Full Paper

Zonisamide Attenuates MPTP Neurotoxicity in Marmosets

Mohammed Emamussalehin Choudhury¹, Takashi Moritoyo¹, Hayato Yabe¹, Noriko Nishikawa¹, Masahiro Nagai¹, Madoka Kubo¹, Seiji Matsuda², and Masahiro Nomoto¹,*

¹Department of Therapeutic Medicine, ²Department of Anatomy and Embryology, Ehime University Graduate School of Medicine, Shitsukawa, Toon-Shi, Ehime 791-0295, Japan

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Abstract. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induces parkinsonism in humans and animals. The effects of zonisamide on dopamine neurons were studied in MPTP-treated common marmosets (Callithrix jacchus). Groups of animals (n = 3) were treated with MPTP (2.5 mg/kg, every 24 h × 3); MPTP plus zonisamide (40 mg/kg administered 1 h before each MPTP dose); MPTP plus selegiline (a known MAO-B inhibitor) (2 mg/kg administered 1 h before each MPTP dose); and saline controls. An immunohistochemical study of the substantia nigra was performed 14 days after MPTP treatment in each group. MPTP reduced the mean number of tyrosine hydroxylase (TH)-positive neurons to 10% of the normal control group and mean cell size was significantly (P < 0.001) reduced from 424 to 159 μm². In the group pre-treated with zonisamide, the mean number of TH-positive neurons was reduced to 26% of that in the normal control group and the mean neuron size was significantly (P < 0.05) increased from 159 to 273 μm² compared with the group treated with MPTP alone. Moreover, in the group pre-treated with selegiline, the mean number of TH-positive neurons was 47% of that in the normal control group and the mean neuron size was increased significantly (P < 0.01) from 159 to 319 μm² compared to the group treated with MPTP alone. This observation suggests that zonisamide reduces MPTP toxicity.

Keywords: MPTP, tyrosine hydroxylase (TH)-positive dopaminergic neuron, selegiline, zonisamide, marmoset

Introduction

Zonisamide (1,2-benzisoxazole-3-methanesulfonamide) was synthesized in Japan in 1972 and is widely used as an anticonvulsant drug throughout the world (1). It has a long elimination half-life (mean t½ = 63 h) and easily crosses the blood-brain barrier (2). Although the mechanism by which zonisamide acts against epilepsy is not fully understood, it is thought to involve antagonism of voltage-dependent sodium ions (3, 4) and T-type calcium ion channels (5). Furthermore, zonisamide has been shown to attenuate neonatal hypoxic-ischemic damage in experimental animals by a mechanism independent of its anticonvulsant properties (6). Zonisamide has also been used for the treatment of Parkinson’s disease (7, 8). The clinical efficacy of zonisamide in the management of Parkinson’s disease has been recently demonstrated in a randomized, double-blind trial (9). Furthermore, a recent experimental study suggested that zonisamide is effective in alleviating the neurotoxicity of dopamine quinones, which causes dopaminergic neuron-specific oxidative stress (10). Zonisamide attenuates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced toxicity in mice by inhibition of MAO-B (11) and also by increasing the amount of tyrosine hydroxylase (TH) in the striatum (12). Zonisamide also increases dopamine turnover (DOPAC+HVA/DA) (13). Dopamine turnover is the compensatory mechanism of MPTP-treated dopaminergic neurons (14, 15). In the present report, zonisamide was used to study the effects on dopaminergic neuronal degeneration induced by MPTP in common marmosets in comparison with the effect of selegiline, an MAO-B inhibitor.
Materials and Methods

Animals and administration

Twelve acclimatized 2-year-old common marmosets of either sex weighing 340 – 440 g were selected. Two animals per cage were housed under controlled temperature (28 ± 1°C) and humidity (50 ± 5%) with a 12-h light/dark cycle. Before starting drug treatment, the animals were maintained with free access to food and water. After drug treatment, each animal was fed with milk artificially twice daily when they were unable to voluntarily ingest sufficient food. This study was approved by the Committee of Animal Experimentation, Ehime University Graduate School of Medicine.

Drug treatment

Common marmosets were divided into four groups, each of which contained three animals. The animals were treated subcutaneously with MPTP at a dose of 2.5 mg/kg, 3 times at intervals of 24 h (MPTP group): the total dose of MPTP was 7.5 mg/kg. In the zonisamide pre-treatment group, animals were given zonisamide at 40 mg/kg, subcutaneously 1 h prior to each dose of MPTP: the total doses of zonisamide and MPTP were 120 and 7.5 mg/kg, respectively. In the selegiline pre-treatment group, animals were injected with selegiline at 2 mg/kg, subcutaneously 1 h prior to each dose of MPTP: the total doses of selegiline and MPTP were 6 and 7.5 mg/kg, respectively. The remaining animals were considered as the control group and treated with normal saline at the same times as the previous groups.

Immunohistochemistry

After high-dose pentobarbital sodium euthanasia, the brains were dissected out and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 days at 4°C and routinely embedded in paraffin for immunohistochemical study. Paraffin blocks were cut into 7-μm-thick sections by a microtome at the level of the substantia nigra. For morphological analysis, brain sections were analyzed at the central portion of the substantia nigra. This was selected at position A5 where the oculomotor nerve enters the brain (16). These sections were processed and stained immunohistochemically using the ABC method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) according to supplier’s recommendations. After deparaffinization by passing through a series dilution of xylene and ethanol solutions, the sections were washed with phosphate-buffered saline (PBS) and incubated with PBS containing 10% methanol and 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. The sections were then incubated overnight at 4°C with primary antibody, rabbit anti-tyrosine hydroxylase polyclonal antibody (Chemicon International, Temecula, CA, USA; 1:250) in a blocking solution containing 5% bovine serum albumin, 1% normal goat serum, 0.1% Triton X-100, and 0.1% sodium azide. After washing with PBS, these sections were incubated with biotinylated secondary antibody, polyclonal swine anti-rabbit immunoglobulin (DakoCytomation, Glostrup, Denmark; 1:500) for 3 h at 4°C and followed by avidin–biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories) for 30 min at 32°C. Finally, the sections were treated with a DAB substrate kit for peroxidase (Vector Laboratories) for color development at 32°C and were counter-stained with hematoxylin.

The numbers of TH-positive neurons in substantia nigra were counted manually under blind conditions, that is, with the observer being unaware of group allocation of the sections, and the cell size of those neurons having nuclei was measured using the NIH Image program (US National Institute of Health, http://rsb.info.nih.gov/nih-image/). Morphological characteristics were evaluated according to the shape of neuronal cell bodies, dendrites, and axons, as described in Fig. 1.

Fig. 1. Guide for the evaluation of the morphology of TH-positive neurons. The presence of dendrites and axons with TH-positive neurons in the substantia nigra were evaluated as: ++++, well-preserved; ++, moderately preserved; +, slightly preserved; and −, absent.
Statistical analyses

Data were analyzed statistically by analysis of variance (ANOVA) with the post hoc Tukey multiple comparison test where P values <0.05 were accepted as statistically significant.

Results

Immunohistochemistry

Cell numbers: In the normal control group, the mean number of TH-positive neurons in the substantia nigra was 238.33 ± 25.86 (mean ± S.E.M., n = 3) per section. With MPTP treatment, the mean number of TH-positive dopaminergic neurons in the substantia nigra was reduced to 23.33 ± 2.03 per section, which is 10% that of the normal control group (Figs. 2A, 3B). Pre-treatment with zonisamide attenuated this dopaminergic neuronal loss, and the mean number of TH-positive neurons was 60.66 ± 1.76 per section, which is 26% that of the normal control group (Figs. 2A, 3C). Selegiline pre-treatment also attenuated the TH-positive neuronal loss and the mean number of TH-positive neurons was 112.33 ± 17.7 per section, which is 47% that of the normal control group (Figs. 2A, 3D).

Cell size: In the normal control group, almost all TH-positive dopaminergic neurons were healthy, having prominent dendrites (Fig. 4A). By contrast, almost all TH-positive dopaminergic neurons were shrunken, and

Fig. 2. Histopathological features of TH-positive neurons in the substantia nigra. The effects of zonisamide and selegiline on the number (A) and area (B) of TH-positive neurons. Average area of 10 neurons were measured by the NIH image technique and expressed as μm². The levels of significance were analyzed by ANOVA, with the post hoc Tukey test, P < 0.05.

Fig. 3. Images of TH-positive neurons with DAB immunostaining in the substantia nigra counterstained with hematoxylin: normal controls (A), MPTP-treated (B), pre-treated with zonisamide prior to MPTP (C), and pre-treated with selegiline prior to MPTP (D). Scale bar, 400 μm.
dendrites and axons had disappeared in the group that was treated with MPTP alone (Fig. 4B). MPTP induced a significant ($P < 0.001$ vs. control) reduction in the size of the remaining TH-positive neurons from 424 to 159 $\mu m^2$ (Fig. 2B). In the group treated with MPTP after zonisamide pre-treatment, approximately half of the TH-positive dopaminergic neurons were shrunken, but healthy neurons were also observed (Fig. 4C). Zonisamide significantly ($P < 0.05$ vs. MPTP group) increased cell size from 159 to 273 $\mu m^2$ (Fig. 2B). In the group treated with MPTP after selegiline pre-treatment, approximately one-third of the existing dopaminergic neurons were shrunken (Fig. 4D). Selegiline also significantly ($P < 0.01$ vs. MPTP group) increased the size of TH-positive neurons from 159 to 319 $\mu m^2$ (Fig. 2B).

**Discussion**

The MPTP-treated marmosets showed a pronounced reduction of the number of dopaminergic neurons in the substantia nigra after 14 days of MPTP treatment. The MPTP neurotoxicity observed in this study was in good agreement with previous studies (17, 18). The acute effects of MPTP end within 2 weeks following administration of MPTP (13, 14), which set the time of taking samples from MPTP-treated marmosets for immunohistochemical study. Zonisamide was administered subcutaneously at a dose of 40 mg/kg in our study. With this dose, the blood zonisamide concentration was 21.3 ± 1.2 $\mu g/ml$ (mean ± S.D., $n = 5$) at 1 h after administration in common marmosets (13). This is well correlated with both the recommended blood concentration of zonisamide (10 – 30 mg/ml) in patients with epilepsy (19) and to a previous report showing the maximum plasma concentration of zonisamide in rhesus monkeys (20).The major result of the present study was to demonstrate the effects of zonisamide on MPTP-induced reduction of TH-positive cells in the substantia nigra (Fig. 2A). The average number of TH-positive neurons in the substantia nigra in the normal control group was about 238 per
slide, which is in agreement with a previous report (21), while MPTP treatment dramatically reduced the number of TH-positive neurons in the substantia nigra to 10% of that in the normal control group (Fig. 2A). MPTP also significantly reduced cell size and the presence of dendrites and axons (Figs. 2B, 4D), which is also in agreement with a previous study (22). In our previous study, we treated the animals with the same doses of MPTP and the dopamine contents decreased to 0.18 μg/mg, which is 1.1% that of the normal controls, and all of the MPTP-treated animals showed complete akinesia (13). Pretreatment with selegiline attenuated the reduction in the number of TH-positive neurons induced by MPTP toxicity (Fig. 2A). Selegiline also significantly (P < 0.01) increased cell area and the presence of dendrites and axons compared to the MPTP group (Figs. 2B, 4D). Moreover, pre-treatment with zonisamide also inhibited the reduction in number of TH-positive neurons in the substantia nigra induced by MPTP toxicity. Therefore, zonisamide rescued dopaminergic neurons from the neurotoxic effect of MPTP with respect to cell size and the presence of dendrites and axons in a similar manner to selegiline (Figs. 2B, 4C). The irreversible and marked inhibitory effects of selegiline on MAO-B activity is well documented in previous studies (23, 24), whereas zonisamide showed reversible, marginal inhibitory effects on MAO-B activity (9, 11) and it increased the amount of TH in the striatum of MPTP-treated mice (12). Furthermore, in a previous biochemical study we demonstrated that zonisamide increased dopamine turnover in striatum treated with MPTP and also reduced akinesia score of MPTP-treated marmosets (13).

In conclusion, we suggest that zonisamide might produce a neuroprotective effect via MAO-B inhibition as well as a neurotrophic effect by increasing TH activity.

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