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(−)-Epigallocatechin gallate attenuates acute stress responses through GABAergic system in the brain

Nami Adachi [a], Shozo Tomonaga [a], Tetsuya Tachibana [a], D. Michael Denbow [a,b], Mitsuhiro Furuse [a,*]

[a] *Laboratory of Advanced Animal and Marine Bioresources, Graduate School of Bioresources and Bioenvironmental Sciences, Kyushu University, Fukuoka 812-8581, Japan*

[b] *Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0306, USA*

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Abstract

(−)-Epigallocatechin gallate (EGCG), a flavonoid, is the principal catechin found in green tea and is distributed in the brain after tea consumption. The aim of the present study was to investigate the effects of EGCG in the chick brain under an acute stressful condition and to clarify the mechanism by which EGCG attenuates stress behavior with special reference to γ-aminobutyric acid (GABA). Intracerebroventricular (i.c.v.) injection of EGCG (50, 100 and 200 μg) suppressed the vocalization which normally occurs during social separation stress. EGCG decreased the time spent in active wakefulness and induced sleep-like behavior in a dose-dependent manner. Additionally, i.c.v. injection of EGCG attenuated plasma corticosterone release under social separation stress. These effects of EGCG on distress-induced vocalization were significantly attenuated by the $GABA_A$ receptor antagonist picrotoxin but not by the $GABA_B$ receptor antagonist CGP 54626 (3-*N*-(1-(3,4-dichlorophenyl) ethylamino)-2-hydroxypropyl cyclohexylmethyl phosphinic acid hydrochloride). These results indicate that EGCG has sedative and hypnotic effects in the brain, partially through $GABA_A$ receptors, and consequently moderates an acute stress response.

Keywords: (−)-Epigallocatechin gallate; γ-Aminobutyric acid receptor; Brain; Intracerebroventricular injection; (Chick)

1. Introduction

Tea is widely consumed throughout the world as black, oolong and green tea. The main components of tea that have several biological actions have been identified as flavonoids. These compounds have antioxidant, antitoxic, anticarcinogenic and antiviral activities as well as vasculo-protector and antispastic actions. Effects of flavonoids on the central nervous system (CNS) have been recognized (Zanoli et al., 2000), and in particular, the potential of flavonoids as therapeutics involving γ-aminobutyric acid $(GABA)_A$ receptors (Huen et al., 2003). The characteristic structure of flavonoids, which have binding capacity for $GABA_A$ receptors, is similar to that of (−)-epigallocatechin gallate (EGCG). Recently, EGCG has been confirmed to bind $GABA_A$ receptors in vitro (Campbell et al., 2004). GABA, the major inhibitory neurotransmitter in the CNS, is essential for the overall balance between neuronal excitation and inhibition, by interacting with specific membrane receptors. $GABA_A$ receptors, mainly located postsynaptically, mediate most of the inhibitory synaptic transmission in the CNS (Whiting, 2003).

EGCG, a catechin, is the most abundant flavonoid in tea (*Camellia sinensis*) (Arts et al., 2000) and particularly green tea (Campbell et al., 2004). Approximately 26% of the solid weight of green tea extract is tea polyphenols, of which 11% are EGCG (Suganuma et al., 1998). Thus, EGCG is primarily responsible for the pharmacological actions of tea.

Green tea is used as a traditional pharmaceutical in Japan and China. One of the beneficial effects of green tea is to induce a feeling of relief. It is conceivable that this function may be regulated by EGCG in the CNS since EGCG is distributed in the brain after oral administration (Suganuma et al., 1998).

* Corresponding author. Tel./fax: +81 92 642 2953.
E-mail address: furuse@brs.kyushu-u.ac.jp (M. Furuse).

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Moreover, frequent consumption of green tea results in high levels of EGCG in the blood and brain (Suganuma et al., 1998). However, information on the central functions of EGCG is limited. The aim of the present study was to examine the effects and mechanism of EGCG in the brain with special reference to its action during an acute stressful situation in the neonatal chick.

2. Material and methods

2.1. Animals and food

Day-old male layer chicks (Julia) were purchased from a local hatchery (Murata Hatchery, Fukuoka, Japan). The chicks were housed in a windowless room at a constant temperature of 30±1 °C. Continuous lighting was provided. Food (Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water were freely accessible. On the day of the experiment, chicks (5 days old) were allocated to experimental groups based on their body weight, so that the average body weight was as uniform as possible within the same experiment. Experimental procedures followed the guidance for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government.

2.2. Preparation of drugs

EGCG, dimethyl sulfoxide (DMSO) and picrotoxin were purchased from Sigma (St. Louis, MO, U.S.A.). CGP54626 (3-*N*-(1-(3,4-dichlorophenyl)ethylamino)-2-hydroxypropyl cyclohexylmethyl phosphinic acid hydrochloride) was purchased from Tocris (Ballwin, MO, U.S.A.). Drugs were dissolved in 0.85% saline containing 0.1% Evans Blue and distilled water containing DMSO in a ratio of 20:1. The mixture was intracerebroventricularly (i.c.v.) administered to the birds using a mycrosyringe according to the method of Davis et al. (1979). The injected volume was 10 μl per bird according to Furuse et al. (1999). The stress and pain caused are minimal, as described elsewhere (Koutoku et al., 2005). Control groups were given the solvent solution (identified as saline) without drugs.

2.3. Experimental procedure

2.3.1. Behavioral experiments

To examine the effect of i.c.v. injection of either saline as a control or three levels of EGCG (50, 100 and 200 μg) on behavior, chicks were divided into four groups in Experiment 1. For 10 min post-injection, chicks were returned to a cage housing 20–25 birds so as to give sufficient time for drug diffusion. Thereafter, the chicks were gently placed individually into a 40×30×20 cm acrylic glass chamber for 10 min. In Experiment 2, the birds were divided into four groups which received either saline, 50 μg of EGCG, 500 ng of picrotoxin, or EGCG plus picrotoxin. Experiment 3 was similar to Experiment 2 except picrotoxin was replaced with 10 ng of CGP54626. In Experiments 2 and 3, since the $GABA_A$ receptor antagonist picrotoxin works in seconds, chicks were immediately placed individually into the chamber for 10 min, following injection. Chick behaviors were recorded by three video cameras positioned in different directions. The number of vocalizations was simultaneously recorded and counted, using a computer with Gretchen software (Excla, Inc.).

The postures during the 10 min after administration were determined from video recordings. According to Takagi et al. (2001), four behavioral categories were distinguished: (1) active wakefulness; (2) standing/sitting motionless with eyes opened; (3) standing motionless with eyes closed; and (4) sitting motionless with head drooped (sleep-like behavior). The monitoring systems were set in a separate room to avoid disturbing the animals. During the monitoring period, chicks were not given food or water.

2.3.2. Plasma corticosterone analysis

Blood was collected in heparinized microtubes from the jugular vein immediately after the behavioral tests. Blood was centrifuged at 4 °C at 10,000 ×*g* for 4 min, and plasma was collected and stored at −30 °C until analysis. Plasma corticosterone concentration was determined using a corticosterone enzyme immunoassay kit (Assay Designs Inc., MI, USA). After the collection of blood, the birds were killed with an overdose of sodium pentobarbital. The brains were removed and the location of the Evans Blue dye was confirmed. Data for chicks without dye in the lateral ventricle were deleted.

2.3.3. Statistical analysis

Data were statistically analyzed by repeated measures two-way analysis of variance (ANOVA) for vocalization and one-way ANOVA for corticosterone concentration and postures in

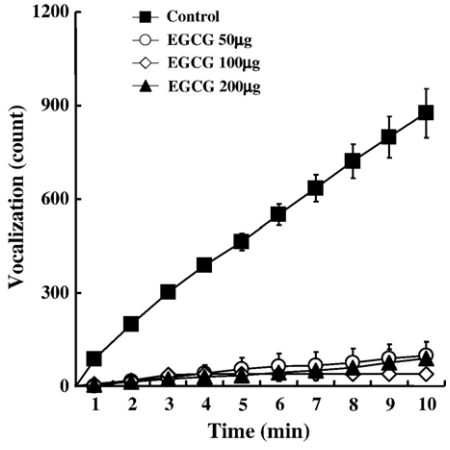

Fig. 1. Effect of i.c.v. injection of EGCG (0, 50, 100 or 200 μg) on cumulative number of vocalizations for 10 min in response to social separation stress in 5-day-old layer chicks. Data are expressed as means±S.E.M. Significant regression equations were obtained between number of vocalizations and EGCG (μg) as follows: vocalization (count)=0.001 (SE 2.063×10^{-4}) $EGCG^3$+0.243 (SE 0.059) $EGCG^2$−26.036 (SE 3.895) EGCG+875.571 (SE 55.016), R^2=0.872, $P<0.0001$. The number of chicks used in each group was control, 7; EGCG 50 μg, 6; EGCG 100 μg, 6; EGCG 200 μg, 8.

Table 1
Effect of i.c.v. injection of EGCG (0, 50, 100 or 200 μg) on various behavioral categories in response to social separation stress for 10 min in 5-day-old layer chicks

EGCG (μg)	0	50	100	200
Active wakefulness	543±15	172±107*	51±51*	13±9*
Standing/sitting motionless with eyes open	57±15	302±101	164±34	193±67
Standing motionless with eyes closed	0±0	0±0	26±26	20±17
Sitting motionless with head drooped (sleep-like behavior)	0±0	126±87	359±44*	374±66*,#
Total	600	600	600	600

Data are expressed as means±S.E.M. Significant regression equations were obtained between behavior and EGCG (μg) as follows: active wakefulness(s)= 0.025 (SE 0.006) EGCG^2−7.604 (SE 1.306) EGCG+529.67 (SE 49.949), R^2=0.730, P<0.0001 and sleep-like behavior(s)=1.868 (SE 0.402) EGCG+ 45.776 (SE 48.602), R^2=0.464, P<0.0001. *,#Significantly different from control and 50 μg of EGCG, respectively, at P<0.05. The number of birds used was control, 7; EGCG 50 μg, 6; EGCG 100 μg, 6; EGCG 200 μg, 8.

Experiment 1. Regression equations were also fitted for corticosterone concentration and postures against EGCG levels. Repeated measures three-way ANOVA was used for evaluation of vocalization in Experiments 2 and 3. Two-way ANOVA was applied for corticosterone concentration. Tukey–Kramer test was used for post hoc analysis of data. Statistical analysis was done using a commercially available package (Stat View, Version 5, SAS Institute, Cary, USA, 1998).

3. Results

In Experiment 1, the i.c.v. injection of EGCG clearly suppressed the number of vocalizations (Fig. 1). Significant effects of EGCG [F(3,23)=71.724, P<0.0001] and time [F(9,207)=70.366, P<0.0001] were detected. A significant interaction between EGCG and time [F(27,207)=38.827, P<0.0001] implied that the cumulative number of vocalizations increased with time, but those of EGCG-treated groups remained low during the experimental period. Behavioral patterns are shown in Table 1. Significant effects of EGCG were observed on active wakefulness [F(3,23)=21.825, P<0.0001] and sleep-like behavior [F(3,23)=10.047, P<0.0005]. Central EGCG increased sleep-like behavior, but the reverse was true for active wakefulness. I.c.v. injection of EGCG significantly [F(3,22)=4.481, P<0.05] decreased plasma corticosterone release compared with the effect of saline (Fig. 2).

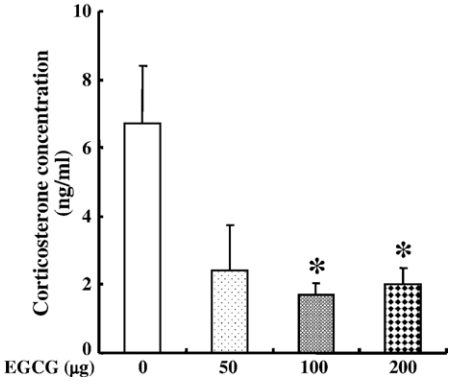

Fig. 2. Effect of i.c.v. injection of EGCG (0, 50, 100 or 200 μg) on the plasma corticosterone concentration after social separation stress for 10 min in 5-day-old layer chicks. Data are expressed as means±S.E.M. *Significantly different from control at P<0.05. The number of chicks used in each group was control, 7; EGCG 50 μg, 6; EGCG 100 μg, 6; EGCG 200 μg, 7.

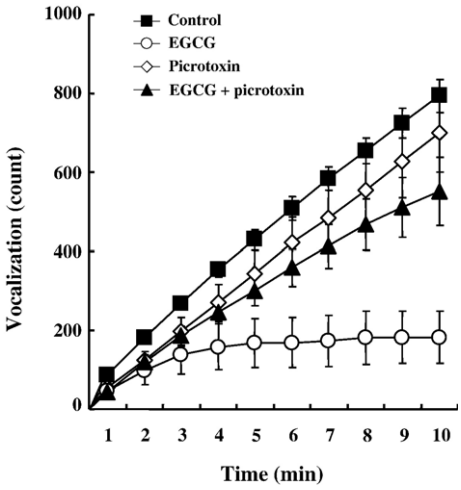

Fig. 3. Effect of i.c.v. injection of either saline, 50 μg of EGCG, 500 ng of picrotoxin, or EGCG plus picrotoxin on cumulative number of vocalizations in response to social separation stress for 10 min in 5-day-old layer chicks. Data are expressed as means±S.E.M. The number of chicks used in each group was control, 7; EGCG, 6; picrotoxin, 9; EGCG+picrotoxin, 8.

Fig. 3 shows the effect of i.c.v. injection of EGCG with or without picrotoxin on the number of vocalizations during the 10 min social separation stress (Experiment 2). Significant effects of EGCG [F(1,26)=13.785, P<0.005] and time [F(9,234)= 163.620, P<0.0001] were detected, but the effect of picrotoxin was not significant [F(1,26)=0.824, P>0.05]. A significant [F (1,26)=6.468, P<0.05] interaction between EGCG and picrotoxin suggested that the suppressive effect of EGCG on

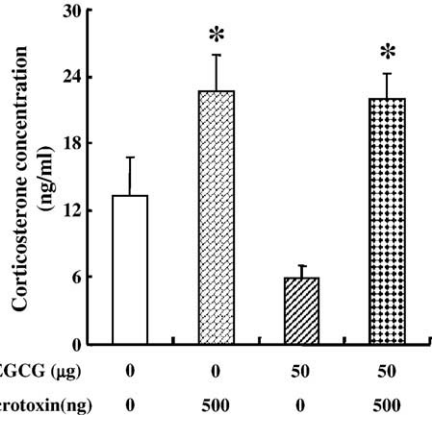

Fig. 4. Effect of i.c.v. injection of either saline, 50 μg of EGCG, 500 ng of picrotoxin, or EGCG plus picrotoxin on plasma corticosterone concentration for 10 min in 5-day-old layer chicks. Data are expressed as means±S.E.M. *Significantly different from 50 μg of EGCG alone at P<0.05. The number of chicks used in each group was control, 7; EGCG, 6; picrotoxin, 9; EGCG+ picrotoxin, 8.

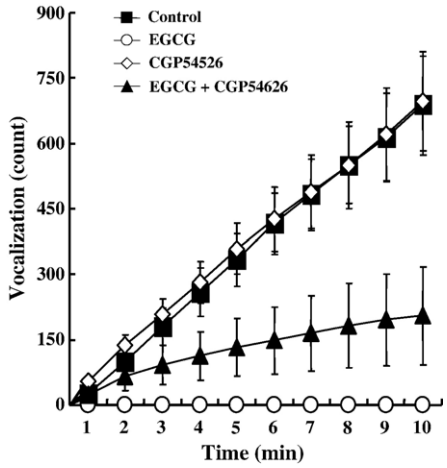

Fig. 5. Effect of i.c.v. injection of either saline, 50 μg of EGCG, 10 ng of CGP54626, or EGCG plus CGP54626 on the cumulative number of vocalizations in response to social separation stress for 10 min observation in 5-day-old layer chicks. Data are expressed as means±S.E.M. The number of chicks used in each group was control, 7; EGCG, 6; CGP54626, 8; EGCG+CGP54626, 7.

distress-induced vocalizations was attenuated by co-administered picrotoxin.

Fig. 4 shows the effect of i.c.v. injection of EGCG with or without picrotoxin on plasma corticosterone concentration during social separation stress (Experiment 2). Picrotoxin significantly [$F(1,26)=19.012$, $P<0.0005$] increased the plasma corticosterone concentration compared with the effect of EGCG [$F(1,26)=1.876$, $P>0.05$]. Furthermore, picrotoxin attenuated the decrease in plasma corticosterone caused by EGCG; however, the interaction was not significant [$F(1,26)=1.228$, $P>0.05$].

Fig. 5 shows the effect of i.c.v. injection of EGCG with or without CGP54626 on the number of vocalization during the 10 min social separation stress (Experiment 3). Significant effects of EGCG [$F(1,24)=26.548$, $P<0.0001$] and time [$F(9,216)=51.725$, $P<0.0001$] were detected, but no significant effect was observed with CGP54626 [$F(1,24)=1.574$, $P>0.05$]. The lack of a significant [$F(1,24)=0.939$, $P>0.05$] interaction between EGCG and CGP54626 implies that the effect of EGCG could not be attenuated by CGP54626.

4. Discussion

Chicks vocalize loudly when socially isolated from their conspecifics (Feltenstein et al., 2003b). The observations in the control group, which displayed a high frequency of vocalization, confirmed this phenomenon. I.c.v. injection of EGCG significantly decreased isolation-induced vocalization compared with the control. This effect was apparent immediately after the start of observation and remained until the end of the observation period (Fig. 1). A curvilinear response of vocalizations to EGCG levels was detected by regression analysis, but vocalizations were almost completely inhibited by the lowest level of EGCG. The reduced vocalizations suggested that central EGCG has a sedative effect under acute stressful conditions. Chicks administered EGCG also showed a curvilinear decrease in active wakefulness and a linear increase in sleep-like behavior (Table 1). The reduction in active wakefulness was also confirmed by spontaneous activity measurements, which were suppressed by EGCG in a dose-dependent manner (data not shown). These results suggest that i.c.v. injection of EGCG induced not only a sedative but also a hypnotic effect. It is conceivable that the dose-dependent decrease in spontaneous activity caused by EGCG was induced by the hypnotic effect. Plasma corticosterone concentration is related to stress and was measured as the primary biological stress marker. Corticotropin-releasing hormone messenger ribonucleic acid is up-regulated and plasma corticosterone concentration is elevated by chronic electroconvulsive shock treatment (Herman et al., 1989). The plasma corticosterone concentration is increased in chicks under separation stress (Feltenstein et al., 2003a). The action of EGCG may be partially explained by an effect on the hypothalamic–pituitary–adrenal axis, since EGCG attenuated corticosterone release enhanced by separation stress (Fig. 2). Orally administered EGCG may have a similar effect since EGCG is distributed in the brain after oral administration (Suganuma et al., 1998). This remains to be examined in the future.

Some flavonoids are linked to $GABA_A$ receptors and induce antianxiety, sedative and hypnotic effects (Marder and Paladini, 2002). Thus, the hypnotic effects and the suppression of the hypothalamic–pituitary–adrenal axis by EGCG may be mediated through the GABAergic system. The carbonyl group of the flavonoid structure is important for flavonoid binding to $GABA_A$ receptors. Although EGCG does not have the carbonyl group of the flavonoid structure, EGCG has a gallate group. The gallate group of EGCG seems to substitute for the carbonyl group (Campbell et al., 2004). Thus, it is hypothesized that EGCG functions through GABAergic neurons in the CNS. In fact, the results of Experiment 2 indicated that the $GABA_A$ receptors antagonist, picrotoxin, attenuated the suppressive effect of EGCG on vocalizations under socially isolated conditions (Fig. 3). We used picrotoxin to confirm the relationship between EGCG and $GABA_A$ receptors in vivo. $GABA_A$ receptors have several binding sites, and the coadministration of EGCG and other $GABA_A$ receptor antagonists is valuable for investigating the biding site of EGCG. The $GABA_A$ receptor is a member of the fast-acting transmitter-gated ion channel superfamily, which includes a pentameric structure with a central ion pore (Whiting, 2003). Accordingly, behavior should be monitored immediately after drug administration. This method of observation was different from that of Experiment 1, in which the experiment started 10 min after EGCG injection. However, the reduction in vocalization was immediate occurring after i.c.v. injection of EGCG in Experiments 2 and 3 as well as Experiment 1. These facts imply that the action of EGCG is very quick when the drug is i.c.v. administered.

EGCG is highly effective against distress vocalizations than corticosterone levels in chicks tested under stress conditions, as shown in Experiment 1. This is the case for central serotonin, since serotonin attenuates corticotrophin-releasing factor-

induced behaviors while stimulating corticosterone release (Zhang et al., 2004). It is conceivable that the dosages of picrotoxin required for attenuation by EGCG of the stress response may be different for different responses. The reduction of plasma corticosterone concentration by EGCG tended to be attenuated, but not significantly, by picrotoxin (Fig. 4). In Experiment 1, 50 μg of EGCG induced a slight, non-significant, hypnotic effect (Table 1). In Experiment 2, EGCG (50 μg) did not show a hypnotic effect.

$GABA_B$ receptors are not involved in the induction of antianxiety (Zarrindast et al., 2001). This concept was confirmed in Experiment 3, since the $GABA_B$ receptor antagonist, CGP54626, did not attenuate the suppressive effect of EGCG on distress-induced vocalization (Fig. 5) or plasma corticosterone concentration (data not shown). Consequently, it is concluded that EGCG has a sedative effect via the $GABA_A$, but not $GABA_B$, receptor system under acute stressful conditions.

The present study revealed a novel hypnotic effect of EGCG. However, green tea consumption does not induce sleep even though green tea contains abundant EGCG. This may be explained by the caffeine in green tea (Kuo et al., 2005). Caffeine acutely stimulates the autonomic nervous system and increases wakefulness (Quinlan et al., 2000). Consequently, it is conceivable that the hypnotic effect of EGCG may be counteracted by the action of caffeine in green tea. EGCG is a member of the promising family of natural flavonoid catechins and its properties suggest that it has a future as a sedative.

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