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Short communications

Theanine improves stress resistance in *Caenorhabditis elegans*

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ABSTRACT

The beneficial effects of theanine in tea are reported on many aspects. Here we report that theanine can extend nematode *Caenorhabditis elegans* lifespan under stress. Theanine (100 mg/mL) significantly increased the mean longevity of *C. elegans* by 12.8% and 21.3% under heat stress and oxidative stress, respectively. However, theanine treatment did not increase survival of *C. elegans* under normal culture condition. Further studies showed that theanine mediated lifespan extension under heat stress was involved in heat shock protein-16.2 (HSP-16.2) up-regulating expression.

1. Introduction

Owing to the delicate taste and the ability to alleviate astringency, theanine plays an important role in determining the quality and characteristics of green tea (Nakagawa, 1975). In addition to the beneficial effects such as enhancement of relaxation and improvement of concentration and learning ability (Egashira et al., 2008; Gomez-Ramirez, Kelly, Montesi, & Foxe, 2008; Juneja, Chu, Okubo, Nagato, & Yokogoshi, 1999; Yamada et al., 2008), theanine has been found beneficial in many aspects including the prevention of certain cancers (Sadzuka, Sugiyama, Miyagishima, Nozawa, & Hirota, 1996) and regulation of blood pressure (Yokogoshi et al., 1995), promotion of weight loss (Zhent et al., 2005) and enhancement of the performance of the immune system (Bukowski & Percival, 2008). It is also linked with neuroprotective effects such as Parkinson disease and Alzheimer disease (Di et al., 2010; Il Kim et al., 2009; Yamada et al., 2009). Recently it has been reported that theanine may inhibit nicotine addiction and possess the antidepressant effect (Egashira et al., 2008; Yan et al., 2010; Yin et al., 2011).

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However, only a few studies have reported on the beneficial properties of theanine on stress resistance. In an earlier study, Kimura, Ozeki, Juneja, and Ohira (2007) reported theanine reduced psychological and physiological stress responses. Unno et al. (2011) suggested in a recent study that theanine intake could improve the shortened lifespan, cognitive dysfunction and behavioral depression that were induced by chronic psychosocial stress in mice.

The stress resistance effects of theanine in the model organism *Caenorhabditis* elegans was investigated in this work. We found that, although theanine could not extend *C. elegans* lifespan under normal culture condition, it could improve the longevity of *C. elegans* under heat and oxidative stress conditions. Furthermore, theanine mediated lifespan extension under heat stress was involved in heat shock protein-16.2 (HSP-16.2) up-regulating expression.

2. Materials and methods

2.1. Reagents and worm strains maintenance

FUDR (5-fluoro-2′-deoxyuridine) and juglone (5-hydroxy-1,4-naphthoquinone) were obtained from Sigma (St. Louis, MO). Theanine was prepared from green tea followed by isolation and purification (Zhang, Chen, Huang, & Shi, 2004). Unless stated otherwise, the worms were cultured at 20 °C on nematode growth medium (NGM) plates seeded with live bacteria (*Escherichia coli*, strain OP50) as food. Bristol N2 (Caenorhaditis Genetics Center; CGC) was used for lifespan assays as well as most stress resistance assays. The transgenic CL2070 (dvIs70) strain, containing a HSP-16.2::GFP fusion protein, obtained as a generous gift from Y. Luo, Ph.D. (Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, College Park, MD, USA), was used to visualize HSP-16.2 expression.

2.2. Lifespan assays and stress resistance

Lifespan assays were performed *C. elegans* wild type N2 at 20 °C. Synchronized worms were prepared by alkaline hypochlorite method (Sulston & Brenner, 1974). The animals were transferred to treatment plates when the young adults began to lay eggs of the indicated genotypes. The worms were then transferred to fresh treatment plates every 3 days of the assays. Treatment plates were prepared using standard NGM with the reproductive suppressant 100 mg/L FUDR for the first 6 days and theanine of various concentrations. Theanine at 50, 100, or 200 μg/mL was diluted into live *E. coli* OP50 suspension and added to the surface of the NGM plates to the indicated final concentrations. Animals were examined every day and scored as dead when they no longer responded to light touch stimulus with a platinum wire.

Thermo-tolerance assays were performed with 2-day-old adults followed by heat stress (treatment at 25 or 30 °C for half an hour and then 35 °C). Juglone-induced oxidative stress assays were performed with 2-day-old adults at 20 °C as for aging assays, except which juglone was added to NGM medium at a final concentration of 500 μmol/L. The number of dead worms for stress resistance was counted and recorded every hour.

For all the above assays, every experiment was repeated three times and conducted in a double-blind manner.

2.3. Fluorescence microscopy and quantitation of hsp-16.2 expression

CL2006 worms were cultivated and maintained at 20 °C, using *E. coli* (OP50) as a food source. The worms were treated with or without theanine on the day before hatching for 48 h. The heat stress resistance was followed with treatment at 25 or 30 °C for half an hour and 35 °C for an hour. After recovery for 24 h, the expression of hsp-16.2 was measured by observing the fluorescence of the reporter protein GFP. The overall fluorescence of GFP-expressing populations was assayed using a Thermo Labsystems Fluoroskan Ascent microplate (Thermo Fisher, Waltham, MA). Twenty control or treated adult animals of the indicated age were transferred in 100 μL of PBS to a well of a Costar 96-wellmicrotiter plate (black, clear, flat-bottom wells), and total GFP fluorescence was measured using 485 nm excitation and 530 nm emission filters.

Quadruple populations were used for each determination. For fluorescence microscopy, the worms were mounted with a drop of levamisole (10 mmol/L) and placed on a cover slip covered with 3% agarose. The GFP pictures of transgenic worms were taken using an Olympus FluoView IX71 microscope (Olympus Corporation, Tokyo, JP).

2.4. Western blot analysis

Following heat shock treatments, the worms were collected from plates by washing with M9 buffer and subsequent centrifugation. After freezing and thawing for three times, pellets debris were boiled at 100 °C for 15 min in sample loading buffer and stored at −70 °C until electrophoresis. The worm lysate was electrophoresed on a 12% SDS–polyacrylamide gel and electro-blotted onto a PVDF membrane (Millipore, Billerica, MA). The membrane was incubated in a blocking solution containing hsp-16.2 antibody (1:500 dilution; a gift from Y. Luo of the University of Maryland, College Park, MD, USA) or actin antibody (1:1000 dilution; Abcam, UK.) at 4 °C overnight, followed by incubation with anti-rabbit horseradish peroxidase antibody (1:5000 dilution; Zhongshan Goldernbridge Biotechnology Co. Ltd., Beijing, CN) for 1 h at 25 °C. The Chemilucent ECL Detection System (Millipore, Billerica, MA) was used to detect the secondary antibodies on the membrane. Mean densities of the hsp-16.2 were analyzed by the FluorChem HD2 gel documentation system (Alpha Innotech, Imgen Technologies, CA, USA).

2.5. Statistical analysis

The data of the lifespan assays and stress resistance assays were processed using the Kaplan–Meir survival analysis of SPSS 13.0 and compared among groups scoring for significance using the log-rank test. Differences between untreated and heat shock treated groups were analyzed for significance using one-way ANOVA (followed by the Tukey test) in the Origin 7.5 software. Data are presented as mean ± SEM. *P*-value <0.05 is considered statistically significant.

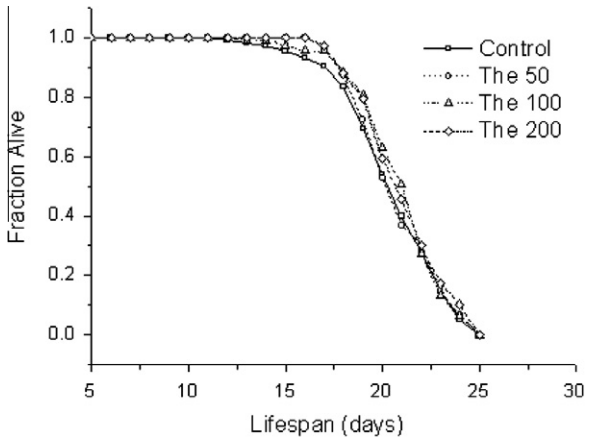

Fig. 1 – Effect of theanine on the lifespan of *C. elegans* under normal culture condition. Survival rate was scored every day. The data were processed with Kaplan–Meir survival analysis (log-rank test). Compared with the control (*N* = 120), theanine did not significant increase the mean lifespan of wild type *C. elegans* N2 at concentrations 50 μg/mL (*N* = 123), 100 μg/mL (*N* = 115) and 200 μg/mL (*N* = 117).

3. Results

3.1. *Theanine cannot significantly extend the lifespan of wild-type C. elegans N2 under normal culture condition*

To determine whether theanine would affect the lifespan of *C. elegans*, synchronized populations of worms were exposed to different concentrations of theanine. Compared with the control, the theanine treated groups at 50, 100 and 200 μg/mL did not associate with any increase in survival of *C. elegans* at 20 °C, as shown in Fig. 1. From this experiment, we conclude that theanine treatment in concentrations ranging from 50 to 200 μg/mL shows no significant benefits on the mean lifespan of *C. elegans* under normal culture condition.

3.2. *Theanine improves stress resistance of C. elegans under stress conditions*

To address the question whether theanine is able to enhance resistance against environmental stress in *C. elegans*, severe stress assays were exposed to a heat shock at 35 °C and 500 μmol/L juglone-induced oxidative stress. In the thermotolerance assay, the worms that had just reached adulthood were pretreated with theanine (100 μg/mL) for 48 h before being exposed to heat shock at 35 °C. The data showed that 100 μg/mL theanine treatment can significantly increase the mean survival rate of the worms by 12.8% (Fig. 2A).

In addition, theanine treatment also improved survival under oxidative stress. Resistance to oxidative stress was examined by exposing animals to juglone, an intracellular free-radical-generating compound. According to the statistical results, the survival rate was significantly increased by 21.32% in the theanine-treated groups compared with the control (Fig. 3A).

Together with these findings, we conclude that although theanine could not increase survival of *C. elegans* under normal culture conditions, it really improve the stress resistance of *C. elegans*.

3.3. *Theanine enhance expression of small heat shock proteins (sHSP16)*

HSP is considered as a very important chaperone protein involved in stress resistance. In order to elucidate whether theanine regulate a specific stress response gene, the transgenic *C. elegans* (CL2070) was used. In this strain, the gene coding for green fluorescent protein (GFP) was fused to hsp-16.2 gene. In this way GFP is used as a reporter of hsp-16.2 expression. Figure 3A shows the images of hsp-16.2/GFP worms induced by a heat shock treatment (temperature shift from 20 to 35 °C, 25 and 30 °C for half an hour and then 35 °C for 1 h, then recover at 20 °C for 12 h). After induction by thermal stress, the expression of hsp-16.2 induced by heat shock was significantly enhanced in CL2070 worms fed with

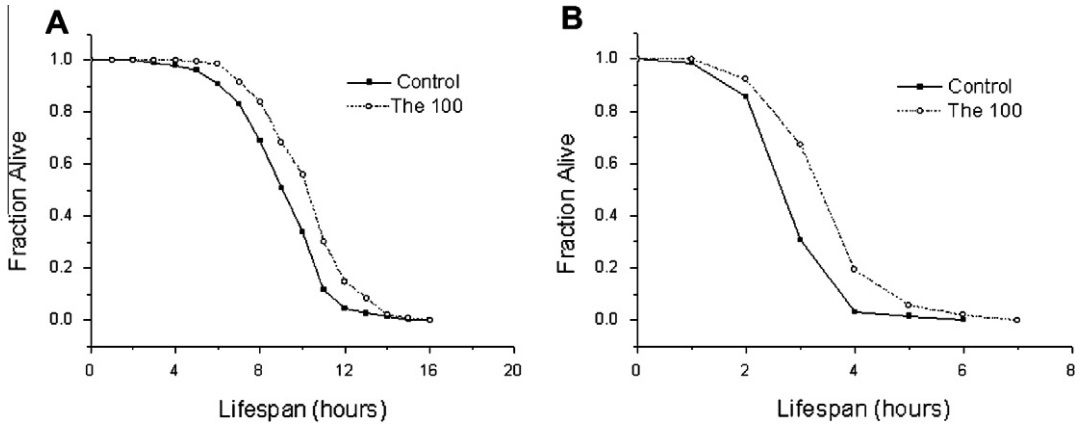

Fig. 2 – Effect of theanine on the lifespan of *C. elegans* under heat stress and oxidative stress. Survival rate was scored every hour. The data were processed with Kaplan–Meir survival analysis (log-rank test). (A) At 35 °C, compared with the control (*N* = 155), theanine extended the worm longevity by 12.8% at 100 μg/mL (*N* = 143), *P* < 0.001. (B) Under oxidative stress, compared with the control (*N* = 60), theanine extended the worm longevity by 21.32% at 100 μg/mL (*N* = 57), *P* < 0.001.

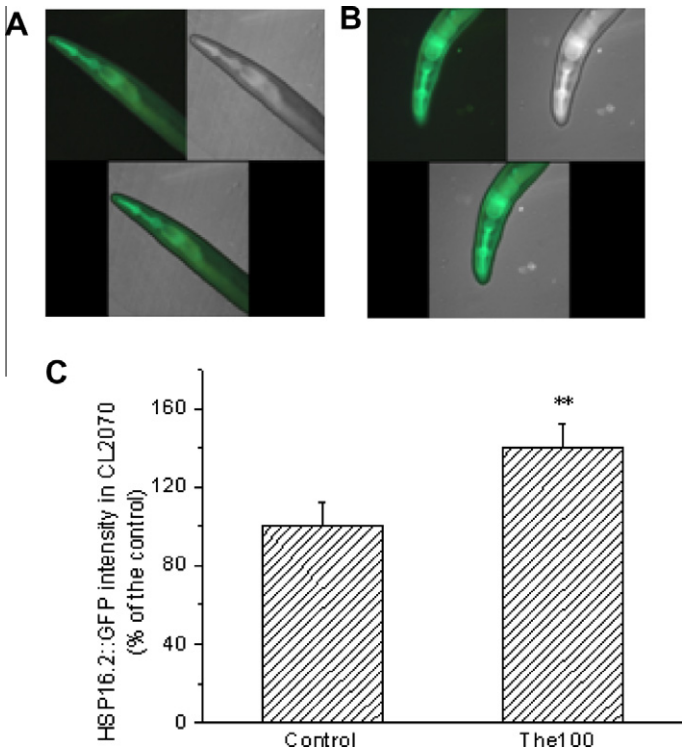

Fig. 3 – Effects of theanine on the expression of heat shock protein HSP-16.2 in CL2070 under heat stress. After treated with theanine 2 d, the CL2070 worm was heated at 35 °C under stress and incubated 24 h. (A) Image of HSP-16.2::GFP expression in the control worms. (B) Image of HSP-16.2::GFP expression in the 100 μg/mL theanine-treated group. The GFP pictures of living transgenic worms were taken on an Olympus FluoView IX71 microscope. (C) Quantified HSP-16.2::GFP intensity with 20 worms in each experiment, detected on a Thermo Labsystems Fluoroskan Ascent microplate reader(means ± S.E., N = 4). The HSP-16.2::GFP expression in theanine-treated worms (B) is higher than that in control worms (A). Compared with the control, theanine increased the expression of HSP-16.2 by 40.2% at 100 μg/mL, $P < 0.01$.

theanine. It is visible at the head of the living animal, including the pharynx and the anterior nerve ring.

The intensity of the fluorescence was quantified by a Thermo Labsystems Fluoroskan Ascent microplate reader. As is shown in Fig. 3B, theanine could significantly up-regulate HSP-16.2::GFP expression by 40.2% in CL2070 ($P < 0.01$ compared with the control). The results showed that theanine was able to induce the synthesis of GFP, indicating their ability to activate the heat shock promoter.

To further validate the expression of HSP-16.2 gene in *C. elegans*, the wild-type *C. elegans* N2 was used for quantification using west blotting. A notable enhancement of HSP-16.2 expression fed with theanine was observed in the wild-type worms following heat shock 35 °C for 1 h (Fig. 4).

Therefore we conclude that theanine could increase the life expectancy of *C. elegans* by up-regulating the expression of the HSP-16.2 gene.

4. Discussion

In previous researches, theanine, a non-protein amino acid that occurs naturally in the tea plant (*Camellia sinensis*), has been mostly used for the memory improvement and relaxation properties, little is known for the stress resistance effect. Since the age-related morbidity and mortality in a population is closely associated with the cause from environmental

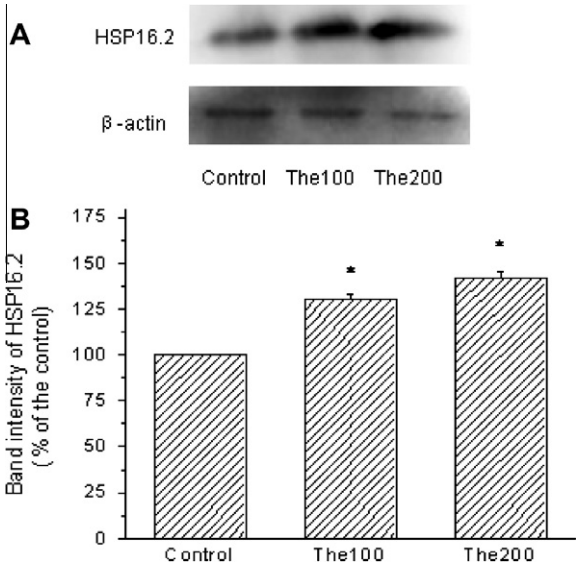

Fig. 4 – Effects of theanine on HSP-16.2 expression of *C. elegans* N_2 under heat stress with west-blotting (after treated with theanine 2 d, the CL2070 worm was heated at 35 °C under stress and incubated 24 h. Compared with β-actin, the statistic results of band intensities were expressed as ratio ± S.E., N = 3. Compared with the control, theanine increased the expression of HSP-16.2 by 39.6% at 100 μg/mL and 44.6% at 200 μg/mL, $P < 0.05$.

stresses, the stress resistance effects of theanine was investigated in the present paper.

Using the short-lived nematode *C. elegans*, we found an increase in stress resistance was observed when worms were incubated in presence of theanine. In order to delineate the antistress mechanisms of theanine, the up-regulating expression of HSP-16.2 gene was investigated in this study.

C. elegans is a well popular model for studying aging and longevity due to the short lifespan, rapid generation time and experimental flexibility (Guarente & Kenyon, 2000). In addition, *C. elegans* share the high conservation of biochemical pathways and the similarity of many aspects of aging from worms to humans (Kenyon, 2010). There are many previous literatures showed that natural products and herbal formulas occurring plant could increase lifespan and slow aging-related decline in *C. elegans* (Fan et al., 2011; Wiegant et al., 2009; Wilson et al., 2006; Yu et al., 2010). Given this evidence, we propose the model for theanine's effects on *C. elegans* lifespan. However, the results showed that theanine could not delay animals survivals under normal culture condition (Fig. 1).

Interestingly, theanine is able to enhance resistance against environmental stress such as heat and oxidative stress (Fig. 2). This is a significant finding that provides a support to previous experiments showing that theanine intake could improve the shortened lifespan that are induced by chronic psychosocial stress in mice.

A strong transcriptional up-regulation of HSP-16 is commonly observed in response to stress, which subsequently can serve as a stress-sensitive reporter to predict longevity in *C. elegans* (Hsu, Murphy, & Kenyon, 2003). In this paper, the transgenic strain CL2070 was used containing a construct in which the gene coding for GFP was coupled to the promoter of the hsp-16 gene (Link, Cypser, Johnson, & Johnson, 1999). This transgene animal allows visualization of the stress response in vivo in which the amount of synthesized GFP is used as a measure of experienced stress. Our results demonstrate that theanine were able to significantly induce the synthesis of GFP, indicating the ability to activate the heat shock promoter (Fig. 3). And it was also validated by west-blotting in wide-type *C. elegans* N2 (Fig. 4).

In conclusion, the theanine's longevity-improving effects in *C. elegans* under stress may be attributed to up-regulating stress resistance-associated gene hsp-16.2. A better understanding of the mechanisms would be considered for further investigation in the future.

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