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L-Theanine prevents ETEC-induced liver damage by reducing intrinsic apoptotic response and inhibiting ERK1/2 and JNK1/2 signaling pathways

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ABSTRACT

L-Theanine (LTA; γ-glutamylethylamide), a peculiar non-protein-derived amino acid isolated from tea, is widely used as a functional ingredient and dietary supplement. L-Theanine has been confirmed to have hepatoprotective effects, but the underlying mechanism remains unknown. This study investigated the protective effect of L-Theanine-in vivo, using an enterotoxigenic *Escherichia coli* (ETEC)-infected mouse model. L-Theanine significantly decreased the elevated serum activities of both aspartate aminotransferase (AST) and alanine aminotransferase (ALT), two biomarkers of hepatic impairment. This was consistent with histopathological images from the microscopic observation of liver tissue. In addition, L-theanine significantly increased the mRNA and protein expression of Bcl-2 and decreased the expression of Bax, anti- and pro-apoptotic molecules, respectively, compared with levels in the ETEC control group. The expression of cleaved caspase-3 protein in the group pre-treated with L-theanine was significantly lower than that in the ETEC group. Additionally, decreases in extracellular signal-regulated kinase (ERK1/2) and c-Jun NH_2-terminal kinase(JNK1/2) MAPK phosphorylation were observed in the L-theanine pre-treated group. Our study demonstrates that L-theanine possesses anti-apoptotic activity, which can be attributed to suppression of the intrinsic mitochondria-mediated apoptosis and MAPK phosphorylation signaling pathways.

1. Introduction

L-Theanine (LTA; γ-glutamylethylamide) is a non-protein-derived amino acid abundantly distributed in tea (*Camellia sinensis*) leaves (Williams et al., 2016). L-Theanine has been extensively studied for its considerable health benefits, including its anti-tumor (Li et al., 2013), neuroprotective (Lardner, 2014; Takehana et al., 2017), antimicrobial (Bansal et al., 2013), immunomodulatory (Kurihara et al., 2013), and hepatoprotective (Perez-Vargas et al., 2016) effects. Because of these benefits, many researchers have undertaken studies to explore the potential applications of L-theanine (Chatterjee et al., 2016; Williams et al., 2016).

Enterotoxigenic *Escherichia coli* (ETEC) are a group of diverse pathogenic microorganisms. ETEC infections are a major cause of almost 400 million cases of diarrhea per year,and they result in large economic losses in the swine industry worldwide (Fairbrother et al., 2007; Walker et al., 2012). While the intestines is the preferential site in which ETEC-induced toxicity develops (Deng et al., 2015; Zanello et al., 2011), ETEC infections are also known to affect liver tissue, resulting in oxidative stress and liver damage, as we have previously reported (Deng et al., 2016). It has been shown that ETEC infections can elevate liver enzyme levels and induce reversible and irreversible liver damage (Yacoub et al., 2017).

Some research has indicated that L-theanine can prevent liver injury (He et al., 2000; Perez-Vargas et al., 2016), mainly by enhancing hepatocyte antioxidant abilities and limiting the inflammatory response and hepatocyte apoptosis (Deng et al., 2016; Jiang et al., 2012; Li et al., 2012). However, the anti-apoptotic effects of L-theanine have not been

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investigated in mouse liver cells exposed to ETEC. We previously reported thatL-theanine modulated the transcription and protein expression of antioxidative enzymes, but the underlying mechanism of L-theanine remains unknown, especially in terms of its effect on apoptosis-related protein expression (Deng et al., 2016).

Oxidative stress and liver damage can induce hepatic apoptosis,and many studies have confirmed that hepatic apoptosis is associated with the activation of mitogen-activated protein kinases (MAPKs) (Ma et al., 2014). MAPKs can be activated by ETEC and are tightly associated with pathological conditions in intestinal epithelial cells (Zanello et al., 2011). We hypothesize that L-theanine exerts its hepatoprotective effects by changing the profiles of apoptosis-related molecules via the MAPK signaling pathway. Thus, in this study, we established an ETEC-induced liver cell apoptosis mouse model to investigate the anti-apoptotic mechanisms of L-theanine. We also determined the biological effects of L-theanine on the intrinsic mitochondria-mediated and MAPK phosphorylation apoptosis pathways and its role in preventing ETEC-induced liver damage.

2. Materials and methods

2.1. Reagents

L-Theanine(purity ≥ 98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The ETEC strainE44813 was provided by the China National Institutes for Food and Drug Control. Aspartate aminotransferase (AST) and alanine aminotransferase(ALT) assay kits were purchased from the Institute of Jiancheng Bioengineering (Nanjing, China). The total protein extraction radioimmunoprecipitation assay (RIPA) kit and bicinchoninic acid (BCA) kit were purchased from Beyotime Institute of Biotechnology Inc. (Jiangsu, China). Polyvinylidene fluoride membranes were purchased from Millipore (Milford, MA, USA). The following antibodies were used in this study: Bcl-2, Bax, cleaved caspase-3, and β-actin from Santa Cruz Biotechnologies (Santa Cruz, CA,USA);extracellular signal-regulated kinase (ERK1/2), p-ERK1/2, c-Jun NH2-terminal kinase (JNK1/2), p-JNK1/2, P38, and p-P38 from Abcam (Cambridge, UK).

2.2. Preparation of ETEC suspensions

ETEC suspensions were prepared by a previously reported method (Deng et al., 2015), and ETEC was cultured in liquid lysogeny broth medium in a shaking incubator at 180 rpm and 37 °C for 12 h. As measured by ultraviolet-visible spectrophotometer (UV-1800, Shimadzu, Japan), the cell density of each ETEC suspension was 0.6×10^8 CFU/ml.

2.3. Experimental animals and experimental design

All experimental procedures and animal care were designed strictly according to the animal care and use standards of Hunan Agriculture University (registry number:015063506). Female specific pathogen-free (SPF)Balb/c mice weighing 21 ± 2 g were purchased from Hunan SJA Laboratory Animal Company (Changsha, Hunan, China). All mice were acclimatized for 1 week before the experiment was conducted and housed in a clean room at 20–25 °C with a relative humidity of 50–70% and a 12-h light/dark cycle. They were allowed free access to regular tap water and animal feed.

All 60 mice were randomly divided into six treatment groups(n = 10 mice/group): normal control, ETEC control, LTA-100 mg/kg + ETEC, LTA-300 mg/kg + ETEC, LTA-500 mg/kg + ETEC, and 5 mg/kg dexamethasone as a positive control. L-Theanine and dexamethasone were adjusted to specific concentrations using physiological saline. In the normal control and ETEC control groups, physiological saline was injected by lavage once a day for 30 days. In the LTA + ETEC and the dexamethasone groups, the given concentrations of L-theanine and dexamethasone were injected by lavage once a day for 30 days. The volume was fixed at 0.3 ml based on the body weight of the mice. The mice were fasted for 12 h after the last lavage administration and were intraperitoneally administered the prepared ETEC suspension(0.2 ml/10 g BW, 0.6×10^8CFU/ml), except for in the normal control group. After 5 h, all animals were euthanized by sodium pentobarbital anesthesia. Liver samples were collected immediately and washed with 4 °C saline, then quick-frozen with liquid nitrogen and stored at –80 °C until further use.

2.4. Biochemical determination

Blood was collected through the extirpation of mouse eyes, and the serum fraction was separated by centrifugation. The activities of AST and ALT were measured using spectrophotometric kits in accordance with the manufacturer's instructions.

2.5. Histopathological examination

Histopathological examination was performed on liver tissue. Livers were dissected and preserved in 10% formaldehyde for 24 h, and then samples were embedded in paraffin and stained with hematoxylin and eosin. All sections were observed under a Leica light microscope (Leica Microsystems AG, Wetzlar, Germany)

2.6. Real-time PCR

Total RNA was extracted from liver samples, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's specifications. The integrity of total RNA was examined by 1% agarose gel electrophoresis, and the concentration was measured by ultraviolet spectrophotometer (UV-1800, Shimadzu, Japan). cDNA was synthesized with a Prime Script RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions.

The expression of targeted genes was analyzed by real-time quantitative PCR using SYBR Green fluorescence dye (Takara, Dalian, China). Briefly, each 10-μL reaction included 5 μL of SYBR Green mix, 2 μL of tenfold diluted cDNA template, 0.5 μL each of the forward and reverse primers, and 2 μL distilled water. Reactions were conducted on an ABI 7900 HT sequence detection system(Applied Biosystems, Carlsbad, CA, USA). The following cycling conditions were used: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s for amplification and quantification, respectively. Synthetic oligonucleotide primers used in this study to examine the expression levels of targeted genes were designed using Primer5 software (Premier Co., Edmonton, Canada) and are listed in Table 1. The threshold cycle (Ct) value of each reaction was determined. Expression levels were normalized to that of β-actin,using the following formula: relative mRNA level of target gene (fold of control) = $2^{-\Delta\Delta CT}$, where ΔΔCT = (Cttarget– Ct β-actin in treatment) – (Cttarget – Ct β-actin in control). Results are expressed relative to control values, which were arbitrarily given a value of 1.

Table 1
Forward and reverse oligonucleotide primer sequences for amplification of apoptosis-related genes.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
β-actin	AACAGTCCGCCTAGAAGCAC	CGTTGACATCCGTAAAGACC
Bcl–2	CGATTGTGGCAGTCCCTTA	CCAGGATGAAGTGCTCAGGT
Bax	GCTACAGGGTTTCATCCAG	TCAGCAATCATCCTCTGC

Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.