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The threshold of pentylenetetrazole-induced convulsive seizures, but not that of nonconvulsive seizures, is controlled by the nitric oxide levels in murine brains

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ABSTRACT

Alterations in the NO pathway play an important role in the development of convulsive seizures via the glutamatergic and GABAergic systems in acute pentylenetetrazole (PTZ) seizure animals. We previously reported that the background NO levels under physiological conditions negatively regulate convulsive seizures, while excess NO levels under pathologic conditions positively regulate PTZ-induced convulsive seizures. In this study, the NO content in various brain regions after a single dose injection of PTZ was quantitatively and directly measured using the *ex vivo* X-band electron paramagnetic resonance method with an NO-trapping agent. Experimental data demonstrated the effects of NO on the convulsive seizure threshold: a 1.5-fold increase in the NO level in all brain regions compared to that observed in the control state showed proconvulsive properties without any involvement with nonconvulsive seizures. The distribution of the background NO content in the normal animals was higher in the temporal region of the cerebral cortex, including the amygdala, than in the hippocampus, cerebellum and other regions of the cerebral cortex. However, the levels of NO after the occurrence of acute PTZ-induced convulsive seizures significantly increased by more than 50% in all brain regions, thus suggesting that the NO levels in all brain regions contribute to PTZ-induced convulsions as a seizure threshold. In a pharmacological study, the inhibitor of neuronal NO synthase and antagonists of ionotropic glutamate receptors prevented PTZ-induced convulsions and excessive NO generation. In addition, therapeutic drugs, such as valproate and ethosuximide used to treat generalized seizures not only inhibited the increase in NO generation induced by PTZ, but also prevented both convulsive and nonconvulsive seizures caused by PTZ. We herein provide novel insight into the involvement of NO in PTZ-seizure susceptibility at the whole-animal level.

Introduction

Approximately 50 million people suffer from epilepsy worldwide, and the disease is one of the most widespread neurological disorders (Chang and Lowenstein, 2003; World Health Organization, 2009). Epileptic seizures have been effectively prevented with antiepileptic drugs (AEDs) since phenobarbital was created a century ago (Shorvon, 2009). Pentylenetetrazole (PTZ) and maximal electroshock seizure tests are widely used as the gold standard to evaluate the effectiveness of AEDs. Although the anticonvulsive properties of most AEDs were discovered using gold standard tests with simple and easy procedures, the underlying mechanisms are only partially understood and thus remain elusive. In this study, we focused on the acute seizures induced by single doses of PTZ as a chemical convulsant. This animal model is well known for studying primary generalized epilepsy (Löscher et al., 1991); however, the pathophysiological mechanisms of PTZ remain unknown. Generally, PTZ-induced convulsive seizures are caused by the enhancement of glutamatergic neuronal activity by antagonism of γ-aminobutyric acid$_A$ receptors (GABA$_A$R) in the brain (Psarropoulou et al., 1994; Rocha et al.,

Abbreviations: 3Br7NI, 3-bromo-7-nitroindazole; AEDs, antiepileptic drugs; AMPA, DL-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, AMPA receptor; CGP39551, (E)-(±)-2-amino-4-methyl-5-phospho no-3-pentenoic acid ethyl ester; CBZ, carbamazepine; CP465022, 3-(2-Chlorophenyl)-2-[2-[6-[(diethylamino) methyl]-2-pyridinyl]ethenyl]-6-fluoro-4(3*H*)-quinazolinone hydrochloride; DMSO, dimethyl sulfoxide; DETC, *N*,*N*-diethyldithiocarbamate Na; EPR, electron paramagnetic resonance; ESM, ethosuximide; GABA$_A$R, gamma-aminobutyric acid $_A$ receptor; L-NNA, Nω-nitro-L-arginine; LTG, lamotrigine; MK-801, (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f] quinoxaline-7 -sulfonamide; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PHT, phenytoin; PTZ, pentylenetetrazole; SMTC, *S*-methyl *L*-thiocitrulline; VPA, sodium valproate.

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1996). Neuron-derived nitric oxide (NO) synthesized from L-arginine by neuronal NO synthase (nNOS) in the brain is considered to be both a neurotransmitter and a neuromodulator, and the involvement of NO in epileptic disorders has been demonstrated in experiments using systemic injections of NOS inhibitors (Banach et al., 2011; Snyder and Bredt., 1991). We previously reported a relationship between NO-nNOS machinery and PTZ-induced convulsion susceptibility. NO generation triggers motor seizures such as clonic and tonic–clonic convulsions. NO was generated by nNOS through the activation of N-methyl-D-aspartate receptors (NMDAR) in brain of PTZ-induced seizure models. Therefore, we showed that PTZ-induced convulsive seizures occur following NO generation through the activation of nNOS induced by hyperexcitability of glutamatergic neurons (Itoh et al., 2004; Kaneko et al., 2002). On the other hand, high doses of nNOS inhibitors have been reported to increase seizure severity in response to subconvulsive doses of PTZ, and PTZ-induced convulsion susceptibility is facilitated in mice lacking the nNOS gene compared to that observed in wild type mice, thus suggesting that the motor seizure threshold is regulated by the background NO levels (Itoh and Watanabe, 2009). Therefore, NO may be a neuromodulator that exhibits dual proconvulsive and anticonvulsive properties under certain circumstances, and the therapeutic modulation of the NO levels to treat epilepsy may be complicated by adaptive responses to this therapy.

In the current study, the NO content in the brain at the time of PTZ-induced convulsive seizures was quantitatively and directly measured using the *ex vivo* X-band electron paramagnetic resonance (EPR) method with an NO-trapping agent due to the method's high resolution and ability to specifically detect only NO (Itoh et al., 2004; Kaneko et al., 2002). We determined whether the background levels of NO in various brain regions are regulated by activation of nNOS induced by excitability of glutamatergic neurons using a pharmacological study and nNOS gene-deficient mice. Next, we investigated the relationship between PTZ-induced generalized convulsive seizures and upregulation of NO generation in various brain regions. This study indicated that the threshold of PTZ-induced convulsive seizures is regulated by certain NO levels in murine brains. In addition, valproate and ethosuximide, chosen treatments for generalized convulsive seizures, inhibited the PTZ-induced high levels of NO and convulsive seizures.

Experimental procedures

Experimental animals

The protocols for all animal experiments were approved by Tokushima Bunri University Animal Care Committees according to the National Institutes of Health (USA) Animal Care and Use Protocol. All efforts were made to minimize the number of animals used and their suffering. Ninety male ICR mice, nine to 14 weeks old, were purchased from Japan SLC (Shizuoka, Japan). Mice homozygous for nNOS deficiency ($nNOS^{-/-}$, B6.129SNOS1tm1Plh/J) were generated by intercrossing heterozygote $nNOS^{+/-}$ mice and genotyped using PCR, as previously described (Itoh and Watanabe, 2009). All mice were maintained with laboratory chow and water *ad libitum* on a 12-hr light/dark cycle.

Administration of NOS inhibitors, NMDAR antagonists, AMPAR antagonists and AEDs

(5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine maleate (MK-801), (E)-(±)-2-amino-4-methyl-5-phospho no-3-pentenoic acid ethyl ester (CGP39551), 3-(2-Chlorophenyl)-2-[2-[6-[(diethylamino)methyl]-2-pyridinyl] ethenyl]-6-fluoro-4(3H)-quinazolinone hydrochloride (CP465022) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) were obtained from Tocris Biosciences (Ellisville, MO, USA). The doses of MK-801, CGP39551, CP465022 and NBQX used in this study were 0.05, 10, 3 and 30 mg/kg, respectively (Itoh and Watanabe, 2009; Löscher and Hönack, 1991; Löscher et al., 1993; Menniti et al., 2003). Nω-nitro-L-arginine (L-NNA, in saline; Sigma-Aldrich Corp.) and 3-bromo-7-nitroindazole (3Br7NI, in dimethyl sulfoxide (DMSO); Calbiochem, San Diego, CA, USA) were used as nNOS inhibitors (Hara et al., 1996; Kaneko et al., 2002). All inhibitors dissolved in saline were administered intraperitoneally (i.p.) 30 min before treatment with PTZ. All AEDs, sodium valproate (VPA, Wako Pure Chem. Ltd., Oosaka, Japan), ethosuximide (ESM, Sigma-Aldrich Corp.), carbamazepine (CBZ, Wako Pure Chem. Ltd.), phenytoin (PHT, Dainippon Sumitomo Pharma Co., Ltd., Oosaka, Japan) and lamotrigine (LTG, Sigma-Aldrich Corp.) were dissolved in 0.5% (w/v) carboxymethyl cellulose 400 solution (Wako Pure Chem. Ltd.). Each AED was orally administered at an injection volume of 0.05 ml/10 g 60 min before PTZ injection. The doses of each AED were selected based on the findings of previous reports (Mandhane et al., 2007; Watanabe et al., 2010).

Acute PTZ-induced seizures protocol

The animals were placed in a plastic chamber (15 × 15 × 30 cm) and their behavior was observed before and after PTZ administration. After the animals displayed a resting posture, they were injected i.p. with varying doses of PTZ (Sigma-Aldrich, Corp., St. Louis, MO, USA). The mice were acutely treated with subconvulsive doses (20 and 40 mg/kg) and convulsive doses (60 mg/kg) of PTZ. The control mice received 0.2 ml/10 g saline injections. After each PTZ injection, the convulsive behaviors of the mice were observed for 10 min and resultant convulsions were classified and scored according to the criteria of a previous report (Itoh et al., 2004; Itoh and Watanabe, 2009) as follows: 0: normal; 1: immobilization; 2: facial, vibrissal and forelimb clonus (short myoclonic jerk); 3: myoclonic jerking consisted of a whole body jerk with or without irregular, bilateral forelimb movements; 4: generalized clonic seizures (GCS) with kangaroo posture; 5: generalized tonic–clonic seizures (GTCS) with loss of posture tone. In this study, “immobilization” and “myoclonic jerking” were given scores ranging from 1 to 3 as a nonconvulsive seizure and “severe GCS and GTCS” were assigned scores of 4 and 5 as convulsive seizures, respectively.

Measurement of NO content with ex vivo X-band EPR

The NO content in brain tissue was measured using *ex vivo* X-band EPR, as previously described (Itoh et al., 2004; Kaneko et al., 2002). Briefly, mice were injected i.p. with 500 mg/kg of *N*, *N*-diethyldithiocarbamate Na (DETC, Wako Pure Chem. Ltd.) and subsequently injected subcutaneously (s.c.) with an iron-citrate complex (50 mg/kg·$FeSO_4 \cdot 7H_2O$ + 250 mg/kg·sodium citrate) 30 min prior to PTZ injection. After undergoing PTZ injection, mice that had also received the NO-trapping agents (DETC and iron-citrate complex) were observed for 10 min and scored for behavioral changes. The mice were then killed, their brains were excised and each cortical region (frontal, temporal, parietal and occipital), the hippocampus and the cerebellum were immediately dissected and stored in liquid N_2 until EPR measurement. The excised sections of brain tissue were weighed and subsequently measured in glass capillaries (Microcaps 100, Drummond Scientific Co., Broomall, PA, USA) at ambient temperature on an Elexsys E500 X-band EPR spectrometer (Bruker BioSpin K.K., Kanagawa, Japan). The typical spectrometer conditions were: microwave power = 20 mW; microwave frequency = 9.84 GHz; modulation frequency = 100 kHz; modulation amplitude = 2.0 G; receiver gain = 60 dB; response time = 20.48 mS; number of points = 1024; sweep time = 40 s; number of scans = 10; central field = 3450 G; sweep width = 100 G. The concentration of trapped NO was determined using double