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原　著（査読あり）

Theanine, an Ingredient of Green Tea, Inhibits the Progression of Electrical Kindling in the Cerebral Cortex in Mice

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緑茶成分テアニンはマウスの大脳皮質電気キンドリングの進行を抑制する

岡村　法宜*

Abstract

The purpose of this study was to examine how the green tea ingredient theanine acts on epilepsy, with a focus on the cerebral cortex, using electrical stimulation in a kindling model of mice. Forty mice were implanted with stimulating electrodes on the frontal dura mater, and electrical stimulation at the after-discharge (AD) threshold was given for 7 weeks. During this time, the mice were divided into two groups: one fed with tap water (control group) and the other fed with tap water containing 2 g/L of theanine (theanine group). We observed that the frequency of AD induced by electrical stimulation was significantly lower in the theanine group than in the control group ($p < 0.0001$). In addition, both the occurrence of neuronal cell death and the appearance of microglia with nuclei that became rod-shaped in the stimulation area were significantly lower in the theanine group than in the control group ($p < 0.0001$). The frequency of neuronal cell death and the appearance of rod-shaped microglia at the stimulation site around the stimulation site were significantly suppressed ($p < 0.0001$). Based on these results, we surmised that excessive excitement of the nerve cell group due to electrical stimulation did not occur in the theanine group, suggesting that secretion of glutamate, an excitatory neurotransmitter, was suppressed, and the neuronal cell death due to excitotoxicity of glutamate was suppressed. We further speculated that suppression of neuronal cell death led to suppression of the appearance of rod-shaped microglia. These results suggest that daily intake of beverages and foods containing theanine may contribute to seizure control in epilepsy through regulation of electrical activity in the cerebral cortex.

Keywords：theanine, kindling, neuronal death, microglia, mice

Introduction

There are many animal models of epilepsy. Kindling animals are appropriate models of epilepsy because these animals and epilepsy patients have strong similarities when undergoing seizure[1]. In both kindling animals and epilepsy patients, there is a typical EEG spike, or spike and wave, that precedes a seizure, and consciousness is often lost. In particular, kindling animals produced by electrical stimulation of the amygdala or hippocampus are considered the most accurate model of human temporal lobe epilepsy, because seizure-related EEG activity is found in the hippocampus for both.

The most important brain changes that lead to epileptic seizures are alterations in synaptic function caused by repeated over-excitement of neurons. However, such changes cannot be easily confirmed microscopically. On the other hand, in the brains of patients with temporal lobe seizures, neuronal death and activation of microglia with immune reaction are observed through general pathological obervations[2]. Due to the characteristics of the brain, most of these observations are confirmed

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postmortem. It is not clear whether these pathological features are generated in kindling animals[3,4].

Theanine (γ-glutamylethylamide) was isolated from Japanese green tea (Camellia sinensis) as a flavor constituent by Sakato[5] in 1949. Oral intake of theanine was reported to induce a feeling of relaxation among the human subjects examined. We show here that theanine inhibits neuronal excitation by caffeine using EEG recordings from mice rats that were administered caffeine and theanine[6]. In addition, we report that administration of theanine can suppress excessive discharge induced by electrical stimulation in the hippocampi of kindling rats[7].

Below, we report the effects of theanine administration on the appearance of the after-discharge (AD) and cerebral histopathology in kindling mice that were electrically stimulated over a long period of time.

Materials and methods

1. Research approval

This study was approved by the President of Ehime Prefectural University of Health Sciences after the review by the Institutional Animal Care and Use Committee (Permission number: 2018-004), and carried out according to the Ehime Prefectural University of Health Sciences Animal Experimentation Regulations.

2. Animal care and surgery

Ten-week-old male ICR mice were housed individually in polycarbonate cages in an air-conditioned room (22 ± 2℃, 55% ± 10% humidity) with a 12-h/12-h light/dark cycle (lighting from 9:00 ㏂ to 9:00 ㎰). The mice had free access to water and basal diet (CLEA Japan, Inc, Tokyo, Japan) for 1 week prior to the experiment as they accustomed to their surroundings. The rats then underwent stereotactic operation using the units for stereotactic coordinates (Narishige Co., Ltd : SR-6M-HT, Tokyo, Japan) under pentobarbital anesthesia (40 ㎎/㎏, i.p.). Two stainless steel screws were placed in contact with the dura over the right and left frontal cortices, and served as cortical electrodes. As shown in Figure 1, the two electrodes were fixed to the skull using dental cement. Electrode implantation was performed on 40 mice.

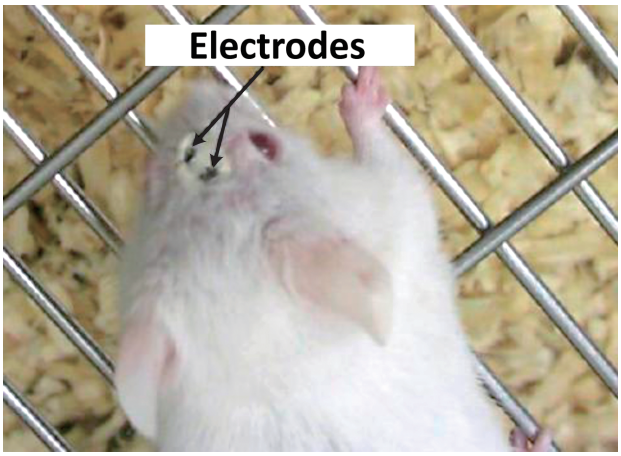

Figure 1. Mouse head after electrode implantation. Electrodes fixed to the skull with dental cement served both for stimulation and recording.

3. EEG measurements and determination of the AD threshold

Mice were allowed seven days to recover. The AD threshold was then measured by stimulating the animal's frontal cortex with 1s trains of bipolar pulses (0.5+0.5㎳, 60Hz) of increasing amplitude, starting from 100㎃. A bioelectric stimulation device (NIHON KOHDEN Co., Ltd: AB-621G, Tokyo, Japan) was used for electrical stimulation. EEGs were amplified by a bioelectric amplifier (NIHON KOHDEN Co., Ltd: SEN-7203, Tokyo, Japan), sampled at a frequency of 200Hz using an A/D converter (TURTLE INDUSTRY Co., Ltd.: TUSB-0412DSM-SZ, Ibaragi, Japan), and recorded on the hard disk of PC. The animals for which AD was evoked by this current for three successive days were used in the experiment.

4. Repeated electrical stimulation

After determination of the AD threshold, all mice were divided into two groups: one that was provided with drinking water containing 2 g/L theanine (theanine group) and one that was provided with tap water (control group). There were 20 mice in each group, and the respective drinking water was provided freely for each group. All mice were electrically stimulated in the frontal cortex once per day for 49 days at the AD threshold intensity, and EEGs of the frontal cortex were recorded before and after stimulation. All mice were checked for AD induction every 7 days and the AD-evoked rate of each group was calculated.

5. Preparation of pathological specimen

Mice were killed with an overdose of pentobarbital after stimulation and EEG recording on the final day. The brains were removed and fixed in 10% formalin. The brains were then dehydrated and embedded in paraffin, and 4-㎛ sections were cut and stained with hematoxylin-

eosin (HE). The tissue sections near the stimulating electrode were observed under a light microscope. The presence of neuronal cell death and microglial activation were assessed. A field of the cerebral cortex in contact with the stimulation electrode and a non-stimulated field were observed with a light microscope at a magnification of 400 times. The neuronal cell death was judged as follows; the shape of neuronal cell deformed thinly and its cytoplasm stained for excess eosinophilia. When much more dead neuronal cells were confirmed than in the non-stimulated area, the neuronal cell death was regarded as positive. The microglia activation was judged as follows; the microglia clearly deformed into rod-like shapes. When much more activated microglias were confirmed than in were observed than in the non-stimulated area, that microglia activation was regarded as positive.

6. Statistical analyses

Differences between the two groups in the average of AD-evoked rates for both groups, calculated a total of seven times every 7 days, were compared using Student's paired t-tests. The frequency of neuronal cell death and microglia activation due to differences in drinking water was tested using chi-square (χ^2) tests.

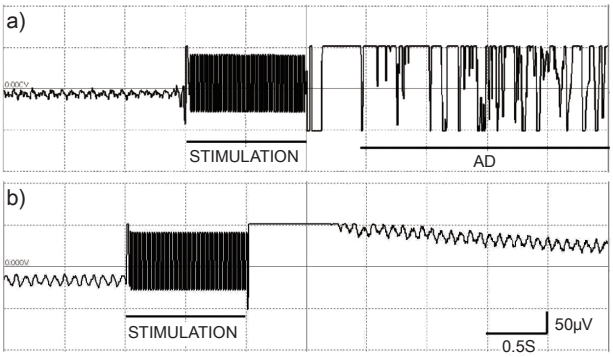

Figure 2. EEGs before and after electrical stimulation of cerebral cortex. a) A case of AD evoked after stimulation. b) A case of non-evoked AD after stimulation.

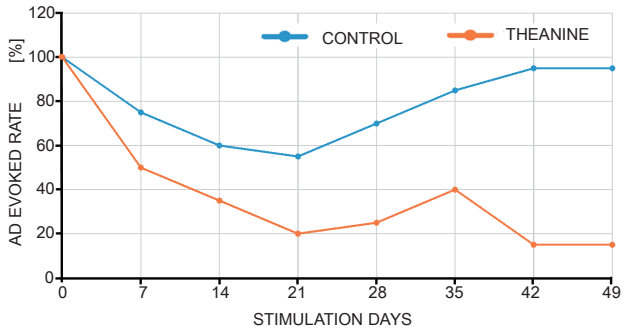

Figure 3. Change in AD-evoked rate over time.

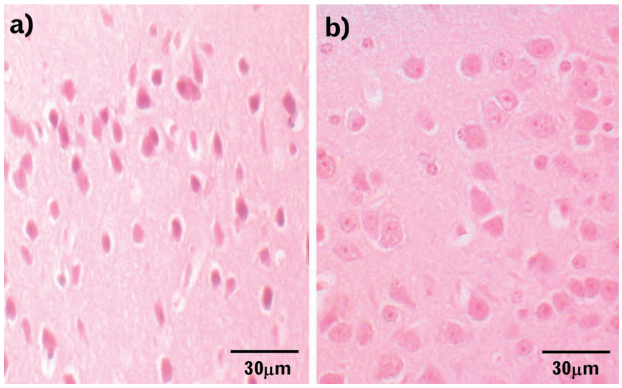

Figure 4. Microscopic images of cerebral cortex in both groups. a) Control group, b) Theanine group.

Results

1. AD-evoked rate

The AD threshold was found to be 250±150mA for all mice. Figure 2 shows EEG traces before and after electrical stimulation in AD-evoked mice a) and non-evoked mice b). Figure 3 shows the changes over time in AD-evoked rate in the control group and theanine group. In both groups, the AD-evoked rate decreased from 7 to 21 days after ascertaining the AD threshold. Thereafter, in the control group, an increase in the AD-evoked rate was observed from the 28th day after ascertaining the AD threshold, while in theanine group, a temporary increase in the AD-evoked rate was observed from the 28th to the 35th day, after which the AD-evoked rate declined sharply. The AD-evoked rate in the control group was 79.4%±17.0% (mean±SD) and the AD-evoked rate in the theanine group was 37.5%±28.2% (mean±SD). The AD-evoked rate in the theanine group was significantly lower than in the control group ($p=0.0035$).

2. Neuronal cell death and microglial activation

Figure 4a was a typical example of a microscopic image of the cerebral cortex of a mouse in the control group. In Figure 4a, many nerve cells have atrophied and the cytoplasm was eosinophils. And many microglia have transformed into rod-like shapes. On the other hand, as shown in Figure 4b, these histopathological views were hardly observed in the theanine group. The incidence of neuronal cell death was 100% (20/20) in the control group and only 20% (4/20) in the theanine group. Independence was rejected regarding the occurrence of neuronal cell death and the difference in drinking water ($p<0.0001$). Also, the incidence of microglia activation was 95% (19/20) in the control group and only 5% (2/20 in the

theanine group. Independence was rejected regarding the occurrence of microglia activation and the difference in drinking water ($p < 0.0001$).

Discussion

Many of the mice in the theanine group did not undergo AD following electrical stimulation of the cerebral cortex. It is known that theanine can suppress the excitement of the central nervous system by caffeine or kainic acid[5,6]. Theanine is glutamic acid methylamide, and its structure is similar to glutamate, as shown in figure 5. Glutamate is water-soluble and does not cross the blood-brain barrier, but because theanine is its methylamide, it becomes lipophilic and crosses the blood-brain barrier[8,9]. At first, therefore, we considered that this phenomenon was a natural result of theanine acting antagonistically on excitatory glutamate receptors in the brain. But, according to Kakuda et al.[10,11], theanine's affinity for glutamate receptors is not very high. Furthermore, their study showed that sustained exposure to theanine led to a significant decrease in the level of extracellular glutamate released from cultured neurons[10,11]. Theanine appears to function as an inhibitor of transporters that transports Gln across the plasma membrane and modulate of the glutamate/Gln cycle required for maintenance of neuronal glutamate pools[10,11]. For that reason, the present results must be due to a decrease in glutamate secretion from excitatory neurons due to daily theanine intake.

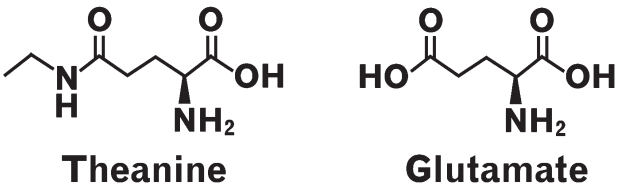

Figure 5. Structures of theanine and glutamate.

The reason why the incidence of AD decreased in both groups from the start of stimulation to the 21st day after the start is considered to be that the frequent stimulation increases the inhibitory mechanism by GABA-operating nerve. It is a well-known phenomenon that the excitability of nerve cells temporarily increases immediately after the suppression of the excitatory psychometer by the inhibitory nerve disappears[12].

In hippocampal and amygdala kindling animals, which are models of temporal lobe epilepsy, neuronal cell death and microglial activation are not always observed[13]. However, in human temporal lobe epilepsy, hippocampal neuronal cell death and microglial activation are routinely observed[2]. We thought that these differences might be due to the period of time that had elapsed since the completion of the seizure resulting from neural hyperexcitability. Neuronal cell death in the brains of epilepsy patients is caused by excitotoxicity of glutamate, an excitatory biotransmitter that can be over-secreted. When a kindling animal is produced by electrical stimulation of the amygdala, the duration of stimulation required to acquire a convulsive response is about 20 days[1]. The more easily a kindling animal acquired seizure readiness, the shorter it was subjected to the experiment. In this case, we considered that there may not have been enough time to induce neuronal cell death and microglia activation in some animals. On the other hand, electrical stimulation of the cerebral cortex does not as easily lead to convulsability as does stimulation of the amygdala and hippocampus[14]. For this reason, it is common to set a longer test period in experiments of cerebral cortex kindling in mice. In this study, as the test period was set longer than the experiment using hippocampal kindling mice, the number of electrical stimulations required to the cerebral cortex increased accordingly. In other words, stimulation of the mouse cerebral cortex in our experiment might be akin to the number of hyperexcitations that occur over many years in the brains of patients with temporal lobe epilepsy. Neuronal cell death and microglial activation were observed in all mice in the control group. Based on these results, we suspect that neuronal hyperexcitation must be repeated many times in order for neuronal cell death and microglial activation to occur in the cerebral cortex. Furthermore, neuronal cell death and microglial activation were not observed in most of the mice in the theanine group. Our results suggest that daily ingestion of theanine can inhibit severe epilepsy, even in epilepsy focused on excessive discharge of the cerebral cortex.

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Conflicts of Interest

The author has no conflict of interest directly relevant to the content of this article.

要　　旨

本研究は，大脳皮質を電気刺激したマウスを用いて，緑茶成分テアニンが大脳皮質に焦点があるてんかんにどのように作用するか検討した。40匹のマウスの前頭葉硬膜上に刺激電極を植え込み，１日１回後発射（After Discharge, AD）閾値上の電気刺激を７週間にわたって，前頭葉皮質に与えた。その結果，水道水を摂取していた対照群に比べ，テアニンを２g/l 溶解した水道水を摂取していたテアニン摂取群では，電気刺激によるAD誘発率は有意に低かった（$p=0.0035$）。さらに，テアニン摂取群では，刺激部位周辺の神経細胞死と核が棍棒状に変化した反応性ミクログリアの出現が有意に抑制された（$p<0.0001$）。以上の結果から，テアニン摂取群では，電気刺激よる過剰な神経細胞群の興奮が生じなかったため，興奮性神経伝達物質であるグルタミン酸の分泌が抑制され，グルタミン酸の興奮毒性による神経細胞死が抑えられたと推測された。さらに神経細胞死の抑制は，ミクログリアの活性化の抑制に繋がったと考えられた。したがって，大脳皮質に焦点を有するてんかんに，テアニンを含有する飲料や食品を日常的に摂取することは，発作の抑制だけでなく重症化の防止に寄与しうることが示唆された。