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Theanine supplementation prevents liver injury and heat shock response by normalizing hypothalamic-pituitaryadrenal axis hyperactivity in mice subjected to whole body heat stress

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ABSTRACT

Thermal stress evokes heat shock response and activates hypothalamic-pituitaryadrenal (HPA) axis. Additionally, liver injury is an important adverse effect of thermal stress. Considering the anti-stress effects of theanine, an amino acid found in tea plants, we hypothesized that theanine may protect against heat stress. Mice were administered intragastrically with theanine prior to whole body thermal exposure. Theanine prevented the heat-induced upregulation of heat shock proteins and reduced the heat-induced liver damage and oxidative stress. Theanine significantly prevented the heat-induced effects on inflammatory and acute phase responses as measured by plasma inflammatory cytokine concentrations, hepatic inflammatory cytokine mRNA levels, plasma nitric oxide and C-reactive protein levels. Additionally, theanine supplementation suppressed heat stress-related disorders associated with normalizing HPA axis hyperactivity. These findings suggest that theanine can have beneficial effects against heat stress and may be an attractive dietary application for people who are at high risk of developing heat stress.

1. Introduction

Thermal stress is typically defined as heat stress, which affects organ function and contributes to the occurrence of a systemic inflammatory response and acute-phase response (Leon, Dineen, Blaha, Rodriguez-Fernandez, & Clarke, 2013). Severe hyperthermia causes thermoregulatory failure and dysregulation of the acute-phase response may contribute to the progression of heat stress into heatstroke (Bouchama & Knochel, 2002). Heatstroke is a fatal disorder characterized by critical hyperthermia, which is associated with systemic inflammatory responses that result in multiple organ dysfunctions, including delirium, convulsion, or coma (Chen, Lin, & Chang, 2013). After the onset of heatstroke, the reduction in blood flow to the brain results in hypothalamic neuronal damage, which induces central nervous system disturbances or multi-organ system injury (Hsuan, Lin, Chang, & Lin, 2016). Heat-induced multi-organ injury includes acute renal failure, gut ischaemia, skeletal muscle and liver injury (Leon & Helwig, 2010). In the Southern Hemisphere, summer temperature can reach 35 °C or higher, causing heat stress to both humans and animals. Heat stress and related illnesses are major concerns in the military, sports, and workers in hot environments. The correction or prevention of whole body heat stress is therefore of major public health importance.

The hormonal response to heat stress has been considered an important mechanism to maintain homeostasis after an unpleasant physical or psychological experience. Heat stress elicits an abundance of neuroendocrine responses, including activation of the hypothalamic–pituitaryadrenal (HPA) axis and release of glucocorticoids (CORT) (Vargas & Marino, 2016). Neuroendocrine-immune interactions are

Abbreviations: ACTH, adrenocorticotropic hormone; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; CORT, glucocorticoids; COX-2, cyclooxygenase-2; CRP, C-reaction protein; GSH, glutathione; HO1, heme oxygenase-1; HPA, hypothalamicpituitaryadrenal; HSPs, heat shock proteins; IL, interleukin; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; NO, nitric oxide; NQO1, NAD(P)H: quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; TNF-α, tumor necrosis factor-α; T-SOD, total superoxide dismutase

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profoundly regulated by CORT, the effects of heat stress on inflammatory/immune responses occur via the activation of the HPA axis (Webster, Elenkov, & Chrousos, 1997). Although the acute activation of the HPA axis in response to stressful stimuli represents an important mechanism to promote survival, however, a prolonged increase in plasma CORT concentrations leading to induction of HPA axis hyperactivity has deleterious consequences for an organism (Chrousos, 2009). The occurrence of HPA axis hyperactivity is a critical step in the pathological development of heatstroke, which is due to the disruption of the negative feedback mechanism after stress exposure (Dallman et al., 1994). Inflammation and heat shock response are hallmarks of heat stress, with elevations in levels of inflammatory cytokines and mediators, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and heat shock proteins (HSPs) (Akerfelt, Morimoto, & Sistonen, 2010; Chang, 1993; Tseng, Chen, Lin, & Lin, 2014).

With the prospect of increasing global warming and the frequency and intensity of heat stress (Bouchama & Knochel, 2002), it is important to investigate preventive measures that can alleviate adverse effects of exposure to high environmental temperatures. Theanine (γ-glutamylethylamide), a unique nonproteinic amino acid found in the tea plant, is gaining attention and can be considered as a functional food ingredient due to its multiple beneficial effects (Kakuda, 2011; Türközü, & Şanlier, 2017; Wang, Gao, Wang, Qian, & Wang, 2017). Theanine as a dietary supplementation has generally been recognized as safe by the US Food and Drug Administration. Recent studies have shown that arginine or glutamine supplementation is associated with preventing severe hyperthermia and heat-related disorders in mice (Costa et al., 2014; Soares et al., 2014). However, it is unknown whether theanine has a similar protective effect against heat stress-induced pathological changes. The aim of this study is to investigate the effects of theanine on heat stress-induced liver injury and heat shock response as well as the underlying mechanisms in mice.

2. Materials and methods

2.1. Solution preparation

L-Theanine (CAS: 3081-61-6) with the purity of 99% was purchased from Yibeijia Tea Technology, Inc. (Hangzhou, China). In this study, theanine will refer specifically to L-theanine. Solutions with concentrations of 10 mg/L 10 mg/mL and 20 mg/mL theanine were prepared, and the solvent used is physiological saline.

2.2. Animals and treatments

Healthy male ICR mice (8 weeks, 20–22 g) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The animals were provided with food and water *ad libitum* and were housed in a controlled environment at 22 ± 2 °C with 40–60% humidity and a 12-h light–dark cycle. All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China), and animal experimental protocol was reviewed and approved by the ethics committee of Anhui Agricultural University (ethical approval code: AHAU 2016-125). A simple mouse model of whole body heat stress was exposed to the elevated ambient temperature (42 °C) with relative humidity of 60% for 2 h in a heating chamber at 9:00 a.m. The mice were randomly divided into the following four experimental groups: (i) control, (ii) heat stress (saline only), (iii) heat stress + 100 mg/kg theanine, and (iv) heat stress + 200 mg/kg theanine (n = 6–8 per group). Prior to heat exposure, the mice in the two intervention groups, theanine (100 or 200 mg/kg b.w.) was administered intragastrically to mice once a day over a 7-day period. After the last pre-treatment with theanine, three heat exposure groups were subjected to whole body heat stress immediately. At the end of thermal stress exposure, the mice were anesthetized and sacrificed by cervical dislocation at 11:00 a.m. Plasma was obtained through centrifugation of blood samples at 3000 rpm for 10 min and stored at −80 °C until analysis. The livers were excised, sectioned transversely or longitudinally, and fixed in 10% (v/v) neutral-buffered formalin, and the remaining tissue was rinsed in ice-cold phosphate-buffered saline and then stored at −80 °C.

2.3. Measurements of plasma aminotransferase levels

Plasma enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) level were determined by the corresponding commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturer's protocols.

2.4. Histopathological analysis

Histopathological changes of liver were observed by haematoxylin and eosin (H&E) staining as previously described (Wang et al., 2015). In brief, the fixed tissues were dehydrated in graded ethanol and embedded in paraffin. Paraffin sections (4 μm) were stained with H&E using a standard protocol. The stained specimens were examined and recorded by a light microscopy with camera (Olympus, Tokyo, Japan) by two pathologists in a blinded fashion.

2.5. Determination of hepatic antioxidant status

Liver tissue was homogenized in ice-cold 150 mM pH 7.2 phosphate buffer solution containing 1 mM $EDTANa_2$ to prepare 10% liver homogenate. The homogenate was centrifuged at 15,000*g* for 15 min at 4 °C to remove cell debris and nuclei, and the resulting supernatant was used to determine the total superoxide dismutase (T-SOD) activity, catalase (CAT) activity, and glutathione (GSH) level per the protocol described in our previous study (Wang, Wang, Wan, Yang, & Zhang, 2015). Malondialdehyde (MDA) content was determined according to the method of Ohkawa, Ohishi, & Yagi (1979). The level of MDA was expressed as nmol/mg protein. Protein levels were determined by Bradford assay with bovine serum albumin as a standard. Values were normalized to hepatic total protein.

2.6. Measurements of plasma cytokines

Plasma levels of IL-1β, TNF-α and IL-6 were determined using the corresponding commercial mouse ELISA kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol.

2.7. Determination of plasma NO and CRP levels

Nitric oxide (NO) production in plasma was measured using the corresponding commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as nitrite–nitrate and was performed according to the protocols provided by the manufacturer, using the Griess Reagent. Plasma C-reaction protein (CRP) level was measured based on antigen-antibody specific agglutination using the corresponding commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and was performed according to the protocols provided by the manufacturer.

2.8. Quantitative RT-PCR

Total RNA was isolated from liver tissue using TRIzol reagent, and reverse-transcribed using PrimeScript™ RT Reagent Kit (Takara Biotechnology, Dalian, China). The gene-specific primers listed in Table 1 were designed from National Center for Biotechnology Information (US National Library of Medicine, www.ncbi.nlm.nih.gov/nuccore/), and synthesized by Generay Biotechnology (Shanghai,