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L-Theanine Improves Immunity by Altering TH2/TH1 Cytokine Balance, Brain Neurotransmitters, and Expression of Phospholipase C in Rat Hearts

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Statistical Analysis C
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Background: This study aimed to investigate the regulatory effects of L-theanine on secretion of immune cytokines, hormones, and neurotransmitters, and mRNA expression of phospholipase C (PLC) in rats, and to explore its regulatory mechanism in immune function.





Material/Methods: Sixty-four Sprague-Dawley rats received daily intragastric infusion of different doses of L-theanine solution [0, 50 (LT), 200 (MT), and 400 (HT) mg/kg BW]. Cytokines, immunoglobulins, and hormones in the serum, neurotransmitters, and mRNA expression of PLC in the relevant tissues were assayed.

Results: L-theanine administration increased the splenic organ index and decreased the contents of ILs-4/6/10 and the ratio of IL-4/IFN- γ in the serum. High-dose L-theanine administration increased the levels of dopamine and 5-hydroxytryptamine in the pituitary and hippocampus, resulting in decrease in corticosterone level in the serum. L-theanine administration decreased the mRNA expressions of PLC isomers in the liver and PLC- γ 1 and PLC- δ 1 in the spleen. Interestingly, mRNA expressions of PLC- β 1 in the spleen and PLC isomers mRNA in the heart were up-regulated by L-theanine administration.

Conclusions: Administration of 400 mg/kg BW L-theanine improved immune function of the rats by increasing the splenic weight, altering the Th2/Th1 cytokine balance, decreasing the corticosterone level in the serum, elevating dopamine and 5-hydroxytryptamine in the brain, and regulating the mRNA expression of PLC isomers in the heart.

MeSH Keywords: **Immunity • L-theanine • Neurotransmitter Agents • Phosphoinositide Phospholipase C • Th1-Th2 Balance**

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Background

Theanine (N-ethyl- γ -glutamine) is a unique non-protein amino acid found in tea. Theoretically, it has 2 chiral isomers, D- and L-theanine, and the biological activity of L-theanine is very high *in vivo* [1]. L-theanine is an important bioactive component of tea, and has many physiological functions, such as regulating immune response [2,3], preventing diseases [4], anti-tumor [5], relaxing neural tension [6], and anti-oxidation stress [7].

Previous studies have shown that L-theanine can enhance innate immune function by regulating the secretion of immune cytokines. Bukowski et al. [8] demonstrated that ingestion of L-theanine by drinking tea induces innate immune response and immunologic memory in humans. The explanation is that L-theanine can be degraded by glutaminase to glutamate and ethylamine *in vivo* [9], and the latter, as a non-peptide alkylamine antigen, can be subsequently recognized by the gamma delta T cells ($\gamma\delta$ T cells) in peripheral blood, and the primed $\gamma\delta$ T cells further participate in a memory response [3]. Such priming also results in a non-memory response to whole bacteria and lipopolysaccharide, which is characterized by interleukin-12 (IL-12)-dependent secretion of interferon- γ (IFN- γ) by $\gamma\delta$ T cells and their proliferation [2]. Further clinical studies [8] have found that oral administration of L-theanine enhances the activity of $\gamma\delta$ T cells, promotes the secretion of IFN- γ , and further inhibits tumor activity. The possible mechanism could be that L-theanine metabolite-ethylamine induces the prenyl pyrophosphate accumulation by inhibiting the mevalonate pathway, and then promotes the proliferation of $\gamma\delta$ T cells [3]. Wen et al. [10] demonstrated that adding 400 mg L-theanine/kg daily in the diet increases the level of secretory IgA in the jejunum and the levels of IL-2 and IFN- γ in the serum of baby chickens. Hwang et al. [11] proposed that treatment of β -glucan (400 mg/kg feed) plus L-theanine (80 mg/kg feed) in weaning piglets lessens the inflammatory responses against Gram-negative bacterial infection via the inhibition of pro-inflammatory cytokines (e.g., IFN- γ and TNF- α) hyperproduction and enhancement of anti-inflammatory cytokine (IL-10) production *in vivo* after lipopolysaccharide exposure, in a time-dependent manner. Kurihara et al. [7] reported that oral administration of L-theanine and L-cysteine can increase IgG level in the blood of mice. Takagi et al. [12] showed out that combined administration of L-theanine and L-cystine before primary immunization enhances the serum antigen-specific IgM and IgG levels in 24-month-old mice, reduces the lung viral titers at day 6, and increases the anti-influenza-virus IgG antibody at day 10 after influenza virus infection in 13-month-old mice, and that the serum IL-10/IFN- γ ratio is elevated in the spleens of mice.

After being absorbed into the body, L-theanine can penetrate the blood-brain barrier and regulate the secretion of neurotransmitters of central nervous system cells [13] and

levels of hormones such as corticosterone (CORT) and adrenaline (EPI) in the serum. Peng et al. [14] demonstrated that L-theanine can increase levels of 5-hydroxytryptamine (5-HT) and noradrenaline, and decrease levels of adrenocorticotropic hormone and CORT in the hippocampus and prefrontal cortex of rats. Yamada et al. [15] found that L-theanine injection into the rat brain striatum increases the concentration of glycine and dopamine (DA) in the perfusate by activating α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. Another study [16] has shown that when mother rats are fed L-theanine ad libitum, the concentrations of DA, 5-HT, glycine and γ -amino butyric acid are increased in the 3-week-old infant rat brain, and the mRNA level of nerve growth factor is increased in the cerebral cortex and hippocampus. Tamano et al. [17] reported that when young rats are provided water containing 0.3% L-theanine for 3 weeks after weaning and are subjected to water-immersion stress for 30 min, the serum CORT level is less in L-theanine-treated rats than that in the control group, even after exposure to stress. Takeda et al. [18] reported that when young rats are provided water containing 0.3% L-theanine after birth, the serum CORT level is also markedly decreased. It has been reported that [19] oral administration of L-theanine decreases the levels of DA, noradrenaline (NA), and CORT, but increases 5-HT content in the brain cortex, hippocampus, and striatum of mice. Hence, L-theanine has a regulatory function in the secretion of immune cytokines, neurotransmitters, and hormones.

According to the amino acid sequence and molecular size, phospholipase C (PLC) isozymes in the mammalian cells are divided into 4 main types: PLC- β , PLC- γ , PLC- δ , and PLC- ϵ [20]. PLC- β is in charge of transmitting neuroendocrine signaling, and causes nerve impulses and the secretion of glands [21]. The PLC- γ is involved in the regulation of cell growth, proliferation, and differentiation by mediating the mitotic signals [22]. PLC- δ regulates the cell growth and its adaptability to the environment and stress [23]. Recent studies [24,25] have suggested that the expression of PLC can broadly reflect antioxidant capacity *in vivo*, and the activation of PLC and protein kinase C (PKC) causes $\gamma\delta$ T cells to react quickly against pathogen invasion, further regulating the immune function. Activation of PLC by L-theanine, hormones, and neurotransmitters combining with cell surface receptors induces cell proliferation, differentiation, secretion, and contraction [26]. However, the role PLC plays in the process of regulating cytokine secretion by L-theanine is unclear.

In this study, based on the premise that L-theanine has the ability to regulate the secretion of immune cytokines, neurotransmitters, and hormones, we further hypothesized that L-theanine could improve the immunity and resistance to stress via the activation of PLC subunits in rats. This experiment was therefore designed to determine the contents of immune cytokines,

immunoglobulins, neurotransmitters, hormones, and mRNA expression of related regulatory protein PLC after the intra-gastric administration of 3 doses (low, middle, and high) of L-theanine solution for 2 weeks, to determine which dose of L-theanine has the best immunomodulatory effect and to explore the possible L-theanine-induced regulation mechanism in immune function in rats.

Material and Methods

This experiment was conducted according to the animal care guidelines of the Animal Care Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha city, Hunan province, China (no. KYNEAAM-2013-0009). All efforts were made to minimize suffering of experimental animals.

Chemicals

L-theanine (assay: 99.2%, chemical synthesis) was purchased from Hongya Yaxing Biotechnology Co., Ltd (Meishan, Sichuan, China). ELISA kits for IL-2, IL-4, IL-6, IL-10, IFN- γ , adrenaline (EPI), CORT, DA, and 5-HT determination were purchased from Huamei Biotechnology Co., Ltd (Wuhan, Hubei, China). All the other chemicals used in this study were of analytical purity.

Animals and management

Thirty-two male and 32 female Sprague Dawley rats (SD rats, 3 weeks old) weighing 74–92.2 g were purchased from Hunan Slack Jingda Laboratory Animal Co., Ltd (Changsha, Hunan, China). The animals were individually housed in plastic cages on the floor under laboratory conditions ($25\pm 3^\circ\text{C}$, $70\pm 5\%$ relative humidity, good ventilation, and a 12-h light/dark cycle), and had free access to food and pure water.

Experimental design

The SD rats were acclimated for 3 days, then L-theanine was administered during fasting (15:00–17:00 h) via gastric intubation. During the whole experiment, the rats were examined for any abnormalities suggestive of health problems, and their body weights (BW) were recorded every day to determine the average daily gain (ADG) for each group. The rats were randomly assigned into 4 groups with 16 rats (8 males and 8 females) each. The control rats (CON) received gastric perfusion of 1 mL of 0.9% NaCl solution once per day. The rats in the other groups – low-dose L-theanine administration (LT), middle-dose L-theanine administration (MT), and high-dose L-theanine administration (HT) – received gastric perfusion of 3 different doses of L-theanine (50, 200, and 400 mg/kg BW/day, respectively) pre-dissolved in 0.9% NaCl solution, and each rat was administered 1 mL of the solution once per day for 14 days.

Blood and tissue samples collection

On completion of the treatment, rats were fasted overnight, then were placed in a sealed specimen jar with ether anesthesia for 4 min, and then the samples were collected. Blood was collected from the jugular vein by the standard procedure into blood collection tubes with potassium EDTA anticoagulant. Then the blood samples were centrifuged at 3500 rpm for 15 min at 4°C , and the serum samples were stored at -80°C until assay.

After flushing out the blood with 0.9% saline, the brains were removed and fractionated into the hypothalamus, pituitary, and hippocampus under ice-cold conditions. At the same time, the heart, liver, spleen, kidney, and thymus were quickly collected and their weights were recorded to calculate the organ index (OI: the ratio of organ weight to body weight, g/kg). All tissue samples were stored at -80°C prior to subsequent analyses.

Analysis of serum and tissue samples

The tissue samples were homogenized, the homogenates were centrifuged at 3500 rpm/min for 10 min at 4°C , and the supernatants were subjected to further measurement. IL-2, IL-4, IL-6, IL-10, IFN- γ , EPI, and CORT were assayed in the serum; IL-2, IL-4, IL-6, and IFN- γ were assayed in the spleen; and DA and 5-HT were assayed in the hypothalamus, pituitary, and hippocampus. The profiles in the serum and tissue samples were determined according to the protocols of the respective ELISA kits. The serum IgM and IgG were determined by automatic biochemistry analyzer (Synchron Clinical system CX4 PRO, Beckman Coulter, USA) according to the instructions.

RNA extraction and real-time quantitative PCR (qPCR)

Total RNA was isolated with the EZNA RNA-Solv reagent (Omega Bio-Tek, Norcross, GA, USA) and cDNA synthesis was performed using the Revert Aid First Strand cDNA Synthesis kit (Applied Biosystems, Thermo Fisher Scientific, USA). For relative quantification of gene expression, the ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster, CA) was used. Primers were designed using the Primer 3 plus program, and sequences are listed in Table 1. The reaction system contained 5 μL SYBR[®] Premix Ex Taq[™] (2 \times), 0.2 μL PCR forward primer (10 μM), 0.2 μL PCR reverse primer (10 μM), 0.2 μL ROX reference dye (50 \times), 1.0 μL cDNA, and 3.4 μL sterilized ddH₂O. The thermal profile for all reactions was 30 s at 95°C , then 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s. At the end of each cycle, fluorescence monitoring was performed for 15 s. Each reaction was completed with a melting curve analysis to ensure the specificity of the reaction. All the samples were analyzed in duplicate, and the relative amount of each specific transcript was obtained after normalization against the endogenous control β -actin.

Table 1. Data on primers used for real-time quantitative PCR.

Target gene	Accession number	Primer sequence (5'- 3')	Primer size (bp)	Product size (bp)
PLC-β1	NM_001077641.1	For GCAGGTCCAAGTGTGATTG	20	103
		Rev TTCTTCTCCGCTCAGGTAGC		
PLC-γ1	NM_013187.1	For CAAGGGCTTAAGTGGCTCA	20	108
		Rev TCCTCTCGGTTACGATCCAC		
PLC-δ1	NM_017035.1	For CCCTACCCAGTCATCCTGTC	20	161
		Rev ATCTTGCCCTTCAGTTGCTC		
β-actin	NM_031144	For TGCACCAACTGGGACGATA	20	165
		Rev GGGGTGTTGAAGGTCTCAA		

For – forward; Rev – reverse; PLC – Phospholipase C.

Table 2. Effects of L-theanine administration on organ index (OI) and average daily gain (ADG) in the rats.

Item	CON	LT	MT	HT	P value	
					Linear	Quadratic
Thymus OI	2.67±0.52	2.71±0.22	2.75±0.43	2.57±0.40	0.424	0.320
Spleen OI	1.92±0.07 ^d	2.16±0.05 ^c	2.33±0.06 ^b	2.51±0.05 ^a	<0.0001	<0.0001
Heart OI	4.01±0.56 ^a	3.72±0.21 ^b	3.83±0.26 ^{a,b}	3.80±0.17 ^{a,b}	0.416	0.372
Liver OI	32.6±1.2	33.1±1.8	32.1±1.5	31.9±2.9	0.132	0.872
Kidney OI	8.62±0.44	8.46±0.45	8.75±0.72	8.57±0.43	0.799	0.412
ADG (g/d)	3.66±1.4	4.24±1.1	4.04±0.90	4.03±0.94	0.658	0.477

CON – control group; LT – low-dose L-theanine administration group; MT – middle-dose L-theanine administration group; HT – high-dose L-theanine administration group. ^{a-d} Means in the same row not bearing a common superscript letter differ ($P<0.05$).

Statistical analyses

Statistical analyses were conducted by one-way analysis of variance (ANOVA) using the Mixed Proc of SAS (version 8.2, SAS Institute, Cary, NC, USA). The main effect tested was the dose of L-theanine. When indicated by ANOVA, means were separated using least significant differences. All data are expressed as means ±SD. Significance was declared at $P<0.05$ or 0.01.

Results

ADG and OI

As illustrated in Table 2, splenic OI of the treatment groups increased (Linear, $P<0.001$; Quadratic, $P<0.001$) with the doses of L-theanine. There were no differences ($P>0.05$) in the OI of liver, heart, kidney, thymus, and ADG among the 4 groups.

Cytokines in the serum and spleen

As shown in Table 3, the content of IL-4 in the serum linearly decreased ($P<0.001$) with the increasing doses of L-theanine. The administration of L-theanine linearly decreased the contents of IL-6 ($P<0.001$) and IL-10 ($P=0.01$) in the serum with the incremental doses of L-theanine. The ratio of IL-4 to IFN-γ in the serum was also linearly decreased ($P<0.001$) with the increasing doses of L-theanine. IgM level in the MT group was higher ($P=0.034$) than that in the LT group. There were no differences ($P>0.05$) in the serum IL-2, IFN-γ, IgG, and splenic IFN-γ, IL-2, IL-4, and ratio of IL-4 to IFN-γ among the 4 groups.

Neurotransmitters in brain tissues and hormonal content in the serum

As depicted in Table 4, the DA content was increased in the pituitary (Linear and Quadratic, $P<0.001$) and hippocampus (Linear, $P<0.001$) with the increasing doses of L-theanine, with

Table 3. Effects of L-theanine administration on cytokines and immunoglobulins in the serum and spleen of rats.

Item	CON	LT	MT	HT	P value	
					Linear	Quadratic
Serum						
IL2 (pg/ml)	54.0±23	57.4±14	59.1±20	61.3±21.4	0.333	0.794
IL4 (pg/ml)	207±53a	165±42 ^b	147±44 ^{b,c}	124±27 ^c	<0.0001	0.144
IL6 (pg/ml)	30.5±4.9 ^a	28.9±4.0 ^{a,b}	26.1±3.5 ^{b,c}	22.1±8.3 ^c	<0.0001	0.881
IL10 (pg/ml)	25.1±7.7 ^a	17.3±11 ^{a,b}	13.4±12 ^{b,c}	12.6±9.8 ^c	0.010	0.101
IFN-g (pg/ml)	1.11±0.14 ^{a,b}	1.09±0.14 ^{a,b}	0.98±0.1 ^b	1.21±0.47 ^a	0.310	0.034
IgG (mg/dl)	16.1±3.9	15.5±3.7	15.9±4.4	16.4±4.3	0.696	0.735
IgM (mg/dl)	8.81±2.6 ^{a,b}	7.06±2.6 ^b	9.31±3.1 ^a	8.57±2.7 ^{a,b}	0.482	0.616
IL4/IFN-g	186±28a	154±25b	144±31b	113±18 ^c	<0.0001	0.381
Spleen						
IL2 (ng/g)	2.85±0.93	3.00±0.89	3.16±1.02	3.35±0.92	0.146	0.822
IL4 (ng/g)	1.65±0.11	1.71±0.18	1.65±0.13	1.60±0.14	0.103	0.487
IFN-g (ng/g)	0.57±0.07	0.57±0.06	0.57±0.07	0.54±0.05	0.160	0.429
IL4/IFN-g	2.92±0.28	2.95±0.13	2.86±0.12	2.97±0.04	0.605	0.155

IL – interleukin; IFN – interferon; Ig – immunoglobulin; CON – control group; LT – low-dose L-theanine administration group; MT – middle-dose L-theanine administration group; HT – high-dose L-theanine administration group. ^{a-c} Means in the same row not bearing a common superscript letter differ ($P < 0.05$).

the highest values found in the MT and HT groups, respectively. The 5-HT content was elevated in the pituitary (Quadratic, $P=0.005$) and hippocampus (Linear, $P=0.003$) with the increasing levels of L-theanine, with the highest values in the MT and HT groups, respectively. However, in the hypothalamus, the 5-HT content decreased (Linear, $P=0.002$) with the inclusive levels of L-theanine, and the lowest value was found in the HT group. There were no differences ($P > 0.05$) in the hypothalamus among the 4 groups.

Serum EPI content increased (Linear, $P=0.007$) but serum CORT content decreased (Linear, $P=0.013$; Quadratic, $P < 0.001$) with the increasing doses of L-theanine; the highest and lowest values were in the HT and MT groups, respectively.

Expression of PLC-β1, PLC-γ1, and PLC-δ1 genes in the liver, spleen, and heart

The mRNA expression of PLC subtypes (PLC-β1, PLC-γ1, and PLC-δ1) in the liver, spleen, and heart are given in Table 5. L-theanine administration decreased the mRNA expression of PLC-β1 (Quadratic, $P=0.002$) and PLC-γ1 (Linear, $P=0.007$; Quadratic, $P=0.010$) in the rat livers. When compared with the CON, the lowest values were in the LT group. However, there

were no differences ($P > 0.05$) in the PLC-δ1 in rat livers among the 4 groups. The administration of L-theanine also depressed mRNA expression of PLC-γ1 (Linear and Quadratic, $P < 0.001$) and PLC-δ1 (Linear, $P < 0.0001$) in the rat spleens. However, the mRNA expression of splenic PLC-β1 was up-regulated ($P=0.038$) in the LT group compared with the CON group. In the heart, the administration of L-theanine stimulated the mRNA expression of PLC-β1 (Linear, $P < 0.0001$), PLC-γ1 (Linear, $P < 0.0001$; Quadratic, $P=0.015$), and PLC-δ1 (Linear, $P < 0.0001$; Quadratic, $P=0.002$), and were the highest in the HT group.

Discussion

In the current study, we found that the intragastric administration of L-theanine only caused a linear increase of splenic OI in the rats, while other organ indices were not affected, indicating that L-theanine administration increased the splenic weight. Because the spleen is an important immune system organ, we thus inferred that L-theanine probably had the potential to further improve the immune function of rats.

In the immune system, cytokines are important regulatory molecules. Specifically, IL-4 stimulates and activates B lymphocytes,

Table 4. Effects of L-theanine administration on neurotransmitters in the brain tissues and hormones in the serum of rats.

Item	CON	LT	MT	HT	P value	
					Linear	Quadratic
Pituitary						
DA (ng/g)	3.26±2.77 ^d	6.89±5.26 ^c	15.3±4.56 ^a	11.3±2.94 ^b	<0.0001	<0.0001
5-HT (ng/g)	10.9±1.47 ^b	11.6±1.60 ^{ab}	13.0±2.87 ^a	11.4±1.12 ^b	0.408	0.005
Hippocampus						
DA (ng/g)	1.99±1.53 ^b	2.09±1.77 ^b	3.55±0.32 ^a	3.96±0.84 ^a	<0.0001	0.202
5-HT (ng/g)	14.9±7.63 ^b	14.6±5.91 ^b	17.3±2.11 ^b	20.7±6.67 ^a	0.003	0.764
Hypothalamus						
DA (ng/g)	17.2±3.62	17.3±3.82	17.5±1.56	18.8±4.09	0.333	0.878
5-HT (ng/g)	13.1±2.65 ^{ab}	14.7±3.24 ^a	11.4±2.69 ^{bc}	10.3±3.73 ^c	0.002	0.930
Serum						
EPI (pg/ml)	13.1±5.9 ^b	15.2±6.0 ^b	13.0±5.9 ^b	25.9±20 ^a	0.007	0.103
CORT (ng/ml)	147±87 ^a	39.4±41 ^b	21.2±18 ^b	64.6±50 ^b	0.013	<0.0001

DA – dopamine; 5-HT – serotonin; EPI – adrenaline, CORT – corticosterone; CON – control group; LT – low-dose L-theanine administration group; MT – middle-dose L-theanine administration group; HT – high-dose L-theanine administration group.
^{a-d} Means in the same row not bearing a common superscript letter differ ($P<0.05$).

Table 5. Effects of L-theanine administration on the mRNA expression of PLC- γ 1, δ 1, and β 1 genes in the liver, spleen, and heart of the rats.

Item	CON	LT	MT	HT	P value	
					Linear	Quadratic
Liver						
PLC- β 1	1.00 ^a	0.32±0.3 ^b	0.45±0.4 ^b	0.52±0.5 ^b	0.062	0.002
PLC- γ 1	1.00 ^a	0.12±0.1 ^d	0.51±0.4 ^b	0.39±0.3 ^c	0.007	0.010
PLC- δ 1	1.00 ^a	0.15±0.1 ^c	0.62±0.6 ^b	0.35±0.2 ^c	0.055	0.355
Spleen						
PLC- β 1	1.00 ^b	2.07±1.9 ^a	1.17±0.9 ^{ab}	0.6±0.3 ^b	0.088	0.588
PLC- γ 1	1.00 ^a	0.30±0.2 ^b	0.37±0.2 ^b	0.28±0.2 ^b	<0.0001	<0.0001
PLC- δ 1	1.00 ^a	0.31±0.1 ^c	0.55±0.5 ^b	0.34±0.1 ^c	0.0001	0.158
Heart						
PLC- β 1	1.00 ^c	2.72±1.9 ^b	2.92±1.5 ^b	6.45±2.3 ^a	<0.0001	0.184
PLC- γ 1	1.00 ^c	3.1±1.6 ^b	4.93±1.4 ^a	6.04±2.1 ^a	<0.0001	0.015
PLC- δ 1	1.00 ^c	6.53±2.0 ^b	7.98±3.5 ^{ab}	10.1±3.7 ^a	<0.0001	0.002

PLC – Phospholipase C; CON – control group; LT – low-dose L-theanine administration group; MT – middle-dose L-theanine administration group; HT – high-dose L-theanine administration group. ^{a-d} Means in the same row not bearing a common superscript letter differ ($P<0.05$).

promotes the proliferation and differentiation of T lymphocytes, and thereby regulates humoral immunity [27]. IL-6 level reflects the degree of tissue damage [28]. IL-10 can inhibit the activation of T lymphocytes and NK cells activity and subsequently decrease cytokines secretion (e.g., IL-2 and IFN- γ), finally suppressing cellular immune response [29]. IFN- γ promotes B lymphocytes to secrete IgG, while it inhibits the secretion of IL-4-induced IgG and IgE [30]. In our study, the IFN- γ level in the serum of the HT group was increased compared to the MT group. L-theanine administration resulted in linear decrease in IL-4, IL-6, and IL-10 in the serum. These results indicate that L-theanine can alleviate the inflammatory response to Gram-negative bacteria infection and regulate IL-4-, IL-6-, and IL-10-mediated humoral immune response. These results were not consistent with those of previous studies. Hwang et al. [11] reported that weaning piglets administered L-theanine (80 mg/kg feed) did not show differences in the levels of IFN- γ and IL-10 *in vivo* regardless of LPS treatment. Kamath et al. [2] reported that delivering 190 mg of L-theanine per day in humans increased the capacity of $\gamma\delta$ T cells to secrete IFN- γ by up to 15-fold in response to challenge with ethylamine or dead bacteria. The differences between our study and previous studies appear to be due to the animal species and the dosage of L-theanine administered.

In healthy mammals the ratio of T-helper lymphocytes Th2/Th1 is kept in balance, which is characterized by IL-4/IFN- γ [31]. Under the impacts of various antigens, cytokines, antigen-presenting cells, and other factors, the Th2/Th1 balance might be upset, shifting towards the conversion of Th1 or Th2 status, termed Th1 or Th2 drift [32]. As a result, the immune homeostasis of cytokines networks is damaged, causing further changes in immune status, as well as the emergence and development of many diseases [33]. In our study, we found that the intragastric administration of L-theanine dramatically reduced the ratio of Th2/Th1 in the serum of the rats and led to a shift in the Th2/Th1 balance towards Th1, enhancing resistance to pathogens [34]. Our results agree with the findings of Kurihara et al. [7], in which the serum Th2/Th1 ratio was decreased by L-theanine administration at 6 h and 24 h, respectively, after antigenic stimulation in mice.

Neuroendocrine-immune network-related studies [35,36] have confirmed that the neuro-endocrine system and the immune system can share cytokines, hormones, and neurotransmitters and generate extensive and close contact. Hormones secreted by the neuroendocrine system can regulate immune function, and immune response can also change the of neuroendocrine system. The results of this study showed that after intragastric administration of L-theanine to the rats for 2 weeks continuously, the secretion levels of DA and 5-HT were elevated to some extent in the pituitary and hippocampus. These results further validated findings of a previous report in which

Peng et al. [14] demonstrated that the gastric perfusion of 20 and 100 mg/kg L-theanine both increased the content of 5-HT in the hippocampus and prefrontal cortex of rats. Yamada et al. [16] reported that DA and 5-HT were significantly increased in the brain tissues of one-week-old rats whose mothers had been fed 2% L-theanine in water *ad libitum* while pregnant. In addition, we noted that the serum CORT content decreased with low-dose and middle-dose administration of L-theanine. This result was in agreement with previous reports in which weaning rats [17,18] or their dams [18] fed 0.3% L-theanine in water for 3 weeks had lower CORT level in the serum of the weaning rats. However, our results are not consistent with the findings of Yamada et al. [37], who reported that oral L-theanine administered at 4 g/kg BW/day increased the CORT concentration in the blood of rats (BW, 60–280g; 7–8 weeks old). In our opinion, this discrepancy can be ascribed to the different L-theanine doses, route of administration, and growth stage of experimental rats. Therefore, our results suggest that L-theanine can promote the secretion of monoamine neurotransmitters (DA and 5-HT) in the pituitary and hippocampus, and change CORT secretion in serum of rats. Further research is needed to explore the effect of L-theanine on interactions between neuroendocrine and immune systems.

In this study, L-theanine decreased the mRNA expression of PLC- β 1, PLC- γ 1, and PLC- δ 1 in the liver and PLC- γ 1 and PLC- δ 1 in the spleen in a dose-dependent manner. The results indicate that L-theanine inhibited the PLC-signal pathway in liver and spleen. In the spleen, 50 mg/kg L-theanine administration stimulated the PLC- β 1 gene expression. Because the spleen is where B and T lymphocytes aggregated, we inferred that L-theanine might promote the proliferation and differentiation of lymphocytes by upregulating PLC- β 1 gene expression. Our current results showed that L-theanine administration increased the mRNA expressions of PLC- γ 1 and PLC- δ 1 in a dose-dependent manner in the heart. Because PLC- γ 1 is involved in the regulation of the cell cycle [38], PLC- δ 1 protein coded by the PLC- δ 1 gene played important roles in the regulation of Ca²⁺ homeostasis and preventing injury to cardiomyocytes [39], we inferred that L-theanine could promote the growth of cardiomyocytes and protect the heart. PLC- β 1 expression was also increased in the rat hearts, indicating that the PLC- β 1 gene might be involved in protection of cardiomyocytes. In our study, the expressions of PLC isomers were different in liver, spleen, and heart, indicating that L-theanine has tissue-specific biological effects in rats and that L-theanine administration regulates the PLC-related pathway. The relationship between PLC isomers and cytokine secretion regulated by L-theanine has not been investigated; therefore, there is a topic needing further investigation.

Conclusions

Intra-gastric administration of 400 mg/kg L-theanine for 2 weeks improved immune function and resistance to stress in rats by increasing the splenic weight, elevating the secretion of IFN- γ in the serum and DA and 5-HT in the pituitary and hippocampus, altering the balance of Th2/Th1, inhibiting the

CORT level in the serum, and up-regulating the mRNA expression of PLC in the heart.

Competing interests

All authors have no conflicts of interest to disclose.

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