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Theanine prevents doxorubicin-induced acute hepatotoxicity by reducing intrinsic apoptotic response

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ABSTRACT

Doxorubicin (DOX) is widely used as an antitumor agent with topoisomerase II inhibiting activity; however, its dosage and duration of administration have been strictly limited due to dose-related organ damage. The present study investigated whether theanine, an amino acid found in green tea leaves, could reduce DOX-induced acute hepatotoxicity and the apoptotic response in mice. Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum, biomarkers of hepatic impairment, were markedly increased after the administration of 20 mg/kg DOX, whereas the degree of these elevations was significantly attenuated by 10 mg/kg theanine, which was consistent with histological hepatic images assessed by microscopic examination. The hepatic expression of Bax and Fas, representative intrinsic and extrinsic apoptotic molecules, respectively, was significantly increased by dosing with DOX. However, the elevation in the hepatic expression of Bax, but not Fas, was suppressed to control levels by theanine. The formation of cleaved caspase-3 protein in the group given DOX with theanine was significantly lower than that in the group treated with DOX alone. These results suggest that theanine can protect against acute hepatic damage induced by DOX, which is attributed to the suppression of intrinsic caspase-3-dependent apoptotic signaling.

1. Introduction

Chemotherapy using antitumor agents plays an essential role in the treatment of cancer; however, this is accompanied by several problems such as the development of severe adverse events and emergence of tumor cells resistant to these drugs. Due to the difficulties associated with discovering novel chemotherapeutic agents, strategic improvements in current therapeutic regimens are considered important for ensuring the effectiveness and safety of cancer treatments (Zhong et al., 2010). Biochemical modulations, which either enhance the pharmacological properties or reduce the toxicities of antitumor agents by another agent, have been accepted as a promising strategy to improve cancer chemotherapeutic outcomes (Hu et al., 2012; Kim et al., 2014; Wang et al., 2011; Yu et al., 2009).

Theanine, a glutamate derivative, contributes to the favorable umami taste of tea and exerts various beneficial effects such as relaxation, as demonstrated by the induction of alpha waves on electroencephalograms (Graham, 1992; Kim et al., 2009). Interest has been growing in theanine as an ingredient for novel functional foods and also as a dietary supplement (Vuong et al., 2011). A previous study reported that the administration of theanine enhanced the cytocidal activity of doxorubicin (DOX), an anthracycline antitumor drug, in mice bearing M5076 ovarian sarcoma cells (Sadzuka et al., 1996). However, this phenomenon paradoxically raised concerns regarding the possibility of increased damage to normal cells when DOX was combined with theanine. Contrary to the above notion, we clarified that acute cardiac impairment caused by DOX was significantly attenuated by a concurrent treatment with theanine in mice (Nagai and Konishi, 2013), showing evidence of the appearance of cardioprotection rather than increases in cardiotoxicity. While the heart is one of the preferential sites in which DOX-induced toxicity develops (Zhang et al., 2009), this antitumor agent is known to adversely affect a number of systemic organs (Bardi et al., 2007; Kalender et al., 2005). Almost 30% of patients receiving DOX have developed liver abnormalities and the hepatotoxicity of DOX has also been confirmed in fundamental studies using experimental animals (Damodar et al., 2014; Zhao et al., 2012). The onset of hepatic disorders occasionally necessitates the withdrawal or dose adjustments to not only DOX, but also the co-administered medicines used to treat disease complications and improve unpleasant symptoms, thereby increasing the risk of chemotherapy failing. Therefore, a practical strategy needs to be established in order to prevent the hepatotoxicity of DOX and achieve a therapeutic success.

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The aim of the present study was to evaluate the preventive effects of theanine on acute hepatic injury in DOX-treated mice by examining changes in serum enzyme activities and the histopathology of liver tissue. The formation of reactive oxygen species is presumed to be involved in the hepatotoxicity of DOX (Kassner et al., 2008); however, changes in the gene expression of various molecules associated with apoptotic responses are also regarded as a potential mechanism responsible for the cardiotoxicity of DOX (Chatterjee et al., 2010). Therefore, we also investigated biological modifications in the hepatic expression profile of apoptosis-related molecules following the administration of theanine to DOX-treated mice.

2. Materials and methods

2.1. Chemicals

DOX hydrochloride was obtained from Meiji Seika Kaisha (Tokyo, Japan). Theanine was purchased from Wako Pure Chemical Ind. (Osaka, Japan). DOX hydrochloride and theanine were dissolved in physiological saline and used for injections to mice. All other reagents were of commercial or analytical grade requiring no further purification.

2.2. Animal treatment

Male B6D2F1 mice aged 5 weeks were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Mice were acclimatized for at least 2 days before assignment to their experimental groups, and were housed in a clean room maintained at 23 ± 2 °C with a relative humidity of 55 ± 10% and 12-h light/dark cycle. They were allowed free access to a regular animal diet and tap water. Mice were divided into four treatment groups, which were designated as DOX, theanine, DOX plus theanine, and control groups. In the case of the DOX group, the indicated doses of DOX (10–20 mg/kg, i.p.) were injected on the 1st day, and physiological saline (i.p.) was administrated once a day from the 2nd day to the 4th day. In the theanine group, theanine (10 mg/kg, i.p.) was injected once a day for four consecutive days (from the 1st day to the 4th day). In the DOX plus theanine group, a dose of 20 mg/kg DOX was injected on the 1st day, and the indicated doses of theanine (2.5–10 mg/kg, i.p.) were administrated once a day from the 1st day to the 4th day. Control mice received physiological saline only at the same time. The volume of vehicle was fixed at 0.025 mL based on the body weight (g) of the mouse. Biochemical and histological examinations using mouse serum and liver tissues were made on the 5th day as described below. Experimental protocols and animal care methods in the experiment were approved by the Animal Experiment Committee at Osaka Ohtani University.

2.3. Biochemical determination

Blood was collected by cardiac puncture under anesthesia and the serum fraction was separated by centrifugation. The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by enzyme-based colorimetric methods using commercial reagent kits run on a biochemistry analyzer (Spotochem™ EZsp-4430 analyzer; ARKRAY Co., Kyoto, Japan).

2.4. Histopathological examination

A histopathological evaluation was made in liver tissues. Livers were dissected immediately and preserved in 10% buffered formaldehyde for microscopic observations. Tissue samples were embedded in paraffin and 4 μm sections were cut using a rotary microtome and stained with hematoxylin and eosin (Muto Pure Chemical Co., Ltd., Tokyo, Japan). A minimum of eight fields for each liver section was examined and evaluated for severity of changes by an observer blinded to the treatment of the animals. All sections were examined with a Leica DM750 light microscope (Leica Microsystems AG, Wetzlar, Germany).

2.5. Real-time quantitative PCR

Total RNA of the livers in mice was extracted with ISOGEN (Wako Pure Chemical Ind.) and purified with a GenElute Mammalian Total RNA miniprep kit (Sigma-Aldrich Co., St. Louis, MO, USA) according to the manufacturer's instructions. Each total RNA was reverse-transcribed into cDNA by means of Oligo-T priming and Moloney murine leukemia virus reverse transcriptase (GE Healthcare, Seattle, WA, USA). The expression levels of targeted genes were analyzed by real-time quantitative PCR with MyiQ2 (Bio-Rad Lab., Inc., Berkeley, CA, USA), using SYBR green as the fluorescence dye (Toyobo Co., Ltd., Osaka, Japan). cDNA was PCR-amplified at 95 °C for 10 s, at 55 °C for 10 s, and at 72 °C for 30 s. In initial experiments, a melting curve analysis was performed to monitor PCR product purity. Relative quantitation of the mRNA expression of targeted genes was calculated using the comparative threshold cycle number for each sample. To adjust variations in the amount of DNA, gene expression of the target sequence was normalized in relation to the expression of an endogenous control, GAPDH. Synthetic oligonucleotide primers (Hokkaido System Science Co., Ltd., Sapporo, Japan) used to investigate the expression levels of targeted genes were designed by Beacon Designer 8 (Bio-Rad Lab., Inc.), and were listed in Table 1. mRNA levels were quantified based on standard curves. Results were expressed relative to control values, which were arbitrarily a value of 1.

Table 1
Forward and reverse oligonucleotide primer sequences of apoptosis-related factors.

	Sequence	
	Forward	Reverse
Bax	TTTGCTACAGGGTTTCAT	GTCCAGTTCATCTCCAAT
Fas	ACATCAAGGAGGGCAAGA	ATCTAAGGTTCTGCGACATTC
GAPDH	AAGAAGGTGGTGAAGCAG	TCATACCAGGAAATGAGC

2.6. Western blotting

Proteins were extracted from hepatic tissues after homogenization in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Nonident P-40, and protease inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan)). Protein samples (10 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane (Bio-Rad Lab., Inc.). The membrane was washed with phosphate-buffered saline containing 0.1% Tween (PBS-T) and blocked with Blocking One solution (Nacalai Tesque Inc.) for 1 hr at room temperature. After washing with PBS-T, the membrane was probed with a rabbit anti-cleaved caspase-3 monoclonal antibody (Funakoshi Co., Tokyo, Japan; dilution 1:1000) or mouse anti-β-actin monoclonal antibody (Sigma-Aldrich Co.; dilution 1:2000) for 1 hr at room temperature. Each membrane was then incubated for a further 1 hr with an anti-rabbit (dilution 1:5000) or anti-mouse (dilution 1:10,000) IgG polyclonal antibody conjugated with horseradish peroxidase (GE Healthcare). Immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) method using ECL reagent (Wako Pure Chemical Ind.), according to the manufacturer's instructions. The expressed amount of cleaved caspase-3 protein was normalized in relation to the expression of β-actin. Results were expressed relative to control values, which were arbitrarily a value of 1.

2.7. Statistical analysis

Data were represented as means ± S.D. Comparisons among groups were made by means of an analysis of variance (ANOVA) followed by Dunnett's test or Tukey's test. Differences with a p value of 0.05 or less were considered significant.

3. Results

3.1. Effect of DOX on serum enzyme activities

The hepatotoxic effect of DOX was assessed by examining changes in levels of AST and ALT in serum. AST and ALT activities were significantly increased by the administration of 20 mg/kg DOX, but not by 10 mg/kg DOX (Fig. 1).

3.2. Effect of theanine on DOX-induced changes in serum enzyme activities

We evaluated the effect of theanine on the elevated serum enzyme activities observed after the DOX treatment. The administration of theanine alone to mice had no effect on enzyme activities (Fig. 2). Elevations in serum enzyme activities by DOX were significantly suppressed by the treatment with 10 mg/kg theanine (Fig. 2). On the other hand, theanine tended to suppress the increase in serum enzyme activities even when it was administered at doses of 2.5 mg/kg and 5 mg/kg, but the improvements did not reach a statistically significant level (Fig. 3).

3.3. Effect of theanine on DOX-induced histopathological changes in liver tissue

The extent of hepatic damage was assessed by a histological examination to support the results of serum biochemical tests. The histology of liver sections from control mice showed normal cell