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Effect of zonisamide co-administration with levodopa on global gene expression in the striata of rats with Parkinson's disease

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ABSTRACT

The anti-epileptic drug zonisamide is reported to exert beneficial effects in patients with Parkinson's disease. To elucidate the pathophysiological mechanisms underlying the anti-parkinsonism effects of zonisamide, we examined the effect of zonisamide co-administered with levodopa in the striata of rats with 6-hydoroxydopamine hemiparkinsonism by using a DNA microarray for genome-wide gene expression profiling. We found that the expression of some genes related to metabolism and nervous system development and function were upregulated by zonisamide; expression of these genes was downregulated by levodopa. Furthermore, many genes related to the immune system and inflammation were downregulated by zonisamide, and their expression was upregulated by levodopa. These results indicate that zonisamide has a protective effect when co-administered with levodopa.

1. Introduction

Parkinson's disease (PD) is a chronic progressive neurological disorder with increasing incidence in the elderly population. Currently, the standard of care for PD patients is levodopa (L-DOPA), which only provides symptomatic relief early in the course of treatment, and whose long-term use is limited by side effects. The development of drugs that can overcome these shortcomings is required for the efficacious treatment of PD patients.

The anti-epileptic drug zonisamide (ZNS) has been reported to improve motor functions in patients [1]; its clinical efficacy in the treatment of PD symptoms is supported by the results of a randomized double-blind study [2]. However, the actual pathophysiological mechanism underlying the anti-parkinsonism effects of ZNS remains uncertain. The present study aimed to uncover the effect of ZNS co-administered with L-DOPA on gene expression in the striata of rats with 6-hydoroxydopamine (6-OHDA)-induced hemiparkinsonism using a DNA microarray for genome-wide gene expression profiling.

2. Materials and methods

2.1. Materials

L-DOPA and benserazide hydrochloride were purchased from Roche, Japan. 6-OHDA hydrobromide were purchased from Sigma, USA, and methamphetamine (METH) and ZNS were obtained from Dainippon-Sumitomo Pharmaceutical, Japan. All other chemicals used were analytical grade.

2.2. Animals

Male Wistar rats were provided by Kyudo Inc. Ltd. and were kept at a controlled ambient temperature of 23 ± 1 °C and 50% ± 10% relative humidity. The Committee for Ethics in Animal Experiments, Faculty of Medicine, University of Miyazaki, Japan, reviewed and approved the experimental design. The rats were housed under a 12-h light/dark cycle with free access to food and water.

2.3. 6-OHDA lesion on medial forebrain bundle

Rats were anesthetized with pentobarbital 240 mg/kg, i.p. and unilateral lesions of the left medial forebrain bundle were made via injection of 8 mg 6-OHDA hydrobromide in 4 ml of sterile saline containing 0.01% ascorbic acid. Motor disturbance was assessed by counting the number of full rotations per min in a cylindrical container of φ30 cm diameter at 10-min intervals for the first 60 min after METH (3 mg/kg, i.p.) administration [3]. Behavioral

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screening was carried out after 2–3 weeks of recovery and animals that performed at least 7 turns/min during METH challenge were certified as hemiparkinsonism rats and included in the study. Seven days after METH challenge, hemiparkinsonism rats were treated with saline, L-DOPA (100 mg/kg, i.p.), or L-DOPA (100 mg/kg, i.p.) and ZNS (50 mg/kg, i.p.) once per day for 7 days. L-DOPA was injected 30 min after benserazide hydrochloride (a peripheral decarboxylase inhibitor). ZNS was injected 30 min after L-DOPA injection. The four experimental groups were classified as follows: the non-lesioned side of saline-treated 6-OHDA-injected rats (NL), the lesioned side of saline-treated 6-OHDA-injected rats (SL), the lesioned side of L-DOPA-treated 6-OHDA-injected rats (DL), and the lesioned side of L-DOPA and ZNS-treated 6-OHDA-injected rats (ZDL). Three animals were assigned to each group.

2.4. RNA preparation

Euthanasia by cervical dislocation was approved by the Committee for Ethics in Animal Experiments at the University of Miyazaki. Rats were decapitated and striata were removed and rapidly frozen in liquid nitrogen. They were then homogenized with a conventional rotor–stator homogenizer. Total RNA was then extracted from tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA). RNA was treated with DNase 1 (Qiagen) and purified using the RNeasy Mini Kit (Qiagen). The purity and integrity of the isolated total RNA were analyzed using both ultraviolet spectrophotometry and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All RNA samples exhibited a 260/280 ratio between 2.0 and 2.2, and a 28S:18S ratio of >1.6; the RNA integrity number was >9.0.

2.5. Microarray experiment

The Agilent SurePrint G3 Rat GE 8 × 60 K G4853A microarray was used for global gene expression analysis. Target RNA labeling and hybridization were performed according to the protocol for one-color microarray-based gene expression analysis using the Low Input Quick Amp Labeling kit (Agilent Technologies). The labeled complementary RNA was purified, fragmented, and hybridized to microarrays in a rotating hybridization oven at 10 rpm for 17 h at 65 °C. After hybridization, microarrays were washed according to the manufacturer's instructions and scanned on an Agilent DNA Microarray using the Scan Control software (Agilent Technologies). The resulting images were processed, and raw data were collected using the Agilent Feature Extraction software 10.7 (Agilent Technologies). This software attaches a flag to each gene feature that identifies different signal quantification issues and can eventually be used to filter out mRNAs. There are three types of flag: detected, marginal, and not detected. Gene expression data were analyzed using GeneSpring GX 12 (Agilent Technologies). The signal intensity of each probe was normalized by a percentile shift, in which each value was divided by the 75th percentile of all values in its array. The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through the GEO Series accession number GSE39980 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39980).

To identify differentially regulated genes, probes were selected for statistical analysis if they had flags "detected" in at least all samples from one group. These data were compared using *t*-tests, with a significance level set at $p < 0.05$. The remaining probes were selected using the criterion of at least a 2-fold change.

2.6. Gene network and biofunctional analysis

Gene network analysis was performed as described previously using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Mountain View, CA) [4]. Briefly, lists of differentially expressed genes were exported into and analyzed with IPA. These genes were overlaid onto a global molecular network developed from information in the Ingenuity knowledge base (IKB), a repository of biological interactions and functional annotations, and assigned to biofunction. Networks of these differentially regulated genes were then algorithmically generated on the basis of their connectivity. The functional analysis identifies the biological functions that are most closely related to the data set.

3. Results

3.1. Analysis of the effect of 6-OHDA lesion on gene expression in the striatum

To identify the genes differentially expressed between the striata of the non-lesioned side and the lesioned side of saline-treated 6-OHDA injected animals, probes were selected for statistical analysis if they had flags "detected" in all samples of NL, SL, or both. A total of 20,366 out of 30,507 probes on the array were selected in this manner. These data were compared using *t*-tests, with a significance level set at $p < 0.05$.

The remaining probes were selected using the criterion of at least a 2-fold change; a total of 347 out of 20,217 probes on the array were therefore selected. These probes were subdivided into upregulated and downregulated probes on the lesioned side (322

Table 1
Number of Genes common between each comparison.

	NL vs SL up	NL vs SL down	SL vs DL up	SL vs DL down	DL vs ZDL up	DL vs ZDL down
NL vs SL up	200					
NL vs SL down	–	25				
SL vs DL up	0	4	267			
SL vs DL down	27	2	–	161		
DL vs ZDL up	1	2	1	28	81	
DL vs ZDL down	4	2	146	1	–	274

Regulations are described based on the latter status. For example, "NL vs SL up" means upregulated in SL compared to NL.

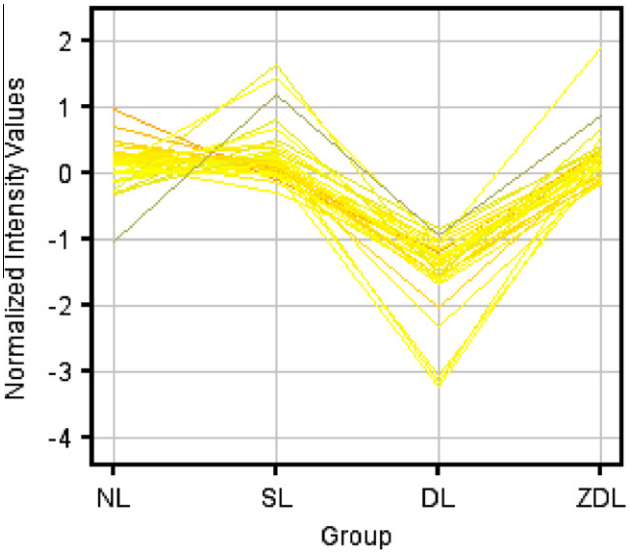

Fig. 1. The profile plot of normalized intensity values of D-down-Z-up genes. NL, non-lesioned side of saline-treated 6-OHDA-injected rats; SL, lesioned side of saline-treated 6-OHDA-injected rats; DL, lesioned side of L-DOPA-treated 6-OHDA-injected rats; ZDL, lesioned side of L-DOPA and ZNS-treated 6-OHDA-injected rats.

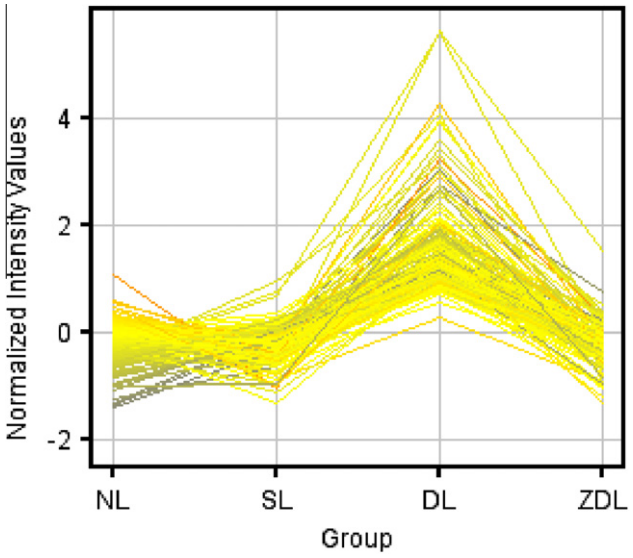

Fig. 2. Profile plot of normalized intensity values of D-up-Z-down genes.

up- and 25 down-regulated). These probes were analyzed using IPA.

Each probe was mapped to a gene in the IKB and used for functional analysis and network generation. Each generated network was scored on the basis of the probability of its generation. After the removal of duplicated entities, 203 upregulated and 25 downregulated probes in SL were successfully mapped, corresponding to 200 upregulated and 25 downregulated genes in SL (Supplementary Tables S1 and S2). Upregulated genes included several PD-related genes such as *Adra2b*, *Rpl13* [5], *Rpl13a* [5], *Rpl17* [5], *Slc25a6* [5], *Th*, *Tpt1* [5], *Fst* [6,7], *Nmu* [6,7], and Tac3 [6,8], while downregulated genes also included *Tac1* [6,9,10], another PD-related gene.

Similarly, microarray data were analyzed to identify changes in gene expression in the 2 group comparisons: SL vs. DL (317 upregulated and 166 downregulated probes assigned to 267 upregulated and 161 downregulated genes in the DL) (Supplementary Tables S3 and S4); and DL vs. ZDL (84 upregulated and 317 downregulated probes assigned to 81 upregulated and 274 downregulated genes in the ZDL) (Supplementary Tables S5 and S6). Table 1 shows the number of common genes for each comparison.

3.2. Characterization of the effect of L-DOPA administration and co-administration of ZNS with L-DOPA on global gene expression on the lesioned side of the striatum

As described in the previous section, 267 genes were upregulated and 161 genes were downregulated in the DL compared to the SL. Of the 267 upregulated genes, 4 (*Chrna5*, *Hist2h4*, *Plagli1*, and *Trh*) were downregulated by dopamine (DA) denervation, whereas 27 of 161 downregulated genes were upregulated by DA denervation (Table 1).

Following co-administration of ZNS with L-DOPA, 81 genes were upregulated and 274 downregulated in the ZDL compared to the DL (Supplementary Tables S5 and S6). Of 81 upregulated genes, 28 (D-down-Z-up genes) were downregulated by L-DOPA administration (SL vs. DL comparison) (Supplementary Tables S7), whereas 146 (D-up-Z-down genes) of 274 downregulated genes were upregulated by L-DOPA administration (Supplementary Tables S8).

The profile plot of normalized intensity values of D-down-Z-up genes shows that most of these genes were upregulated or not affected by DA denervation (Fig. 1). The only exception was *Kcng1*, which was downregulated (fold change = 2) by dopamine denervation. D-down-Z-up genes include some genes related to nervous system development and function, such as *Bdnf* and *Gpr143*, and metabolism-related genes such as *Agmat*, *Akr1b7*, *Aldh1a7*, and *Dhdh*. Gene network analysis using IPA revealed that 18 out of 28 D-down-Z-up genes were involved in one gene network with a network score of 52, suggesting that these genes are functionally related or close to one another (Supplementary Fig. S1).

The profile plot of the normalized intensity values of D-up-Z-down genes indicates that most of these genes were not strongly

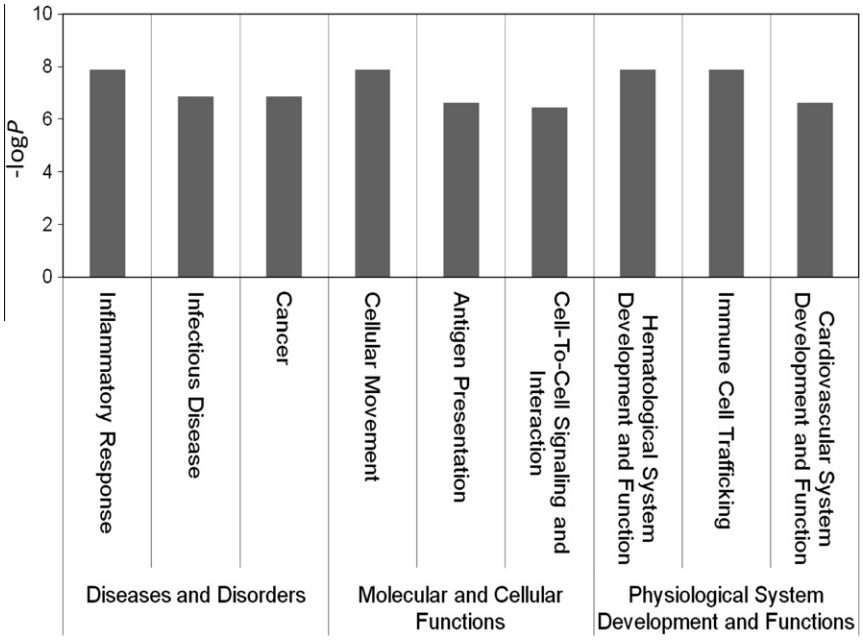

Fig. 3. Top 3 significant biofunctions from 3 major categories. The Functional Analysis identified the biological functions and/or diseases that were most significant to the data set. Right-tailed Fisher's exact test was used to calculate a *p*-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

affected by DA denervation compared to L-DOPA administration and ZNS co-administration (Fig. 2). The result of the functional analysis of D-up-Z-down genes using IPA is summarized in Fig. 3 (All lists are Supplementary tables S9-S11). The most significant molecular or cellular function was cellular movement, whose most significant function was monocyte cell movement, followed by the homing of mononuclear leukocytes. These results reveal that D-up-Z-down genes were comprised inflammation-related genes.

4. Discussion

In this study, we used 6-OHDA hemiparkinsonism rats to examine the effects of DA denervation and L-DOPA administration, and the effects of DA denervation and L-DOPA and ZNS co-administration on gene expression in the striatum using DNA microarray genome-wide gene expression profiling.

In the comparison between the DA-denervated side and the non-denervated side of the striata, we found significant upregulation of several PD-related genes including *Fst*, *Nmu*, and *Tac3*, and significant downregulation of a PD-related gene, *Tac1*. These results are consistent with previous reports of upregulation of *Fst* [6,7], *Nmu* [6,7] and *Tac3* [6,8], as well as downregulation of *Tac1* [6,9,10]. Cadet et al. have reported upregulation of *Nts* [6]. The results of our analysis also indicated upregulation of *Nts* with a fold change of 1.7 (data not shown), slightly shorter than our criterion (at least a 2-fold change).

In the comparison between L-DOPA administration and L-DOPA and ZNS co-administration to DA-denervated striata, we found that many genes were upregulated by co-administration of ZNS with L-DOPA, whiles their expression was downregulated by L-DOPA administration alone. Those genes include some related to nervous system development and function and metabolism-related genes. We also found from the same comparison that many genes that were downregulated by co-administration of ZNS with L-DOPA were upregulated by l-DOPA administration alone. Functional analysis revealed those genes to comprise inflammation-related genes.

It has been reported that chronic administration of L-DOPA to rats enhances hydroxyl radical production [3] and increases lipid peroxidation [11] in the DA-denervated striatum. Upregulation of genes related to the immune system and inflammation, downregulation of genes related to nervous system development and function, and metabolism by L-DOPA administration can be attributed to the neurotoxicity of L-DOPA, which is probably caused by hydroxyl radical production. Furthermore, the adverse effect of co-administered ZNS with L-DOPA on gene expressions can be attributed to the neuroprotective effect of ZNS, probably through its free radical scavenging effect [12–14].

In conclusion, our results show that compared to administration of L-DOPA alone, co-administration of ZNS with L-DOPA has adverse effects on the expression of many genes. This provides clues regarding the underling molecular mechanisms of the anti-PD effects of ZNS. More studies are needed in order to reveal all the molecular aspects of L-DOPA toxicity and the neuroprotective effect of ZNS.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.067.

References

[1] M. Murata, E. Horiuchi, I. Kanazawa, Zonisamide has beneficial effects on Parkinson's disease patients, Neurosci. Res. 41 (2001) 397–399.
[2] M. Murata, K. Hasegawa, I. Kanazawa, The Japan Zonisamide on PD Study Group, Zonisamide improves motor function in Parkinson disease: A randomized, double-blind study, Neurology 68 (2007) 45–50.
[3] Y. Ishida, H. Hashiguchi, K. Todaka, Y. Ishizuka, Y. Mitsuyama, Repeated administration of high dose levodopa enhances hydroxyl radical production in the rat striatum denervated with 6-hydroxydopamine, Neurosci. Lett. 290 (2000) 33–36.
[4] T. Kojima, Y. Ueda, N. Adati, A. Kitamoto, A. Sato, M.C. Huang, J. Noor, H. Sameshima, T. Ikenoue, Gene network analysis to determine the effects of antioxidant treatment in a rat model of neonatal hypoxic-ischemic encephalopathy, J. Mol. Neurosci. 42 (2010) 154–161.
[5] J.M. Kim, K.H. Lee, Y.J. Jeon, J.H. Oh, S.Y. Jeong, I.S. Song, J.M. Kim, D.S. Lee, N.S. Kim, Identification of genes related to parkinson's disease using expressed sequence tags, DNA Res. 13 (2007) 275–286.
[6] J.L. Cadet, C. Brannock, I.N. Krasnova, B. Ladenheim, M.T. McCoy, J. Chou, E. Lehrmann, W.H. Wood, K.G. Becker, Y. Wang, Methamphetamine-induced dopamine-independent alterations in striatal gene expression in the 6-hydroxydopamine hemiparkinsonian rats, PLoS ONE 5 (2010) e15643.
[7] B.H. Meurers, G. Dziewczapolski, T. Shi, A. Bittner, F. Kamme, C.W. Shults, Dopamine depletion induces distinct compensatory gene expression changes in darpp-32 signal transduction cascades of striatonigral and striatopallidal neurons, J. Neurosci. 29 (2009) 6828–6839.
[8] J.M. Burgunder, W.S. Young III, Distribution, projection and dopaminergic regulation of the neurokinin B mRNA-containing neurons of the rat caudate-putamen, Neurosci. 32 (1989) 323–335.
[9] C.R. Gerfen, J.F. McGinty, W.S. Young, Dopamine differentially regulates dynorphin, substance P, and enkephalin expression in striatal neurons: in situ hybridization histochemical analysis, J. Neurosci. 11 (1991) 1016–1031.
[10] C.R. Gerfen, T.M. Engber, L.C. Mahan, Z. Susel, T.N. Chase, D(1) and D(2) dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons, Science 250 (1990) 1429.
[11] N. Ogawa, M. Asanuma, Y. Kondo, Y. Kawada, M. Yamamoto, A. Mori, Differential effects of chronic L-DOPA treatment on lipid peroxidation in the mouse brain with or without pretreatment with 6-hydroxydopamine, Neurosci. Lett. 171 (1994) 55–58.
[12] A. Mori, Y. Noda, L. Packer, The anticonvulsant zonisamide scavenges free radicals, Epilepsy Res. 30 (1998) 153–158.
[13] Y. Noda, A. Mori, L. Packer, Zonisamide inhibits nitric oxide synthase activity induced by N-methyl-D-aspartate and buthionine sulfoximine in the rat hippocampus, Res. Commun. Mol. Pathol. Pharmacol. 105 (1999) 23–33.
[14] Y. Ueda, T. Doi, J. Tokumaru, A. Nakajima, K. Nagatomo, In vivo evaluation of the effect of zonisamide on the hippocampal redox state during kainic acid-induced seizure status in rats, Neurochem. Res. 30 (2005) 1117–1121.