498-5029
E-mail: kamei@hoshi.ac.jp

basement membrane thickening, can also occur in experimental models of diabetes (10 – 13). These pathological changes in cerebral microvessels in diabetes may contribute to morphological changes in the BBB and may impair the delivery of essential nutrients to neuronal tissue. Using immunohistochemical techniques, Stauber et al. (14) found that albumin could easily enter the cerebral cortex in experimental models of diabetes. Therefore, it is possible that second-generation histamine-H_1-receptor antagonists may easily penetrate the BBB and cause potent sedation in diabetics. In addition, it has been suggested that a P-glycoprotein (P-gp) drug efflux pump that is expressed on the luminal surface of cerebral endothelial cells plays some role in the penetration of histamine-H_1-receptor antagonist into the CNS (5, 15 – 17). Recently, van Waarde et al. (18) reported that hepatic gene and protein expression of multidrug-resistant P-gp type 2 were modulated by diabetes. Thus, it is possible that the function of P-gp at the BBB may be modulated by diabetes, and this modulation may contribute to altering the penetration of the second-generation antihistamines into the CNS.

To test these possibilities, we investigated the effects of diphenhydramine (a first-generation antihistamine) and epinastine and cetirizine (second-generation antihistamines) on pentobarbital-induced loss of righting reflex (LORR), as an index of the CNS-depressant effect, in streptozotocin-induced diabetic mice. In addition, we examined the effect of diabetes on the P-gp expression level in the mouse whole brain by Western blot analysis. Furthermore, the effect of diabetes on BBB permeability was also examined using the Evans blue extravasation test, since Evans blue does not easily pass across the intact BBB (19 – 21).

Materials and Methods

Animals

Male ICR mice (Tokyo Animal Laboratory Inc., Tokyo), aged 4 weeks and weighing about 20 g at the beginning of the experiments, were used. They had free access to food and water in an animal room that was maintained at $24 \pm 1°C$ and $55 \pm 5\%$ humidity with a 12 h light-dark cycle (light phase 8:00 – 20:00). The streptozotocin-induced hyperglycemic state has been used as an animal model of type 1 diabetes mellitus (22) because streptozotocin selectively destroys pancreatic islet beta-cells and causes hypoinsulinemia (23). We used mice rendered hyperglycemic by an injection of streptozotocin (200 mg/kg, i.v.) prepared in citrate buffer at pH 4.5 as the animal model of type 1 diabetes. Age-matched control mice were injected i.v. with the vehicle. The experiments were conducted 2 weeks after

the injection of streptozotocin or vehicle. Mice with blood glucose levels above 400 mg/dl were considered diabetic. Blood glucose levels were determined using a glucose analyzer (ANTSENSE II; Sankyo Co., Ltd., Tokyo). All experiments were performed between 11:00 and 17:00 each day. The animals were used only once. This study was carried out in accordance with the guide for the care and use of laboratory animals as adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology.

Measurement of the duration of pentobarbital-induced LORR

The duration of the loss of the righting reflex was measured according to the procedures described by Marley et al. (24). Mice were given an i.v. injection of pentobarbital (50 mg/kg). When the mice become ataxic, they were placed in the supine position in a V-shaped plastic trough and the time was recorded. Mice were judged to have regained the righting response when they could right themselves three times within 30 s.

Examination of BBB permeability

BBB permeability was assessed by measuring Evans blue extravasation according to the procedures described by Hendryk et al. (25) with a minor modification. Evans blue solution (2 w/v%) was injected i.v. into the mouse tail vein. Thirty minutes later, mice were killed under deep ether anesthesia, and then the thorax was opened and a 23-gauge needle was inserted into the left heart ventricle. Mice were transcardially perfused with 1000 ml/kg of heparinized saline solution (10 IU/ml). After perfusion, the brain was removed, and the diencephalon and cerebellum were separated. These regions were weighed and put into 800 μl of formamide. Evans blue was extracted by heating the samples at 55°C in formamide overnight. The supernatant was assayed spectrophotometrically at 630 nm by comparison with readings obtained from standard solutions. Results were expressed as micrograms per mg tissue.

Western blot analysis

Mice were killed under ether anesthesia and the brain was quickly removed. Each whole brain was homogenized in ice-cold buffer A containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, protease inhibitor cocktail [2 mM 4-(2-aminoethyl)benzene-sulfonyl fluoride hydrochloride, 130 μM bestatin, 1 μM leupeptin, 0.3 μM aprotinin, 1 mM EDTA, and 14 μM E-64; Sigma Chemical Co., Ltd., St. Louis, MO, USA], 0.3% mercaptoethanol, 50 μg/ml phenylmethyl-sulfonyl fluoride (PMSF)/dimethylsulfoxide (DMSO),

and 0.25 M sucrose. The homogenate was centrifuged at $1,000 \times g$ for 10 min at 4°C and the supernatant was ultracentrifuged at $100,000 \times g$ for 30 min at 4°C. The pellets were washed with buffer B (buffer A without sucrose) and homogenized in buffer B with 1% Triton X-100. After being incubated for 45 min at room temperature, soluble fractions were obtained by ultracentrifugation at $100,000 \times g$ for 30 min at 4°C and then retained as membrane fractions for Western blotting. The protein concentration in the solution was measured by the Bio-Rad Protein Assay (Bio-Rad Labs., Richmond, CA, USA) using bovine serum albumin (BSA) as a standard.

An aliquot of tissue sample was diluted with an equal volume of $2 \times$ sodium dodecyl sulfate (SDS) sample buffer containing 0.5 M Tris-HCl (pH 6.8), 10% SDS, 12% mercaptoethanol, 20% glycerol, and 1% bromophenol blue. Proteins (80 μg/lane) were separated by electrophoresis on $4 – 9\%$ SDS-polyacrylamide gradient gel. After electrophoresis, the proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in phosphate-buffered saline containing 0.1% Tween-20 and 5% dried skim milk for 60 min at room temperature and washed three times for 15 min in phosphate-buffered saline containing 0.1% Tween-20. Then the membrane was incubated with the primary monoclonal antibody C219 (DAKO, Glostrup, Denmark), diluted 250-fold in phosphate-buffered saline containing 0.1% Tween-20 and 2% BSA, overnight at 4°C. After washing the membrane with phosphate-buffered saline containing 0.1% Tween-20, it was incubated with the secondary polyclonal antibody, mouse anti-immunoglobulin G (IgG) peroxidase conjugate (Amersham Biosciences, Tokyo), was diluted 5,000-fold in phosphate-buffered saline containing 0.1% Tween-20, at room temperature for 45 min. P-gp protein in crude membranes was detected by enhanced chemiluminescence according to the manufacturer's instructions and visualized by exposure to Amersham Hyperfilm (Amersham Biosciences). Film autoradiograms were analyzed and quantified by computer-assisted densitometry using the NIH imaging system.

Drug treatment

The drugs used in this study were streptozotocin (Sigma Chemical Co.), sodium pentobarbital (Nembutal®; Dainippon Pharmaceutical Co., Osaka), diphenhydramine hydrochloride (Sigma), epinastine hydrochloride (Boehringer Ingelheim KG, Ingelheim/Rhein, Germany), and cetirizine dihydrochloride (UCB Japan, Tokyo). Diphenhydramine, epinastine, and cetirizine were dissolved in saline. We conducted preliminary experiments and determined the doses of antihistamines

that were sufficient to prolong the duration of LORR in non-diabetic and diabetic mice. The drug doses used in the present study referred to the respective salt. Subcutaneous (s.c.) injections of saline or drug solutions were given in a volume of 10 ml/kg body weight. One day before beginning the intracerebroventricular (i.c.v.) injections of drugs or saline, mice were anesthetized with ether and a 3-mm double-needle (tip: 27 gauge × 3 mm and base: 22 gauge × 5 mm; Natsume Seisakusho Co., Ltd., Tokyo) attached to a 25-μl Hamilton microsyringe was used at a unilateral injection site to make a hole, according to the method described by Aoki et al. (26). The unilateral injection site was 2 mm from either side of the midline between the anterior roots of the ears. On the day of the intracerebroventricular injection, the head of the mouse was held against a V-shaped holder without any anesthetics, and the drugs were injected into the hole. The intracerebroventricular injection volume was 5 μl for each mouse. The placement for the injection was confirmed by the injection of dye solution after all experiments. The subcutaneous injections of drugs were given 60 min before the injection of pentobarbital. The intracerebroventricular injections of drugs were given 30 min before the injection of pentobarbital.

Statistical analyses

Data are expressed as means with S.E.M. The statistical significance of differences between groups was assessed with one-way and two-way analysis of variance (ANOVA) for factorial comparisons and by the Bonferroni test for multiple comparisons. Student's *t*-test was used to evaluate differences between two groups. Significance was accepted at $P<0.05$.

Results

Effect of diphenhydramine on the duration of pentobarbital-induced LORR in non-diabetic and diabetic mice

As shown in Fig. 1, intravenous injection of pentobarbital (50 mg/kg) produced a LORR in both non-diabetic and diabetic mice. Diabetic mice showed a shorter duration of LORR compared to non-diabetic mice ($P<0.001$). Systemic treatment with diphenhydramine (3 – 30 mg/kg, s.c.) produced dose-dependent and significant increases in the duration of LORR in both non-diabetic and diabetic mice (Fig. 1).

Effects of i.c.v. treatment with epinastine and cetirizine on the duration of pentobarbital-induced LORR in non-diabetic and diabetic mice

I.c.v. treatment with epinastine (0.03 – 0.3 μg/mouse) and cetirizine (0.03 – 0.3 μg/mouse) dose-dependently
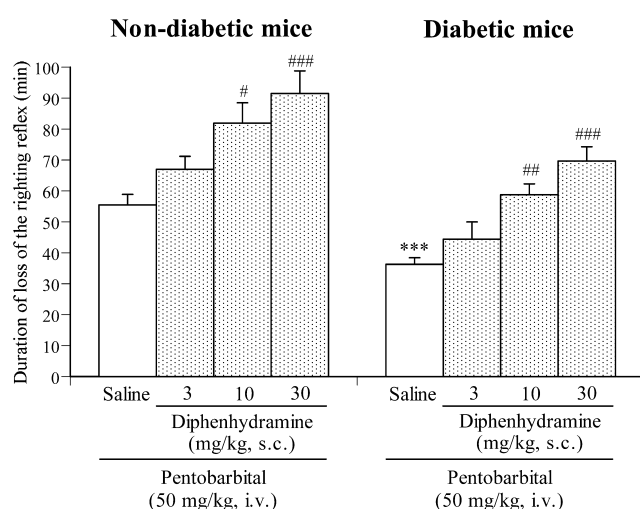
Fig. 1. Effect of diphenhydramine on the duration of the pentobarbital-induced loss of the righting reflex in non-diabetic and diabetic mice. Diphenhydramine was injected subcutaneously 60 min before the test. Each column represents the mean ± S.E.M. of 9 – 10 mice. ***$P<0.001$ vs saline-treated non-diabetic mice (Student's t-test). #$P<0.05$, ##$P<0.01$, ###$P<0.001$ vs respective saline-treated mice (Bonferroni test).

and significantly increased the duration of pentobarbital-induced LORR in both non-diabetic and diabetic mice (Fig. 2: A and B).

Effects of s.c. treatment with epinastine and cetirizine on the duration of pentobarbital-induced LORR in non-diabetic and diabetic mice

Systemic treatment with epinastine (3 – 30 mg/kg, s.c.) and cetirizine (3 – 30 mg/kg, s.c.) had no significant effect on the duration of pentobarbital-induced LORR in non-diabetic mice. However, epinastine (3 – 30 mg/kg, s.c.) and cetirizine (3 – 30 mg/kg, s.c.) dose-dependently and significantly increased the duration of pentobarbital-induced LORR in diabetic mice (Fig. 3: A and B). Two-way ANOVA revealed that the duration of LORR was significantly affected by the interaction of diabetes × epinastine ($F_{(3, 68)} = 3.941$, $P<0.05$). Furthermore, the interaction of diabetes × cetirizine significantly affected the duration of LORR ($F_{(3, 72)} = 4.208$, $P<0.01$).

Effect of diabetes on Evans blue extravasation in mouse diencephalon and cerebellum

As shown in Table 1, Evans blue content in the diencephalon and cerebellum was slightly but not significantly higher in diabetic mice than in non-diabetic mice ($P = 0.206$ and $P = 0.131$, respectively).
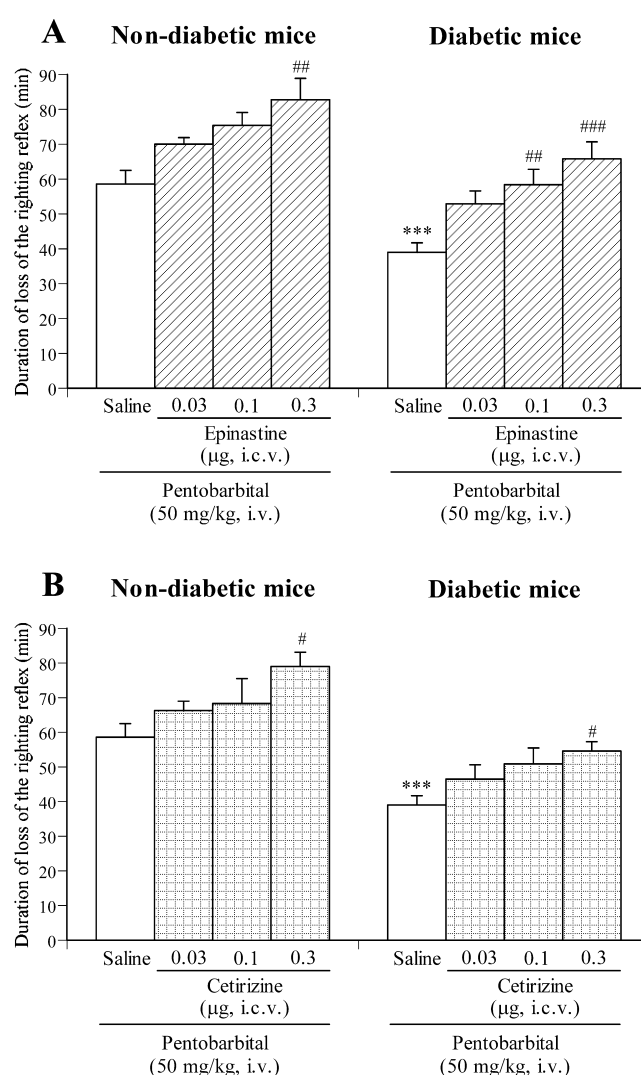

Fig. 2. Effects of i.c.v treatment with epinastine (A) and cetirizine (B) on the duration of the pentobarbital-induced loss of the righting reflex in non-diabetic and diabetic mice. Epinastine and cetirizine were injected intracerebroventricularly 30 min before the test. Each column represents the mean ± S.E.M. of 8 – 10 mice. ***$P<0.001$ vs saline-treated non-diabetic mice (Student's t-test). #$P<0.05$, ##$P<0.01$, ###$P<0.001$ vs respective saline-treated mice (Bonferroni test).

Effect of diabetes on P-gp expression levels in the mouse whole brain

P-gp expression levels in the mouse whole brain were significantly decreased by diabetes, as compared to those found in non-diabetic mice ($62.2 ± 9.1\%$, $P<0.05$; Fig. 4: A and B).

Discussion

In the present study, diabetic mice showed a shorter duration of pentobarbital-induced LORR compared to non-diabetic mice. Although certain mechanisms
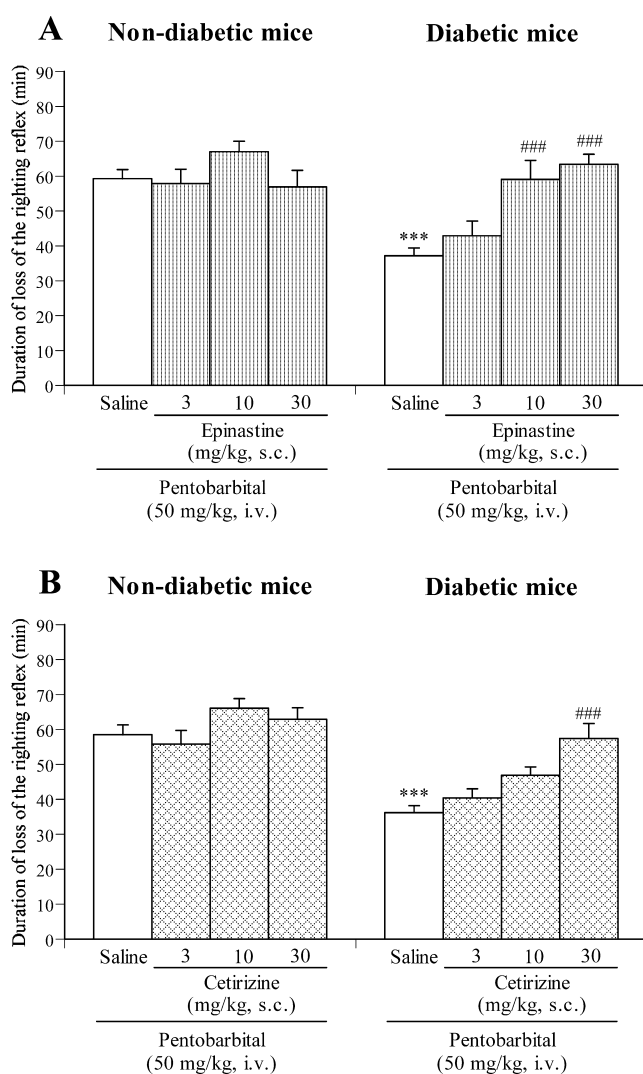
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Fig. 3. Effects of s.c. treatment with epinastine (A) and cetirizine (B) on the duration of the pentobarbital-induced loss of the righting reflex in non-diabetic and diabetic mice. Epinastine and cetirizine were injected subcutaneously 60 min before the test. Each column represents the mean ± S.E.M. of 8 – 10 mice. ***P<0.001 vs saline-treated non-diabetic mice (Student's t-test). ###P<0.001 vs respective saline-treated mice (Bonferroni test).

Table 1. Effects of diabetes on Evans blue extravasation in mouse diencephalon and cerebellum

	Evans blue extravasation (ng/mg tissue)	
	Diencephalon	Cerebellum
Non-diabetic mice	29.1 ± 4.3	31.3 ± 3.6
Diabetic mice	38.8 ± 6.0	47.0 ± 8.9

Data represent the mean Evans blue extravasation ± S.E.M. of 10 mice. Evans blue extravasation was slightly but not significantly increased in mouse diencephalon and cerebellum by diabetes (Student's t-test).
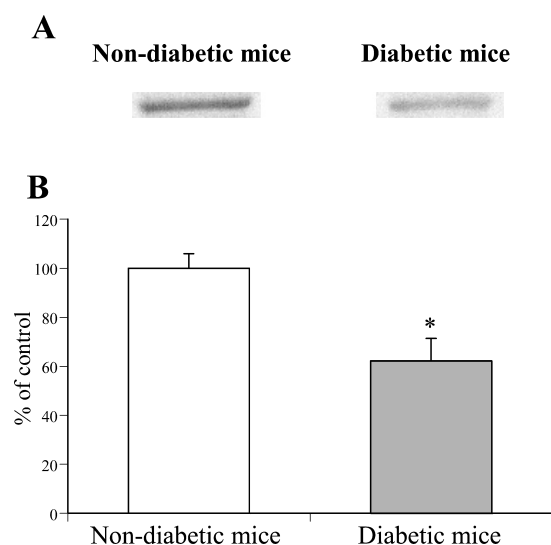

Fig. 4. Effect of diabetes on the protein levels of P-gp in mouse whole brain. Representative Western blot of P-gp protein (A) and ratio of relative staining intensity for P-gp (B) in non-diabetic and diabetic mice are described. Each band corresponding to 165 kDa was observed. Each column represents the mean ± S.E.M. of 4 samples. *P<0.05 vs non-diabetic mice (Student's t-test).

remain unclear, pentobarbital is thought to act mainly on central γ-aminobutyric acid (GABA)$_A$/benzodiazepine receptors. Guyot et al. (27) indicated that chronic hyperglycemia reduces extracellular GABA levels. Furthermore, we previously reported that the anxiolytic effect of diazepam in an unfamiliar environment was decreased by diabetes in mice (28). Therefore, it is possible that the decreased efficacy of pentobarbital in diabetic mice may be due, at least in part, to the changes in GABAergic systems.

In the present study, we observed that the duration of pentobarbital-induced LORR was prolonged by

systemic treatment with diphenhydramine and intra-cerebroventricular treatment with epinastine or cetirizine in both non-diabetic and diabetic mice. These findings indicate that central histamine-H$_1$-receptor blockade may cause a CNS-depressant effect in both non-diabetic and diabetic mice. A previous clinical report suggested that second-generation antihistamines produce little somnolence (29). In this regard, Yanai et al. (30) demonstrated using positron emission tomography that histamine-H$_1$-receptor occupancy in the human brain by oral treatment with epinastine was less than 20% of the total histamine-H$_1$-receptors. Thus, it is possible that systemic treatment with second-generation antihistamines, epinastine and cetirizine, weakly suppresses the activity of the CNS due to limited brain penetration. Indeed, in the present study, we demonstrated that systemic administration of epinastine and

cetirizine had no effect on the duration of pentobarbital-induced LORR in non-diabetic mice.

In contrast to non-diabetic mice, systemic administration of epinastine and cetirizine dose-dependently and significantly increased the duration of pentobarbital-induced LORR in diabetic mice. These results indicate that the CNS-depressant effects of second-generation antihistamines in diabetic mice are greater than those in non-diabetic mice even though pentobarbital has less of an effect in diabetic mice than in non-diabetic mice. It has been reported that BBB permeability is increased in diabetic patients (31). Furthermore, some of the common disease states associated with diabetes, such as transient cerebral ischemia (32) and hypertension (33) can disrupt the integrity of the BBB. This disruption allows molecules that are commonly confined to the blood to enter the brain parenchyma. Therefore, we speculated that the function of the BBB was altered by diabetes. Measurement of Evans blue extravasation is useful to assess the disturbance of BBB integrity because Evans blue strongly binds to the albumin fraction of proteins to give rise to a high-molecular complex, and it is difficult for this complex to penetrate the BBB. In this study, extravasation of Evans blue was slightly, but not significantly, increased in the diencephalon and cerebellum in diabetic mice. This observation is consistent with the previous reports (34). Based on these findings, it is possible that BBB permeability is either normal or tends to increase in the diabetic state.

It has been suggested that a P-gp drug efflux pump plays a role in the penetration of histamine-H_1-receptor antagonist into the CNS (5, 16). A previous report suggested that second-generation antihistamines such as terfenadine might interact with P-gp (35). On the other hand, Chishty et al. (5) reported that first-generation antihistamines such as diphenhydramine and mepyramine are not recognized by P-gp. Therefore, it has been suggested that non-sedating second-generation antihistamines might fail to penetrate the BBB in amounts sufficient to cause sedation due to active efflux mediated by the P-gp drug efflux pump. In the present study, we observed significant decreases in the P-gp protein expression levels in diabetic mice. Therefore, it is possible that the function of the P-gp efflux system may be impaired by diabetes, and this impairment of P-gp efflux function may be involved in the CNS-suppressive effects of the systemic administration of epinastine and cetirizine in diabetic mice.

In conclusion, our present results suggest that systemic treatment with second-generation antihistamines, as with first-generation antihistamines, might produce a CNS-depressant effect in diabetes.

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