Anti-diarrheal effect of Galla Chinensis on the Escherichia coli heat-labile enterotoxin and ganglioside interaction

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Abstract

Enterotoxigenic Escherichia coli (ETEC) is the most frequently isolated enteropathogen, accounting for approximately 210 million diarrhea episodes annually. ETEC-induced diarrhea is initiated by the binding of B subunit of heat-labile enterotoxin (LTB) to the ganglioside G M1 on the surface of intestinal epithelial cell. Therefore, we evaluated the inhibitory effects of 297 Chinese medicinal herbs on the LTB and G M1 interaction by G M1 -enzyme-linked immunosorbent assay. Galla Chinensis extract (GCE) exhibited anti-LT-induced diarrheal effect in the patent mouse gut assay, with IC 50 value of 4.7 ± 1.3 mg/ml. GCE also inhibited the binding of LTB to G M1 , suggesting that GCE suppressed the LT-induced fluid accumulation by blocking the binding of LTB to G M1 . Furthermore, the ethyl acetate (EA) soluble fraction was the most active fraction of Galla Chinensis that inhibiting the binding of LTB to G M1 with an IC 50 value of 153.6 ± 3.4 μg/ml. The major components of the EA fraction should be phenolic derivatives according to a thin-layer chromatography analysis. Gallic acid, the major component of EA fraction, blocked the binding of LTB to GM1, resulting in the suppression of LT-induced diarrhea. In conclusion, these data suggested that Galla Chinensis and gallic acid might be potent drugs for the treatment of LT-induced diarrhea.

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1. Introduction

Diarrheal diseases caused several million deaths in the world annually (Field, 2003). Enterotoxigenic Escherichia coli (ETEC) is the most frequently isolated enteropathogen, which is responsible for approximately 380,000 deaths annually (WHO, 2002). ETEC diarrhea is a primary cause of death in children less than 5 years old (Black, 1993; Orndorff et al., 1996). It is also a significant cause of disease among travelers (Adachi et al., 2001).

The major virulent factor of ETEC is the heat-labile enterotoxin (LT) (Hodmgen and Svennerholm, 1992). LT is a kind of AB toxins, which comprise one A subunit and five identical B subunits (Merritt and Hol, 1995). The mechanism of diarrhea induced by LT is initiated by the binding of B subunit (LTB) to the receptor, ganglioside GM1, on the surface of intestinal epithelial cells (Pickens et al., 2002; Spangler, 1992). Binding of LTB to GM1 induces a conformational change in the toxin molecule, followed by the translocation of A subunit (LTA) into the cell. Inside the intestinal cell, LTA catalyzes the ADP-ribosylation of the stimulatory GTP-binding protein, resulting in increased intracellular levels of cyclic AMP. The resulting elevated levels of cyclic AMP in the cell causes massive loss of fluid and ions from the cell, then leading to the symptom of diarrhea (Spangler, 1992). There is currently no prophylaxis against diarrhea caused by LT. The binding of LT to GM1 is so tight that only the 5- to 7-day turnover of the epithelium eliminates toxicity (Field, 2003). Because the first step of diarrhea induced by LT is the interaction between toxin and receptor, the binding of LT to GM1 is therefore an attractive target for developing drugs or prophylactics for the treatment and prevention of LT-induced diarrhea.
diarrhea (Minke et al., 1999a; Pickens et al., 2002; Mitchell et al., 2004).

In this study, we set up an in vitro model to evaluate the binding ability of LT to GM1. By screening 297 controlled Chinese medicinal herbs supervised by Committee on Chinese Medicine and Pharmacy at Taiwan, we found that Galla Chinensis (Wu-Pei-Tzu) was capable of inhibiting the binding of LT to GM1. Galla Chinensis is a gall caused by the Chinese sumac aphid (Schlechtendalia chinensis Bell) on several Rhus species (Anacardiaceae) including Rhus chinensis Mill. Galla Chinensis has been used in traditional Chinese medicine and others oriental medicine systems for years. Galla Chinensis has long been used for the treatment of diarrhea, prolonged coughing, and spontaneous perspiration in China (ChPC, 2000). It contains several tannin-derived components, such as gallic acid and methyl galate (Ahn et al., 1998). It inhibits the growth of intestinal bacteria and Helicobacter pylori (Ahn et al., 1998; Bae et al., 1998). It also inhibits tumor invasion by inhibiting the transcription of matrix metalloproteinases (Ata et al., 1996). Moreover, aqueous extract from the gall of Rhus chinensis might exert anti-diabetic effect by suppressing carbohydrate absorption from intestine, and thereby reducing the postprandial increase of blood glucose (Shim et al., 2003). Although the Galla Chinensis has been applied in the treatment of diarrhea in China, the anti-diarrheal mechanism of Galla Chinensis remained to be clarified. Therefore, the aim of this study was to examine the anti-LT-induced diarrheal efficacy of Galla Chinensis by GM1 enzyme-linked immunosorbent assay (ELISA) and patent gut assay. The active components of Galla Chinensis responsible for the anti-diarrheal activity were further analyzed.

2. Materials and methods

2.1. Plant materials

The Galla Chinensis was purchased from a local TCM apothecary in Taichung, Taiwan. The plant material was identified by Nien-Yung Chiu Technical Specialist (Institute of Chinese Pharmaceutical Sciences) and subjected to the thin layer chromatography (TLC) analysis according to the Pharmacopoeia Commission of People’s Republic of China (ChPC, 2000). The voucher specimen has been deposited in the Institute of Chinese Pharmaceutical Sciences, China Medical University.

2.2. Extraction and fractionation of Galla Chinensis

Dried Galla Chinensis (100 g) was extracted with 70% acetone by percolation until complete exhaustion. The Galla Chinensis extract (GCE) was concentrated under reduced pressure at a temperature less than 40°C, divided into small aliquots, and kept at −30°C until use.

2.3. Expression and purification of Escherichia coli LT and LTB

Recombinant LT and LTB were expressed in Escherichia coli BL21(DE3)pLysS strain and purified by affinity chromatography (Minke et al., 1999b). Briefly, cells were grown in 100 ml Luria-Bertani broth agitated at 37°C until OD600 reached 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cells were collected 3 h after induction. The cell pellet was resuspended in 4 ml 1× TEAN buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 200 mM NaCl, 3 mM NaN3), lysed by sonication, and centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was collected and mixed with α-galactose resin (PIERCE), and the recombinant LT and LTB were then eluted by 1× TEAN buffer containing 1 M galactose. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantified with a Bradford assay (Bio-Rad, Hercules, CA, USA).

2.4. Patent mouse gut assay

Female BALB/c mice (8 weeks old, 20 ± 1 g weight) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mouse experiments were conducted under ethics approval from the China Medical University Animal Ethics Committee.

In vivo LT-induced diarrheal ability was determined by the patent mouse gut assay as described previously (Baselski et al., 1997; Guidry et al., 1997). Briefly, five mice per group were starved with water only for 16 h. Each mouse was inoculated intragastrically with 0.5 ml of 10^4 LT alone or in conjunction with various amounts of GCE. Six hours latter, the mice were sacrificed. The entire intestine from duodenum to rectum was carefully removed to retain any accumulated fluid, and residual mesentery was removed prior to weighing. The carcass was weighed separately. LT-induced diarrheal ability was presented as a gut carcass weight ratio as followed:

\[
gut(G) \text{ ratio} = \frac{\text{gut weight (g)}}{\text{carcass weight (g)}} = \frac{\text{gut weight (g)}}{\text{carcass weight (g)}}
\]

2.5. Biotinylation of LTB

LTB was mixed with Sulfo-NHS-LS-biotin (Pierce, Rockford, IL, USA) in a ratio of 1–10. After a 2 h-incubation on ice, the unincorporated biotin was removed by centricon-10 (Millipore, Bedford, MA, USA), and the biotinylated LTB was stored at 4°C until further analysis. Sulfo-NHS-LS-biotin should be prepared freshly by dissolving in water.

2.6. Competitive GM1-ELISA

Microtiter plates (MaxiSorp Nunc-Immuno™ plates, Nunc, Denmark) were coated at 4°C overnight with 100 µl of
2 ng/μl GM1 (Sigma, St. Louis, MO, USA), which was diluted in phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH2PO4, 4.3 mM Na2HPO4, 2.7 mM KCl, pH 7.2). The wells were washed with 200 μl washing buffer (0.5% Tween 20 in PBS), blocked with 200 μl blocking buffer (1% bovine serum albumin (BSA) in PBS) at 37 °C for 1 h, and then incubated with 100 μl of biotinylated LTB/compound mixture at 37 °C for 1 h. After three washes with washing buffer, 50 μl of diluted peroxidase-conjugated avidin (PIERCE) was added to each well and incubated at 37 °C for 1 h. Following three washes, 50 μl of chromogenic substrate, 2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid) (Sigma), was added to each well and incubated at 37 °C for 15 min. The absorbance was read at 405 nm in an ELISA plate reader.

For competition assay, biotinylated LTB (16 ng) was mixed with various amounts of compound and incubated at 4 °C with shaking. After a 3 h-incubation, the mixture was added to wells, which were coated with GM1, and incubated at 37 °C for 1 h. Following three washes, peroxidase-conjugated avidin and chromatic substrate were sequentially added. The absorbance was read at 405 nm in an ELISA plate reader. The inhibitory ability (%) was calculated by \[1 - \left(\frac{\text{OD value of mixture containing LTB and compound}}{\text{OD value of mixture containing LTB only}}\right)\] × 100.

2.7. TLC analysis

Samples were submitted to the characterization by thin layer chromatography (TLC; silica gel 60 F254, Merck; development twice with the same mobile phase: chloroform/ethyl acetate/glacial acetic acid, 10:20:1). Chromatograms were evaluated under visible light. To confirm the presence of phenolics, TLC was sprayed with 10% FeCl3 aqueous solution and also evaluated under visible light.

2.8. Statistical analysis

Data were presented as mean ± standard error. Student’s t-test was used for comparisons between two experiments. A value of \(p < 0.05\) was considered statistically significant.

3. Results

3.1. Expression, purification, and characterization of Escherichia coli LT and LTB

*Escherichia coli* LT and LTB were expressed from *Escherichia coli* BL21(DE3)pLysS strain transformed with a pET plasmid carrying LT gene. After induction with IPTG, LTA and LTB with 28 and 11.5 kDa, respectively, were observed by TLC analysis.

![Fig. 1. Expression and characterization of *Escherichia coli* LT and LTB proteins. (A) SDS-PAGE analysis. The preparations of uninduced *Escherichia coli* (lane C), induced *Escherichia coli* (lane I), and purified recombinant LT (lane LT) or LTB (lane LTB) were analyzed by 15% SDS-PAGE and stained by Coomassie brilliant blue. The molecular masses of protein standard (lane M) are shown at the left. The 28 kDa LTA and 11.5 kDa LTB are indicated by arrows. (B) The binding ability of LTB to GM1. The wells were coated with 200 μg of GM1 and challenged with various amounts of biotin-labeled LTB. Following three washes, peroxidase-conjugated avidin and chromatic substrate were sequentially added. The absorbance was read at 405 nm in an ELISA plate reader. Values are mean ± standard error of four independent assays.]
3.2. GCE suppressed the LT-induced fluid accumulation in mice

Since one of the biological activities of LT is the induction of fluid accumulation in the intestine, we analyzed the anti-diarrheal effect of GCE by patent mouse gut assay. As shown in Fig. 2, LT stimulated the fluid accumulation in the gut, with the gut/carcass weight ratio of approximately 0.15. GCE significantly suppressed LT-induced fluid accumulation in a dose-dependent manner, with a concentration-dependent decrease in the gut–carcass ratio. These results indicated that GCE was able to inhibit LT-induced diarrhea, with the IC_{50} value of 4.7 ± 1.3 mg/ml.

3.3. GCE suppressed the LT-induced fluid accumulation by blocking the binding of LT to G_{M1}

The inhibitory ability of GCE on the binding of LT to G_{M1} was evaluated by competitive G_{M1}-ELISA. Various amounts of GCE were mixed with 16 ng of biotin-labeled LT, incubated at 4°C for 3 h, and added to G_{M1}-coated wells. GCE significantly inhibited the binding of LT to G_{M1} (Fig. 3). The inhibitory effect of GCE displayed a concentration-dependent manner, with the IC_{50} value of 0.17 ± 0.02 mg/ml. Therefore, these data indicated that GCE blocked the binding of LT to G_{M1}, resulting in the suppression of LT-induced diarrhea.

3.4. EA fraction of GCE blocked the binding of LT to G_{M1}

We analyzed the effects of GCE fractions on the binding of LT to G_{M1} by G_{M1}-ELISA. As shown in Fig. 4, EA fraction was the most active fraction of GCE. EA fraction inhibited the binding of LT to G_{M1} in a concentration-dependent manner, with the IC_{50} value of 153.6 ± 3.4 μg/ml.
Fig. 5. Identification of phenolics in the EA fraction of GCE by TLC. (A) Direct visualization of chromatogram by visible light without development. (B) Visualization of chromatogram sprayed with FeCl₃. S represents EA fraction of Galla Chinensis, M represents mixture containing EA fraction of Galla Chinensis and gallic acid, and G represents gallic acid.

3.5. Gallic acid exhibited the anti-LT-induced diarrhea ability by blocking the binding of LTB to GM₁

Because Galla Chinensis is a phenolic-rich plant (Ahn et al., 1998), we determined the presence of phenolics in the EA fraction of GCE by TLC. Fig. 5 shows that the major components of EA fraction were phenolic derivatives. Gallic acid is one of the phenolics from Galla Chinensis (Rui, 1997) and we also determined that gallic acid was the major component of EA fraction by high-performance liquid chromatography (data not shown). Therefore, the gallic acid might be the major active component of phenolics from Galla Chinensis for the anti-diarrheal effect.

To address this question, we analyzed the inhibitory effect of gallic acid by GM₁-ELISA and patent mouse gut assay. Gallic acid significantly blocked the binding of LTB to GM₁, with the IC₅₀ value of 10.9 ± 0.3 mM (Fig. 6). Gallic acid also suppressed the LT-induced fluid accumulation in a concentration-dependent manner, with the IC₅₀ value of 25.4 ± 11.6 mM (Fig. 7). Therefore, these findings indicated that gallic acid was the major component of Galla Chinensis for the suppression of LT-induced diarrhea.

4. Discussion

The most commonly principle of management for diarrhea is the fluid replacement combined with pharmacologic therapy (Ahlquist and Camilleri, 2001). Fluid and electrolyte replace-
ment is used to replace fluid losses by the administration of sugar-electrolyte solutions; however, it does not facilitate the re-adsorption of secreted fluid and therefore does not lessen diarrhea. Antibiotics and antimotility agents are used to control symptom. Antibiotics kill bacteria; however, it cannot inhibit the toxicity of bacterial toxin. Moreover, antibiotic therapy is not a viable solution because of the rapid increase in antibiotic resistance, particularly in endemic areas (Garg et al., 2000). Antimotility agents like loperamide can lessen stool frequency and volume in mild diarrheas. However, such agents are unsuitable in severe diarrhea because they might cause pooling of large fluid volumes in paralyzed bowel loops (Field, 2003). There are another potential pharmacological strategies (enkephalinase inhibitors and CFTR inhibitors) to reduce intestinal fluid loss in diarrhea (Thiagarajah and Verkman, 2005). Enkephalins are endogenous opiate substances that prevent fluid secretion through binding to delta opioid peptide receptors on enterocytes. Enkephalins activates the inhibitory G proteins, resulting in the reduced levels of intracellular cAMP and consequent deactivation of apical membrane cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channels and basolateral K+ channels (Turvill and Farthing, 1997). Enkephalins are degraded rapidly in the gastrointestinal tract by endogenous enkephalinases. Enkephalinase inhibitors such as racecadotril increase the levels of intestinal enkephalin, resulting in the reduced secretion of salt and fluid. However, clinical studies have shown that enkephalin treatment is not effective in Vibrio cholerae- and Escherichia coli-related diarrheas in adults (Salazar-Lindo et al., 2000; Alam et al., 2003). CFTR is the final rate-limiting step for intestinal Cl− and fluid secretion in cholera and other enterotoxin-mediated secretory diarrheas. SP-303, an oligomeric proanthocyanidin extracted from the bark latex of the tree Croton lechleri, inhibits CFTR-mediated Cl− secretion and inhibits CT-induced fluid accumulation in human intestinal cells and rodent models (Gabriel et al., 1999). However, CFTR inhibitor cannot specifically block toxin effects and has no application for the prevention of fluid secretion (Thiagarajah and Verkman, 2005).

So far, there is currently no specific prophylaxis against diarrhea caused by bacterial toxin, the discovery of agents that can block the function of toxin is very important for the prevention and treatment of bacterial toxin-induced diarrhea. To develop the agents that inhibit the toxin-induced diarrhea with specificity, we set up the LT-induced instead of castor oil-induced diarrheal model to mimic the diarrhea caused by bacteria. Castor oil is used for experimentally induced diarrhea in animal model (Mascolo et al., 1992; Mascolo et al., 1993). However, the mechanism of diarrhea induced by castor oil is totally different from LT-induced diarrheal. Castor oil administration stimulates the liberation of ricinoleic acid from castor oil, results in the irritation and inflammation of the intestinal mucosa, and consequently leads to the release of prostaglandin, which in turn stimulates the intestinal motility and secretion (Pierce et al., 1971; Chitime et al., 2004). The fluid accumulation assay and patent mouse gut assay are frequently used as the toxin-induced diarrheal models (Gorbach et al., 1971; Hinoctsubashi et al., 1992; Guidry et al., 1997). We set up the patent mouse gut assay as the diarrheal model in this study because the induction of diarrhea is through the oral administrations instead of surgical operation. Results of this study indicated that GCE and gallic acid significantly inhibited the LT-induced diarrhea in animal model.

By GMA-ELISA, we found that GCE, EA fraction of Galla Chinensis, and gallic acid significantly blocked the binding of LTB to GM1. So, gallic acid and relative components from GCE and EA fraction might interact with LTB to block the binding of LTB to GM1. The terminal galactose of exposed oligosaccharide head group of GM1 is important for the binding of LT through several hydrogen bonds (Pickens et al., 2002). Therefore, we speculated that gallic acid might interact with LTB through similar hydrogen bonds. LT and CT share 83% amino acid sequence homology and both consist of an A subunit for enzymatic activity and five B subunits for receptor recognition (Spangler, 1992; Rappuoli et al., 1999). Both toxins attack intestinal epithelia cells via binding to the GM1 on the cell surface (Merritt et al., 1997). In this study, we demonstrated that gallic acid suppressed the LT-induced diarrhea by blocking the binding of LTB to GM1. Other studies also indicated that epigallocatechin galate, RG-tannin, and synthesized gallate derivatives inhibited CT-induced diarrhea (Oi et al., 2002; Toda et al., 1992). Because LT and CT are two closely related toxins, these data suggested that GCE and GCE-derived compounds might not only inhibit LT-induced diarrhea but also inhibit CT-induced diarrhea. Additionally, GCE has been reported to exhibit the growth-inhibitory effect of intestinal bacteria (Ahn et al., 1998). Therefore, we speculated that GCE might exhibit the anti-diarrheal ability by both the suppression of intestinal bacterial growth and the blocking of binding of toxin to cellular receptor.

5. Conclusions

We demonstrated that GCE suppressed the LT-induced fluid accumulation by blocking the binding of LTB to GM1. Furthermore, gallic acid, the core structure of phenolics from GCE, blocked the binding of LTB to GM1, resulting in the suppression of LT-induced diarrhea. Therefore, these data suggested that Galla Chinensis and gallic acid might be potent inhibitors for the treatment of LT-induced diarrhea.

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References


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