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No Self-Injurious Behavior Was Found in HPRT-Deficient Mice Treated With 9-Ethyladenine

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EDAMURA K., AND H. SASAI. *No self-injurious behavior was found in HPRT-deficient mice treated with 9-ethyladenine.* PHARMACOL BIOCHEM BEHAV **61**(2) 175–179, 1998.—It has been reported that 9-ethyladenine (9-EA) is an efficient inhibitor of APRT (adenine phosphoribosyltransferase) and that its administration causes self-injurious behavior (Lesch-Nyhan Syndrome–like symptoms) in HPRT (hypoxanthine-guanine phosphoribosyltransferase)-deficient mice. In contrast, we found neither any self-injurious behavior (SIB), such as visible injury or hair loss, nor any apparent decrease in APRT activity in HPRT-defecient mice treated with 9-EA. We also found that 9-EA has little irreversible or competitive inhibitory effect on APRT in vitro, even at a concentration of 10^{-2} M. In light of the negative finding of SIB in APRT/HPRT double-deficient mice, it seems unlikely that SIB in HPRT-deficient mice is caused by lowered APRT activity. It is concluded that 9-EA is not a sufficient APRT inhibitor and cannot be used in experiments that mimic lowered APRT status in an animal model. © 1998 Elsevier Science Inc.

9-Ethyladenine APRT HPRT-deficient mouse Self-injurious behavior Lesch-Nyhan Syndrome

LESCH-NYHAN syndrome (12) is an X-linked neurodevelopmental disorder characterized by choreoathetosis, dystonia, self-injurious behavior, and hyperuricemia. It results from one defective gene that encodes the purine salvage enzyme HPRT. Over the past few years, a great deal of effort has been devoted to characterizing genetic mutations in patients with Lesch-Nyhan syndrome (16). With regard to a neurological deficit in Lesch-Nyhan syndrome, Lloyd and his coworkers reported reduced levels of dopamine in patients with Lesch-Nyhan syndrome (13). This finding was subsequently corroborated by the direct demonstration of reduced dopamine in a patients with Lesch-Nyhan syndrome through PET analysis of a compound binding to the dopamine transporter (4,17). Despite these efforts to understand the molecular and neurological basis of Lesch-Nyhan syndrome, current treatment remains largely unsatisfactory.

In recent years, several attempts have been made to generate animal models for Lesch-Nyhan syndrome as useful tools to investigate new methods of treatment, including gene therapy. As one of these attempts, HPRT-deficient mice were produced from HPRT-deficient mouse embryonic stem (ES) cells (6,10). However, they were essentially normal and displayed no obvious neurobehavioral abnormalities, in spite of a specific deficit in basal ganglia dopamine systems that emerges during the first 2 months of postnatal development (7). There have been some attempts to produce pharmacological models for Lesch-Nyhan syndrome based on treatment with drugs like 6-hydroxydopamine, caffeine and amphetamine (1,8,9,15). But these models have yielded little information on the relationship between HPRT deficiency and abnormalities in the dopamine systems in the brain. In 1993, Wu and Melton reported that the administration of 9-ethyladenine (9-EA), which was thought to be an APRT inhibitor, to HPRT-deficient mice induced persistent self-injurious behavior (SIB) (18). APRT, a purine salvage enzyme similar to HPRT, converts adenine to adenosine monophosphate. In their study, they stated that APRT was much more dominant than HPRT in the purine salvage system of mice compared with humans. So they interpreted their results as meaning that the partial inhibition of APRT activity in the brain of HPRT-

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TABLE 1
HPRT ACTIVITY IN MICE BRAIN CELL EXTRACTS

Strain	HPRT	HPRT Activity (nmol/min/mg Protein)
C57BL/6	+	9.5 ± 1.1
C57/3B1	−	0.01 ± 0.01*
Inactive protein		0.02 ± 0.02

HPRT activity in mice brain cell extracts of wild-type ($n = 3$) and HPRT-deficient ($n = 3$) male mice.
Heated E14.1 cell extracts are used as inactive protein.
*$p < 0.05$ compared with the wild-type group by Student's t-test.

deficient mice treated with 9-EA (80% of APRT activity relative to the saline-treated control group) induced SIB.

We are interested in an animal model for Lesch-Nyhan syndrome and have reexamined whether or not SIB occurs in 9-EA–treated HPRT-deficient mice. Contrary to our expectations, we observed no SIB in HPRT-deficient mice treated with 9-EA, and our treatment of the mice did not result in any apparent decrease in brain APRT activity. What is more, we found that 9-EA has little inhibitory effect on APRT in vitro .

RESULTS

We produced HPRT-deficient mice derived from a spontaneous HPRT-deficient ES cell, E14.1TG3B1 (5), which has a 203-bp insertion of a transposon sequence in exon 3 of the HPRT gene. This mutation caused the HPRT gene to cease functioning due to the frame shift, and the mice derived from E14.1TG3B1 carry a nonfunctional HPRT gene. Indeed, the incorporation of [^{14}C]-hypoxanthine into cell extracts of E14.1TG3B1 and the brain of HPRT-deficient mice was measured, and no HPRT activity in either material was found (Table 1). These mice have none of the spontaneous behavioral abnormalities that were previously reported. Next, we examined whether the administration of 9-EA to HPRT-deficient mice induced persistent SIB. Three 8-week-old wild-type male and HPRT-deficient male mice were placed in separate cages and injected three times per week for 5 weeks with a saline solution containing 2.5×10^{-6} (Experiment 1) or 1.25×10^{-5} (Experiment 2) mol of 9-EA. During this time, the frequency of stereotypic behavior was scored. Our definition of stereotypic behavior includes all grooming with fore or hind legs, nibbling, and biting. There was no significant difference in the frequency of stereotypic behavior between wild-type and HPRT-deficient mice injected with a saline solution contaning 2.5×10^{-6} mol of 9-EA (Table 2) . Furthermore, none of the animals injected with 9-EA developed tissue injury or hair loss within 120 days after the injections were started.

In Experiment 2, the frequency of stereotypic behavior could only be scored during the first 3 weeks after the injections were started, and there was no significant difference in the frequency of stereotypic behavior between wild-type and HPRT-deficient mice injected with a saline solution contaning 1.25×10^{-5} mol of 9-EA (Table 2) . After 4 weeks, we could not continue the experiment, because they became too sick to move around during recording sessions, and one mouse died, probably due to the chronic toxicity of 9-EA. Within 120 days after the injections were started, none of the animals injected with 1.25×10^{-5} mol of 9-EA developed tissue injury or hair loss, which is the same as the results obtained in Experiment 1. These results do not support Wu and Melton's data. So we examined whether 9-EA inhibited APRT activity in vitro and in vivo.

To determine if 9-EA has an irreversible effect on APRT activity in vitro, we preincubated E14.1 (11) cell extracts with 9-EA prior to the [^{14}C]-adenine incorporation assay. An inhibition of the incorporation of [^{14}C]-adenine was found after incubation with at least 1.5×10^{-1} M 9-EA for 1 h (Table 3). The concentration of 1.5×10^{-1} M is 500 times greater than the concentration of [^{14}C]-adenine, which is a substrate of APRT. Then we examined the competitive effect of 9-EA on APRT activity in vitro. There was no significant reduction in the incorporation of [^{14}C]-adenine into ES cell extracts compared with the control value, even in the presence of 1.5×10^{-1} M 9-EA (data not shown). These data indicate that 9-EA has little irreversible or competitive inhibitory effect on APRT.

We also examined the effect of 9-EA on APRT activity in mouse brain. Eight-week-old wild-type male mice were injected intraperitonealy with 9-EA on alternate days and sacrificed after the fourth injection. The mice received 2.5×10^{-6}, 1.25×10^{-5}, or 2.5×10^{-5} mol of 9-EA. However, four out of the five male mice that received 2.5×10^{-5} mol died after the first injection, and the remaining one died after the second in-

TABLE 2
COUNTS OF STEREOTYPIC BEHAVIOR AND SIB IN WILD-TYPE AND HPRT-DEFICEINT MICE AFTER INJECTION WITH 9-EA

Strain	HPRT	Treatment	Stereotypic Behavior/min	Incidence of Injury	Hair Loss
<Exp. 1>					
C57BL/6	+	saline	0.17 ± 0.09	0/3	0/3
C57BL/6	+	2.5×10^{-6} moles 9-EA	0.09 ± 0.08	0/3	0/3
C57/3B1	−	saline	0.1 ± 0.08	0/3	0/3
C57/3B1	−	2.5×10^{-6} moles 9-EA	0.12 ± 0.07	0/3	0/3
<Exp. 2>					
C57BL/6	+	saline	0.19 ± 0.01	0/3	0/3
C57BL/6	+	1.25×10^{-5} moles 9-EA	0.11 ± 0.01	0/3	0/3
C57/3B1	−	saline	0.07 ± 0.01	0/3	0/3
C57/3B1	−	1.25×10^{-5} moles 9/EA	0.18 ± 0.02	0/3	0/3

Counts of stereotypic behavior in wild-type ($n = 3$) and HPRT-deficient ($n = 3$) male mice after injection with 9-EA and saline.

TABLE 3

THE IRREVERSIBLE EFFECT OF 9-EA ON APRT ACTIVITY IN ES CELL EXTRACTS

9-EA	APRT Activity (nmol/min/mg Protein)	Control (nmol/min/mg Protein)
1.5×10^{-4} M	2.6 ± 0.2	2.7 ± 0.1
1.5×10^{-3} M	2.3 ± 0.1	2.7 ± 0.1
1.5×10^{-2} M	2.8 ± 0.1	3.0 ± 0.1
1.5×10^{-1} M	0.01 ± 0.01*	3.5 ± 0.1

The irreversible effect of 9-EA on APRT activity in E14.1 cell extracts ($n = 3$).

*$p < 0.05$ compared with E14.1 cell extracts not treated with 9-EA by Student's *t*-test.

jection, probably due to the cytotoxity of 9-EA. We measured the incorporation of [^{14}C]-adenine into cell extracts from the brain of HPRT-deficient mice injected with 2.5×10^{-6} or 1.25×10^{-5} mol of 9-EA. There was no significant reduction in the incorporation at either concentration compared with a control group treated with saline solution (Table 4). This indicates that 9-EA does not act as an APRT inhibitor at these concentrations in vivo.

DISCUSSION

In the present study, we could not reproduce Wu and Melton's finding that SIB was induced in HPRT-deficient mice treated with 9-EA. We only noticed that some mice treated with 9-EA were very sensitive to sound and the presence of humans, even though there was no decrease in APRT activity. Wu and Melton postulated that HPRT/APRT double deficiency in rodents could be a good model for Lesch-Nyhan syndrome, which is based on the hypothesis that APRT is more important than HPRT in rodents. Because APRT-deficient mice were not available at that time to investigate the role of APRT in SIB, they used purine analogues to inhibit APRT activity. They used the purine analogue 9-EA to try to inhibit APRT activity because they expected it to be less toxic from its chemical structure, but still act as a competitive inhibitor of the purine salvage pathway. In the present study, however, it was found that 9-EA had little inhibitory effect on APRT either in vitro or in vivo. In our experiments, the incorporation of [^{14}C]-adenine was found to be irreversibly inhibited after incubation in at least 1.5×10^{-1} M 9-EA for 1 h. This concentration is 500 times greater than that of [^{14}C]-adenine, which forms the substrate of APRT. So it is clear that such a concentration of 9-EA caused an apparent reduction in [^{14}C]-adenine incorporation. In addition, no competitive inhibitory effect on APRT was observed, even at this concentration. We believe that our direct evaluation of APRT activity in cell extracts is more straightforward than Wu and Melton's indirect APRT assay of adenine incorporation by cultured cells. Considering the minimum concentration (1.5×10^{-1} M) of 9-EA needed to inhibit APRT in vitro, one should give mice a dose of 2.5×10^{-4} mol, which is about 100 times greater than the one they reported and is far greater than the lethal dosage.

TABLE 4

APRT ACTIVITY IN MICE BRAIN CELL EXTRACTS AFTER TREATMENT OF MICE WITH 9-EA

Strain	Treatment	APRT Activity (nmol/min/mg Protein)
Experiment 1		
C57BL/6	saline	0.25 ± 0.05
C57BL/6	2.5×10^{-6} mol 9-EA	0.26 ± 0.04
Experiment 2		
C57BL/6	saline	0.12 ± 0.02
C57BL/6	1.25×10^{-5} mol 9-EA	0.11 ± 0.02

APRT activity in mice brain cell extracts after treatment of mice ($n = 5$) with 9-EA and saline.

To test the hypothesis that a decrease in the APRT activity of HPRT-deficient mice may induce persistent SIB, we created APRT +/− (heterozygote) mice from APRT-deficient ES cells, which carry a large chromosomal deletion around the APRT locus, and crossed them with HPRT-deficient mice. We observed no SIB for 260 days in HPRT−/APRT+/− (heterozygote) mice, which had approximately half the normal APRT activity (unpublished data). Engle generated APRT-knockout mice and found that these mice develop 2,8-dihydroxyadenine (DHA) kidney stones and renal failure, just as APRT-deficient humans do (2). They bred the nonfunctional APRT allele into an HPRT-deficient mouse background (3). HPRT/APRT double-deficient mice excreted adenine and DHA but did not exhibit any obvious neurobehavioral abnormalities. They concluded that HPRT/APRT deficiency has nothing to do with SIB and is not a good model for Lesch-Nyhan syndrome, as previously suggested. On the basis of our data and Engle's results, Wu and Melton's hypotheses that 9-EA is an APRT inhibitor and that lowered APRT activity causes persistent SIB in HPRT-deficient mice is unlikely.

The question of why SIB was found in 9-EA–treated HPRT-deficient mice in Wu and Melton's study still remains unanswered. With regard to this question, one possibility is that different results might come from mice with different genetic backgrounds. In fact, we used a hybrid strain, of mice that were a cross between C57BL/6 and 129/Ola, while they used mice with a 129/Sv background.

A reexamination of the effects of 9-EA on other strains of HPRT-deficient mice is needed to determine whether 9-EA really causes SIB or not. We still do not know the mechanism by which HPRT deficiency causes peculiar symptoms, such as athetose, self-injurious behavior, and mental retardation, nor do we know an effective treatment for its severe symptoms. Further efforts are needed to establish a more sophisticated model to elucidate the etiology of, and a therapy for, Lesch-Nyhan syndrome.

METHOD

Embryonic stem cell culture

E14.1, a wild-type ES cell line, and E14.1TG3B1, a spontaneous HPRT-deficient derivative of E14.1, were cultured in Dulbecco's Modified Eagle Medium with 15% (v/v) FCS, 0.1 mM β-mercaptoethanol and 1000 U/ml of leukemia inhibitory factor (LIF).

Production of Germline Chimeras and Homozygous HPRT-Deficient Mice

E14.1TG3B1 was expanded and injected into C57BL/6 blastocysts. The resulting male chimeric animals were mated to C57BL/6 females to test for germ line transmission of the HPRT mutation. Offspring derived from ES cells were identified by coat color, and germline transmission was identified by PCR gene typing. After two backcrosses with C57BL/6,

homozygous and hemizygous HPRT-deficient mice were created and the colony was maintained by breeding among heterozygous female and hemizygous male mice. Genetically, they are a hybrid between 129/Ola and C57BL/6.

Administration of 9-EA to Mice and Measurement of Stereotypic Behavior

C57BL/6 male mice and HPRT-deficient male mice (6–8 weeks old) were caged individually and maintained on a 12 L:12 D cycle.

9-EA (Sigma) (2.5×10^{-2} M) solution dissolved in sterile normal saline was stored at 4°C, and mice received 2.5×10^{-6} or 1.25×10^{-5} mol of 9-EA by intraperitoneal injection three times a week. To make video recordings, mice were transferred to a clear cage with bedding but no food and water for 10 min before treatment. Recording was initiated 10 min after the animals had been returned to the cage following injection, and the frequency of stereotypic behavior was determined over a 20-min period by scoring all grooming with fore or hind legs, nibbling, and biting. Recording was made on three mice in each treatment group three times a week for 5 weeks (a total of 15 times/mouse), and the stereotypic behavior index was calculated each day that 9-EA was administered.

Incorporation of Radiolabeled Purines into ES Cell Extracts

ES cells ($1–2 \times 10^7$) were grown to 80% confluency. The cells were trypsinized to release them from the plates, and the cell pellets were washed twice with PBS on ice. The cells were collected by centrifugation, and the final pellet was resuspended in 3 ml of 0.03 MTris-HCl, pH7.4, for the APRT and HPRT assays. The cells were homogenized in a Polytron (Kinematica), and the suspension was spun at 12000 rpm for 20 min at 4°C in a microfuge. The supernatant was put into a clean tube; this crude cell extract was used directly in assays. The protein concentration was determined by using the Bradfold assay (Pierce Coomassie protein assay). The APRT and HPRT assays were performed as described by Meuth et al. (14).

The Irreversible and Competitive Effect of 9-EA on [^{14}C]-Adenine Incorporation in ES Cell Extracts

For irreversible assays, the crude cell extract from E14.1 was used. 9-EA was added to a reaction mixture without [^{14}C]-adenine for the APRT assay and incubated for 1 h. Then [^{14}C]-adenine was added and incubation continued for 1 more hour. The labeled AMP was absorbed by DE81 filters, and the ^{14}C count was determined with a scintillation counter.

Incorporation of Radiolabeled Purines Into Mouse Brain Cell Extracts

Mouse brain cell extracts were obtained by sacrificing animals, removing the brain and homogenizing it, as described above. To determine the effect of 9-EA on APRT activity in mouse brain, 8-week-old wild-type (C57BL/6) male mice were used. Five wild-type male mice received 2.5×10^{-6} or 1.25×10^{-5} mol of the drug by intraperitoneal injection three times a week. Five wild-type male mice were injected in parallel with saline. After the fourth injection, brain cell extracts were prepared from each animal. The APRT assay was performed as described by Meuth et al.

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REFERENCES

1. Breese, G. R.; Criswell, H. E.; Duncan, G. E.; Mueller, R. A.: A dopamine deficiency model of Lesch-Nyhan disease—the neonatal-6-OHDA-lesioned rat. Brain Res. Bull. 25:477–484; 1990.
2. Engle, S. J.; Stockelman, M. G.; Chen, J.; Goivin, G.; Yum, M. N.; Davies, P. M.; Ying, M. Y.; Sahota, A.; Simmonds, H. A.; Stambrook, P. J.; Tischfield, J. A.: Adenine phosphoribosyltransferase-deficient mice develop 2,8-dihydroxyadenine nephrolithiasis. Proc. Natl. Acad. Sci. USA 93:5307–5312; 1996.
3. Engle, S. J.; Womer, D. E.; Davies, P. M.; Boivin, G.; Sahota, A.; Simmonds, H. A.; Stambrook, P. J.; Tischfield, J. A.: HPRT-APRT-deficient mice are not a model for Lesch-Nyhan syndrome. Hum. Mol. Genet. 5:1607–1610; 1996.
4. Ernst, M.; Zametkin, A. J.; Matochik, J. A.; Pascualvaca, D.; Jons, P. H.; Hardy, K.; Hankerson, J. G.; Doudet, D. J.; Cohen, R. M.: Presynaptic dopaminergic deficits in Lesch-Nyhan disease. N. Engl. J. Med. 334:1568–1572; 1996.
5. Hirohisa, T.; Catherina, E. M.; Laura, H. R.; Takayuki, Y.; Koji, E.; Nobuyo, M.; Oliver, S.; Aya, J. L.: Inactivation of the mouse HPRT locus by a 203-bp retroposon insertion and a 55-kb gene-targeted deletion: Establishment of new HPRT-deficient mouse embryonic stem cell lines. Genomics 42:413–421; 1997.
6. Hooper, M.; Hardy, K.; Handyside, A.; Hunter, S.; Monk, M.: HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 326:292–295; 1987.
7. Jinnah, H. A.; Wojcik, B. E.; Hunt, M.; Narang, N.; Lee, K. T.; Goldstein, M.; Wamsley, J. K.; Langlais, P. J.; Friedmann, T. J.: Dopamine deficiency in a genetic mouse model of Lesch-Nyhan disease. Neurol. Sci. 14:1164–1175; 1994.
8. Jinnah, H. A.; Gage, F. H.; Friedmann, T.: Animal models of Lesch-Nyhan syndrome. Brain Res. Bull. 25:467–475; 1990.
9. Jinnah, H. A.; Gage, F. H.; Friedmann, T.: Amphetamine-induced behavioral phenotype in hypoxanthine-guanine phosphoribosyltransferase-deficient mouse model of Lesch-Nyhan syndrome. Behav. Neurosci. 105:1004–1012; 1991.
10. Kuehn, M. R.; Bradley, A.; Robertson, E. J.; Evans, M. J.: A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. Nature 326:295–298; 1987.
11. Kühn, R.; Rajewsky, K.; Müller, W.: Generation and analysis of Interleukin-4 deficient mice. Science 254:707–710; 1991.
12. Lesch, M.; Nyhan, W. L.: A familial disorder of uric acid metabolism and central nervous system function. Am. J. Med. 36:561–570; 1964.
13. Lloyd, K. G.; Hornykiewicz, O.; Davidson, L.; Shannak, K.; Farley, I.; Goldstein, M.; Shibuya, M.; Kelly, W. N.; Fox, I. H.: Biochemical evidence of dysfunction of brain neurotransmitters in the Lesch-Nyhan syndrome. N. Engl. J. Med. 305:1106–1111; 1981.
14. Meuth, M.; Harwood, J.: Selection of cells defective in pyrimidine (TK-) and Purine (APRT- and HPRT-) salvage. In: Murray, E. J., ed. Methods in molecular biology: Gene transfer and expression protocols. Clifton, NJ: Humana Press Inc.; 1991: 257–267.
15. Mueller, K.; Saboda, S.; Palmour, R.; Nyhan, W. L.: Self-injurious behavior produced in rats by daily caffeine and continuous amphetamine. Pharmacol. Biochem. Behav. 17:613–617; 1982.
16. Sege-Peterson, K.; Nyhan, W. L.; Page, T.: Lesch-Nyhan disease and HPRT deficiency. In: Rosenberg, R. N.; Prusiner, S. B.; DiMauro, S.; Barchi, R. L.; Kunkel, L. M., eds. The molecular

and genetic basis of neurological disease. Boston: Butterworth-Heinemann; 1992: 241–260.
17. Wong, D. F.; Harris, J.; Yung, G.; Dannals, R. F.; Ravert, H. T.; Chen, C.; Yaster, M.; Neumeyer, J.; Carpenter, R.; Nyhan, W.; Wagner, H. N., Jr.; Kuhar, M. J.; Breese, G. R.: Dopamine transporters are markedly reduced in Lesch-Nyhan disease in vivo. Proc. Natl. Acad. Sci. USA 93:5539–5543; 1996.
18. Wu, C.-L.; Melton, D. W.: Production of a model for Lesch-Nyhan syndrome in hypoxanthine phosphoribosyltransferase-deficient mice. Nat. Genet. 3:235–240; 1993.