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The Roles of Dominance of the Nitric Oxide Fractions Nitrate and Nitrite in the Epilepsy-Prone EL Mouse Brain

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Background: Oxidative stress is thought to be closely related to epileptogenesis. We have previously reported that nitric oxide (NO) levels are higher in epilepsy-prone EL mice between the ages of 3 and 8 weeks than in control mice. However, NO is divided into two fractions, nitrite (NO_2) and nitrate (NO_3), which appear to play different roles in epileptogenesis.

Methods: NO_2 and NO_3 levels were measured, in EL mice and the control mice, in the parietal cortex, which is thought to be the primary epileptogenetic center in EL mice, and measured in the hippocampus, which is thought to be the secondary center.

Results: NO_3 levels in the hippocampus and parietal cortex of the immature EL mice (3 to 8 weeks of age) were significantly higher than those in the control mice; NO_2 levels were significantly higher in the EL mice throughout the study period. The NO_3 levels were significantly higher than the NO_2 levels in the immature EL mice, but after the onset of ictogenesis at 10 weeks of age, the relative levels of the two fractions reversed.

Conclusion: The reversal of the NO fraction distribution at the onset of seizures that we observed may be related to the developmental process of seizure susceptibility in the neural network of EL mice.

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Key words: nitric oxide (NO), nitrite (NO_2), nitrate (NO_3), EL mice, seizure susceptibility

Introduction

Oxidative stress is closely related to the pathogenesis of epilepsy during ictogenesis in human cerebrospinal fluid (CSF)[1]. However, the role of up-regulated antioxidative agents in the central nervous system of patients with epilepsy still remains unknown. Experimental methods have demonstrated hippocampal antioxidant ability in mutant animal models of epilepsy (EL mice)[2–5]. The EL mouse is an inbred, epileptic mutant model of secondarily generalized seizures[6]. Several lines of evidence indicate that in EL mice, the parietal cortex is the seizure initiation site, while the hippocampus is responsible for seizure generalization[7]. The developmental formation of the focus complex, which consists mainly of the parietal cortex and the hippocampus, has been hypothesized to be the key to epileptogenesis in EL mice. Nitric oxide (NO) has been identified as a source of free radical scavengers; NO is rapidly metabolized by oxidation to nitrite (NO_2) and nitrate (NO_3). Because NO_2 reportedly had a potent neuroprotective effect[8], we decided to measure NO fraction concentrations in an attempt to solve how the redox condition was during the onset of ictogenesis in EL mice.

Materials and Methods

Mutant epilepsy-prone EL mice manifesting no seizures before 5 weeks of age were used, with ddY mice serving

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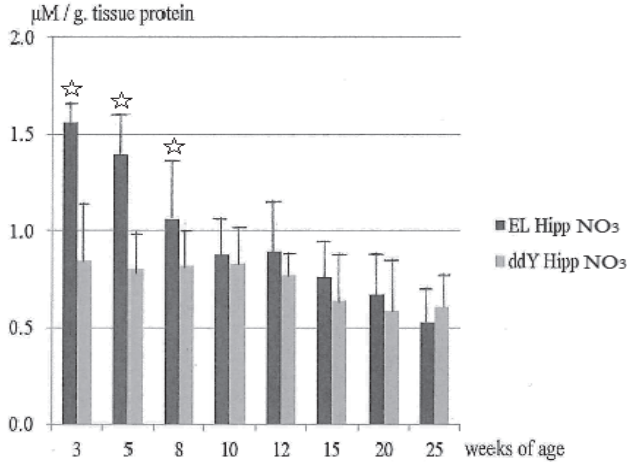

Fig. 1 Time course of NO_3 concentrations in the hippocampus of EL and ddY mice
Each figure represents a comparison of NO_3 or NO_2 concentrations in the hippocampus or parietal cortex of EL and ddY mice. Asterisks indicate statically significant differences ($p<0.01$) on two way ANOVA used to compare the means of each parameter between the two types of mice at the same age.

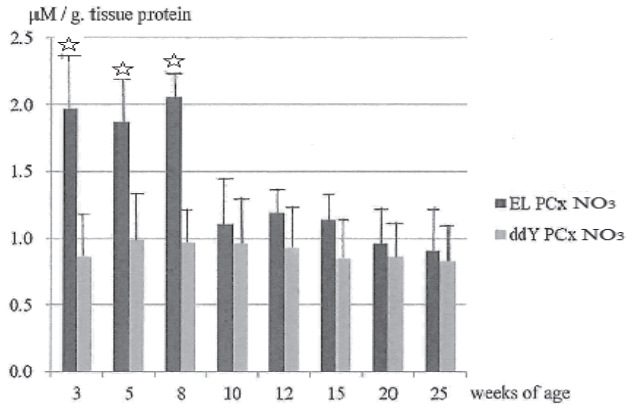

Fig. 2 Time course of NO_3 concentrations in the parietal cortex of EL and ddY mice
Each figure represents a comparison of NO_3 or NO_2 concentrations in the hippocampus or parietal cortex of EL and ddY mice. Asterisks indicate statically significant differences ($p<0.01$) on two way ANOVA used to compare the means of each parameter between the two types of mice at the same age.

as controls (The El mouse was established in Japan as a genetically predisposed epilepsy model from the hydrocephalic mutant of the ddY strain[9]). The brain redox was studied.

Five EL mice and 5 control ddY mice were sacrificed by decapitation, and the brains were removed and placed on ice. The parietal cortex and the hippocampus were excised and weighed (8-20 mg). To obtain brain tissue homogenates, 20 mM Tris-HCl (pH 8.0) was added.

An NO_2/NO_3 Assay Kit-CII (Dojindo, Kumamoto, Japan) was used to determine NO levels. Briefly, the Griess reaction was used to determine NO_2 levels spectrophotometrically at 540 nm[10]. For NO_3 reduction, samples were incubated in the presence of nitrate reductase, NADPH and FAD. Since all NO fractions in our samples were converted to NO_2, their levels were determined spectrophotometrically to serve as total NO (NO_2+NO_3) levels. The Bradford assay[11] was used to measure total protein concentrations in each sample with a Bio-Rad reagent (Bio-Rad, Richmond, California, United States).

Data are presented as mean ± standard error of the mean, and two way ANOVA was used to determine the statistical significance of differences in each parameter in mice of the same age; a level of $p < 0.01$ was considered significant.

Results

1. NO_3 Concentrations in the EL and ddY Mice (Fig. 1, 2)

NO_3 concentrations in the hippocampus and parietal cortex were significantly higher in the EL mice between 3 and 8 weeks of age than they were in the ddY mice at the same age. Between 10 and 25 weeks of age, however, the levels were almost the same in both mouse groups.

2. NO_2 Concentrations in the EL and ddY Mice (Fig. 3, 4)

NO_2 concentrations in the hippocampus and parietal cortex were significantly higher in the EL mice between 3 and 25 weeks of age than in the ddY mice.

3. Comparison of NO_3 and NO_2 Concentrations in the EL Mice (Fig. 5, 6)

When the EL mice were between 3 and 5 weeks of age, the NO_3 concentrations in the hippocampus and parietal cortex were significantly higher than those of NO_2, but the relative levels of the two fractions reversed when the mice were aged between 10 and 25 weeks; the levels were almost the same at 8 weeks of age.

4. Comparison of NO_3 and NO_2 Concentrations in the ddY Mice (Fig. 1～4)

The concentrations of the two fractions were relatively stable throughout the time course, with only slight fluctuations.

Discussion

NO is generated by NO synthetase (NOS) in most cells

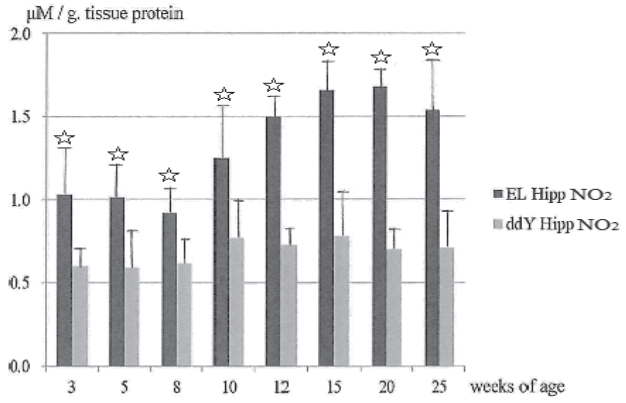

Fig. 3 Time course of NO_2 concentrations in the hippocampus of EL and ddY mice
Each figure represents a comparison of NO_3 or NO_2 concentrations in the hippocampus or parietal cortex of EL and ddY mice. Asterisks indicate statically significant differences ($p<0.01$) on two way ANOVA used to compare the means of each parameter between the two types of mice at the same age.

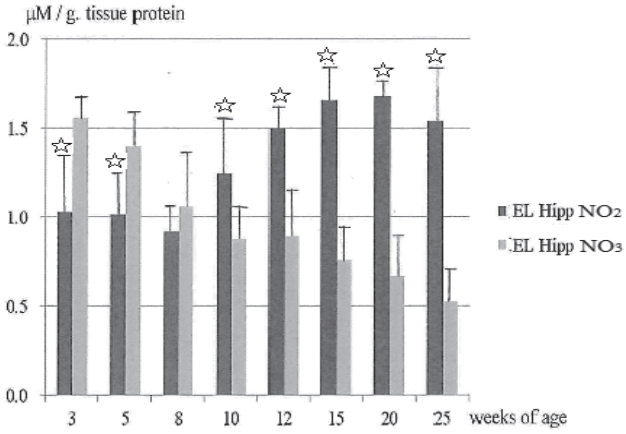

Fig. 5 Time course of NO_2 and NO_3 in the EL mouse hippocampus
Each figure represents a comparison of NO_2 and NO_3 concentrations in the hippocampus or parietal cortex of EL mice. Asterisks indicate statically significant differences ($p<0.01$) on two way ANOVA used to compare the means of each parameter between the hippocampus and parietal cortex in EL mice of the same age.

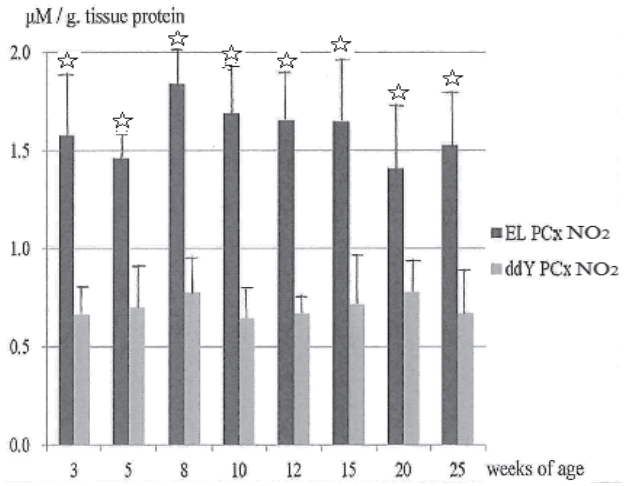

Fig. 4 Time course of NO_2 concentrations in the parietal cortex of EL and ddY mice
Each figure represents a comparison of NO_3 or NO_2 concentrations in the hippocampus or parietal cortex of EL and ddY mice. Asterisks indicate statically significant differences ($p<0.01$) on two way ANOVA used to compare the means of each parameter between the two types of mice at the same age.

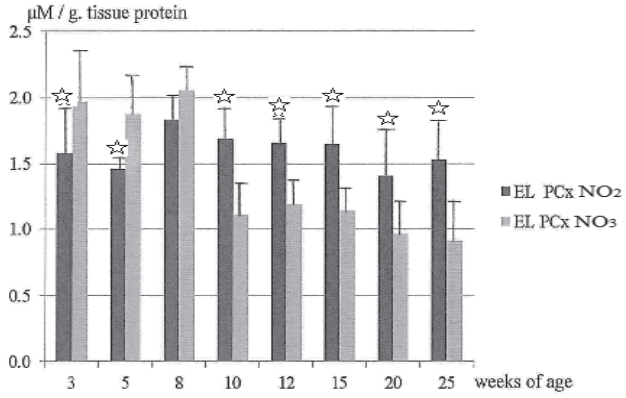

Fig. 6 Time course of NO_2 and NO_3 concentrations in the EL mouse parietal cortex
Each figure represents a comparison of NO_2 and NO_3 concentrations in the hippocampus or parietal cortex of EL mice. Asterisks indicate statically significant differences ($p<0.01$) on two way ANOVA used to compare the means of each parameter between the hippocampus and parietal cortex in EL mice of the same age.

of the body. It is regulated by its rapid oxidation to NO_2 and then, in the presence of oxyhemoglobin, to NO_3. In the presence of carbonic anhydrase, vitamin C, or polyphenols, NO_2 is reduced to NO, thus systemizing the nitrogen cycle in the body[12].

We found that NO_3 and NO_2 levels were almost constant during the growth process in the ddY mouse brain, however, in the EL mouse brain, NO_3 predominated over NO_2 in the early weeks of age (i.e. during the process of epileptogenesis acquisition), that the difference in the levels of the two fractions shrank around the time of seizure onset, and that NO_2 dominated after seizure onset. This seems very interesting and important. NO circulates in the living body and is not localized in the brain. However, it has been reported that it is possible various fac-

tors related to acquisition of epileptogenicity of EL mice containing NO fraction are regulated and act locally in the brain[13] and as in this report, the present data were obtained using local homogenate at a local site in the brain. NO_3 is supposed to act as an oxidative agent[14], NO_2, on the other hand, is supposed to act as a reducing agent[15]. Considering the properties of free radicals, while generality is not lost in this situation, it is recognized that oxidizing agents are neurodestructeve and reducing agents are neuroprotective. So it seems reasonable to think that in the EL mouse brain NO_3 exerts a relatively harmful or proconvulsant effect before 10 weeks of age, while NO_2 plays a relatively neuroprotectve or anticonvulsant role after 10 weeks of age.

It has been reported that epileptogenesis in EL mice is caused either by antioxidant protection or excessive free radical formation, especially in the hippocampus[3,4,16]. In seizure susceptibility regulation, some studies have indicated that the effect of total NO is anticonvulsant[17,18], while others have suggested that it is proconvulsant[19,20]. In this way, NO in vivo has been reported to work in neuroprotective or neurodestructeve[21].

The hypothesis of NO_3 and NO_2 dominance that switches at the onset of seizures could explain previous reports of the duality of anticonvulsant or proconvulsant effects in NO.

As reported previously, the parietal cortex has been thought to be the seizure initiation site of EL mice[7], so it seems reasonable that NO_3 (as neurotoxic) levels are upregulated in the parietal cortex during their early age of weeks before the acquisition of seizures. However, NO_3 was also enhanced in the hippocampus of EL. From this observation it is possible that the hippocampus is functioning as an amplifier for paroxysmal discharges generation, not just a secondary center for seizure propagation in EL. If so, from the viewpoint of maintaining homeostasis in EL, of which ictogenesis is acquired, it seems there is no contradiction that NO_2 levels increase not only in the parietal cortex but also in the hippocampus after 10 weeks of age for the same reason.

We have previously reported that oxidized glutathione (GSSG) levels in the hippocampus and parietal cortex of EL mice between 3 and 8 weeks of age are higher than those between 10 and 25 weeks of age[22], which matches the time course of NO_3 concentrations in the EL mouse brain. It is possible, therefore, that GSSG contributes to the process of epileptogenesis acquisition in EL mice in synergic action with NO_3. We have also reported that glutathione peroxidase (GPX) activities in the brains of EL mice between 5 and 8 weeks of age tend to be higher than those between 10 and 25 weeks of age[22], and since this matches the time course of NO_2 concentrations in the EL mouse brain, it is similarly possible that GPX exerts a neuroprotectve effect in synergic action with NO_2. Moreover, the cellular environment of oxidative stress, causing structural changes in glutamate transporters[23] or changes in neuronal membrane permeability[24], has been reported to increase intracellular glutamate levels.

Taken together with the results of these previous studies, the present data suggest that free radical changes resulting from NO-mediated scavenger potency are related to the acquisition of epileptogenesis in EL mice. Our observation of the predominance in NO_3 and NO_2 reversing the switch at the onset of seizures seems consistent with biological response in EL mice brain at both the hippocampus and the parietal cortex. It should be noted, however, that the present study is only a preliminary and observational one. It seems to be very interesting that the balance of NO_3 (oxidant effect) and NO_2 (antioxidant effect) is changed according to the time course before and after the acquisition of ictogenesis.

Some factors[12] may delicately regulate the dominance of NO_3 and NO_2 in the brain region. Further elucidation of the mechanism is awaited in the future.

Conflict of Interest: The authors have no conflicts of Interest to declare.

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