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Enteral Infusion of Green Tea Extract Selectively Enhances the Biliary Secretion of ^{14}C-Benzo[a]pyrene in Rats without Affecting Other Biliary Lipids

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Abstract

Recently, we have demonstrated that green tea extract (GTE) decreases the intestinal absorption of benzo[a]pyrene (BAP), which is an extremely lipophilic food contaminant. The present study was conducted to examine if an enteral infusion of GTE would influence the biliary secretion of BAP and lipids in rats. Female rats were fed an AIN-93G diet with or without (control) GTE at 5 g/kg diet for 4 week. Following the 4-week dietary treatment, rats with bile duct cannula were infused continuously for 8 hr at 3.0 mL/hr via a duodenal catheter with a lipid emulsion containing 4.0 μmol BAP labeled with ^{14}C (^{14}C-BAP), 20.7 μmol cholesterol, 452 μmol triolein, and 3.1 μmol α-tocopherol, and 396.0 μmol Na-taurocholate with or without 76.1 mg GTE powder in PBS buffer (pH, 6.4). Bile was collected hourly via bile cannula for an 8 hr period. Our results showed that bile flow did not differ between groups. However, the biliary secretion of ^{14}C-BAP was significantly enhanced by GTE infusion, compared with those infused with the lipid emulsion alone. However, GTE did not affect the biliary outputs of cholesterol, fat, phospholipid and α-tocopherol. These findings indicate that GTE has a profound stimulatory effect on the biliary excretion of BAP in rats, without affecting other biliary lipids. The mechanism(s) by which GTE enhances the biliary secretion of BAP remains to be investigated.

Key words: benzo[a]pyrene, bile, green tea, rats, biliary secretion

INTRODUCTION

Benzo[a]pyrene (BAP; Fig. 1), a member of the polycyclic aromatic hydrocarbon class with a potent carcinogenicity, is a ubiquitous lipophilic food contaminant that tends to accumulate in humans. Food ingestion is the major route to its human exposure, mainly via barbecued meats and dairy products, which contribute up to 97% of human's daily intake of BAP (1-4). Based on Koreans' daily food intake, their average daily intake of BAP is shown to be 124.55 ng/day (5).

Green tea is processed from the fermented dried leaves of *Camellia sinensis*. The major polyphenols in green tea are catechins, which comprise up to one-third of its extractable solids. Green tea catechins consist mostly of (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epicatechin, with EGCG being the highest in concentration (6). Green tea catechins have been shown to exhibit potential antioxidant, anti-inflammatory and anti-lipidemic properties as well as an inhibitory effect on the intestinal absorption of lipids (6,7).

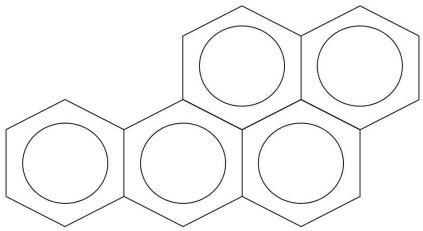

Fig. 1. Chemical structure of benzo[a]pyrene.

While the majority of studies have focused on BAP toxicity, little attention has been directed toward the possible use of bioactive food components for BAP elimination at the enterohepatic tract, the first site to have direct interface with the food contaminant. Studies indicate that dietary approach may be an attractive means of protecting against this lipophilic food contaminant. Studies have shown that green tea extract (GTE) or its catechins, particularly EGCG, inhibit luminal emulsification, micelle formation and lymphatic absorption and also increase the fecal excretion of lipids and lipid-soluble organic compounds such as biphenyls, dibenzofurans and dioxins (8-15). These findings indicate that GTE and its catechins may influence the luminal and intestinal processes of lipids and lipid-soluble organic compounds, thereby lowering their intestinal absorption. Our recent study also showed that GTE markedly lowers the lymphatic absorption of BAP, with a simultaneous decrease in the intestinal absorption of other lipids (16). At pres-

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ent, it is unclear how GTE inhibits the intestinal absorption of BAP. However, available evidence indicates that BAP is emulsified in luminal lipids due to its extreme lipophilicity and is absorbed through the intestine following the transport processes for lipids. Studies have shown that, just like other luminal lipids, the intestinal absorption of BAP is affected by the presence of dietary fat (17) and the luminal conditions influencing luminal hydrolysis, micellization, and transfer of lipids across the unstirred water layer (8,18,19). This lipid lowering effect was in line with our previous findings that green tea catechins inhibit pancreatic phospholipase A_2 and also lower the lymphatic absorption of ^{14}C-phosphatidylcholine (12). Fat and fat-soluble compounds, once taken up by the enterocyte, are packaged into the intestinal lipoprotein, chylomicrons and secreted into the circulation via the lymphatics (8,17,18). Taken together, these data clearly indicate that luminal GTE has a profound inhibitory effect on the intestinal absorption of BAP in rats.

Bile is a complex fluid consisting of water, electrolytes and organic molecules including bile acids, cholesterol, phospholipid, and xenobiotic substances, which flow through the biliary tract from the liver into the small intestine (20). Studies also show that lipophilic organic food contaminants including BAP are secreted into the small intestine via the enterohepatic tract and added into the feces (21). However, thus far, it is not known whether GTE would affect the biliary secretion of BAP and lipids from the body. Using an *in vivo* animal model with bile duct cannula, the present study, therefore, was designed to investigate whether GTE would affect the biliary excretion of ^{14}C-BAP and other biliary lipids in rats.

MATERIALS AND METHODS

Animals and diets

Ten adult female Sprague-Dawley rats (Harlan Sprague Dawley, Japan SLC, Inc., Shizuoka, Japan), weighing 146～154 g, were placed individually in stainless-steel wire bottomed cages in a room maintained at 22°C, with a 12-hr light/dark cycle (the light period from 0330 to 1530 hr). All animal care and experimental procedures were approved by the Changwon National University Institutional Animal Care and Use Committee. The rats had free access to deionized water and a nutritionally adequate rodent diet (Dyets Inc, Bethlehem, PA, USA) without (control) or with GTE at 5.0 g/kg diet for 4 week (Table 1). GTE was fed to activate various efflux transporters for BAP in the enterohepatic tract. Body weights were recorded weekly and food intake was measured twice a week for each rat by determining pre- and postweights of food jars. During the dietary treatment period, there were no significant differences in body weight and food intake between the groups.

Table 1. Composition of green tea extract (GTE) added diet[1] (g/kg)

Ingredient	Amount
Egg white	200.0
Cornstarch	396.5
Dextrinized cornstarch	132.0
Dextrose	100.0
Cellulose	50.0
Soybean oil	70.0
Mineral mix	35.0
Vitamin mix	10.0
Biotin (1 mg/g biotin sucrose mix)	4.0
Choline bitartrate	2.5
GTE	5.0

[1]Formulated and supplied by Dyets (Bethlehem, PA, USA) according to the recommendations of the American Institute Nutrition (22,23).

Cannulation of the common bile duct

Following the 4-week dietary treatment, rats from each group weighing 252～296 g were used to cannulate the common bile ducts (24,25). After 16-hr starvation, the rats were anesthetized with isoflurane (2.0% isoflurane in 2.0 L O_2/min). After midline abdominal incision, the common bile duct was cannulated with PE-10 polyethylene tubing (0.28 mm i.d., 0.61 mm o.d.; Becton Dickinson, Clay Adams Brand, Sparks, MD, USA) and secured in situ with sutures. Silicone tubing (1.02 mm i.d., 2.16 mm o.d.; Silastic® medical grade tubing, Dow Corning Medical Products, Dow Corning Co., Midland, MI, USA) was inserted into the duodenum approximately 2～3 cm below the pylorus for infusion of lipid emulsion and saline solutions as described below and then secured with purse-string suture. The bile duct cannula and the infusion catheter were exteriorized through the right flank. After the incision was closed, rats were placed in individual restraining cages and allowed to recover for 18～22 hr in a recovery chamber maintained at 30°C. Immediately following surgery, glucose-phosphate buffered maintenance solution [277.0 mmol/L glucose in phosphate buffered saline (PBS), which contains 6.8 mmol/L Na_2HPO_4, 16.5 mmol/L NaH_2PO_4, 115 mmol/L NaCl, and 5 mmol/L KCl, pH 6.4], was infused at 3.0 mL/hr through the intraduodenal catheter by an infusion pump (NE-1600, New Era Pump Systems, Inc., Farmingdale, NY, USA).

Measurement of biliary ^{14}C-BAP secretion

Following the overnight postoperative recovery, each rat was infused at 3.0 mL/hr via the duodenal catheter

with a lipid emulsion. The emulsion contained 27.8 kBq [4-^{14}C]-BAP (^{14}C-BAP; specific activity, 3.8 GBq/mmol; Dupont-New England Nuclear, Boston, MA, USA), 4.0 μmol BAP, 20.7 μmol cholesterol (Sigma-Aldrich Co., St. Louis, MO, USA), 452 μmol triolein (Sigma-Aldrich Co.), 3.1 μmol all-rac-α-tocopherol (Sigma-Aldrich Co.), and 396.0 μmol Na-taurocholate with or without (control) 76.1 mg GTE powder in 24 mL PBS (pH, 6.4). The emulsion was prepared using an ultrasonicator (UW 2200, Bandelin Electronic, Berlin, Germany). We analyzed the GTE powder (Indena Inc., Seattle, WA, USA) for catechin and caffeine content by HPLC, as described previously (11). The GTE contained 5.6% caffeine (wt/wt) and 29.2% (wt/wt) catechins with a relative distribution (%) of 47.7 EGCG, 31.2 epigallocatechin, 13.4 epicatechin gallate, and 7.6 epicatechin. The amount of GTE contained 22.2 mg total catechins/76.1 mg GTE, which is equivalent to 2～3 cups/day of green tea in humans on the basis of energy consumption (11). The triolein amount was estimated to be approximately 29% of the daily fat intake of a rat consuming 20.0 g/day of the AIN-93G. The amounts of cholesterol and α-tocopherol were set at a moderate high intake and at 100% of the daily intake of the vitamin in the diet, respectively. Bile was collected hourly under subdued light for 8 hr in pre-weighed ice-chilled conical centrifuge tubes containing 10 μg of *n*-propyl gallate as antioxidant. From the bile collected at the hourly intervals, 100-μL was mixed with scintillation fluid (Ready Safe^{TM}, Beckman Coulter Inc., Brea, CA, USA) and the ^{14}C-radioactivity was determined by scintillation spectrometry (Wallac 1414, Perkin Elmer Inc., Waltham, MA, USA). ^{14}C-radioactivity appearing in the hourly bile samples was expressed as percentage of the total ^{14}C-BAP infused.

Bile lipid analyses

For total cholesterol analysis, bile (100 μL) was saponified with 33% ethanolic KOH for 15 min at 60°C (26,27). The upper phase was obtained, dried under a nitrogen stream, and reconstituted with a chloroform: methanol mixture (1:4, v/v). Cholesterol in the extracts was separated by HPLC (Beckman HPLC with System Gold, Beckman Instruments, Fullerton, CA, USA) equipped with a C-18 reverse-phase column (Alltima C18, 5 μm, 4.6×150 mm; Alltech Associates, Deerfield, CA, USA). The mobile phase was isopropanol/acetonitrile/water (60:30:10, v/v/v) at 1.5 mL/min. Detection was monitored at 292 nm.

α-Tocopherol in the bile was analyzed by HPLC (28). Briefly, α-tocopherol acetate was added as an internal standard. α-Tocopherol and α-tocopherol acetate were separated with a Beckman HPLC instrument with System Gold software (Beckman HPLC with System Gold, Beckman Instruments) equipped with a C-18 reverse-phase column (Alltima C18, 5 μm, 4.6×150 mm; Alltech Associates, Deerfield, CA, USA).

To determine the total fatty acids in bile, total lipids were extracted from 100 μL bile (29). Fatty acids were analyzed by gas chromatography (30). Following addition of an internal standard (17:0), fatty acid methyl esters, generated by an alkali-catalyzed reaction (methanolic NaOH and BF_3), were separated by gas chromatography (Model 7890A, Agilent Technologies, Wilmington, DE, USA) using a DB-23 capillary column (60.0 m×0.25 mm×0.15 μm, Agilent J&W Scientific, Inc., Santa Clara, CA, USA). Fatty acid standards (Nu-Chek-Prep Inc., Elysian, MN, USA) were used for analysis. Total phospholipid in bile was determined by a colorimetric method (31).

Statistical analysis

Values were expressed as means±SD throughout the text and figure. Comparisons between treatment groups were performed using GraphPad Prism (Version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Repeated measures ANOVA with a post hoc Bonferroni multiple-comparison was performed to compare group means and time-dependent changes within groups at each time point. Statistical probability of $p<0.05$ was considered significant.

RESULTS

Biliary secretion of ^{14}C-BAP

In response to the lipid emulsion containing ^{14}C-BAP, bile flow was increased significantly in both groups. The average rates of bile flow were 0.93±0.03 mL/hr in GTE-infused rats and 0.91±0.04 mL/hr in their respective controls with no significant difference between groups. GTE infusion did not significantly affect the hourly rate of bile flow or the 8-hr total bile volume (Table 2). However, the hourly rate of the biliary ^{14}C-BAP secretion was significantly higher at 3 hr and thereafter in rats infused with GTE than in those with lipid emulsion alone and peaked at 4～5 hr in both groups (Fig. 2). The average rates of ^{14}C-BAP secreted into the bile over the 8-hr period were 3.94±0.10% dose/hr in GTE- infused rats and 3.03±0.05% dose/hr in control rats, which was a significant difference (Table 2). The cumulative biliary amount (% dose) of ^{14}C-BAP secreted into the bile also increased significantly in rats infused with GTE at 4 hr and thereafter, compared with control (Table 3). The 8-hr total biliary secretion of ^{14}C-BAP by GTE infusion was increased by 30.0% of the control

Table 2. Cumulative biliary secretion of ^{14}C-benzo[a]pyrene (^{14}C-BAP) and other biliary lipids in rats infused with a lipid emulsion without (control) or with green tea extract (GTE) for 8 hr[1)]

Biliary lipids	Control	GTE
Bile, mL	7.26±0.28	7.39±0.22
^{14}C-BAP, % dose	24.24±0.43	31.51±0.83*
% dose/hr	3.03±0.05	3.94±0.10*
α-Tocopherol, nmol	93.74±26.35	73.14±6.31
Total cholesterol, μmol	4.28±0.97	3.43±0.25
Phospholipid, μmol	49.45±7.73	43.21±4.20
Oleic acid, μmol	1.80±0.46	1.67±0.19
Total fatty acid, μmol	136.2±35.7	118.4±11.6

[1)]Mean±SD, n=5. Asterisks (*) denote significant differences at $p<0.05$.

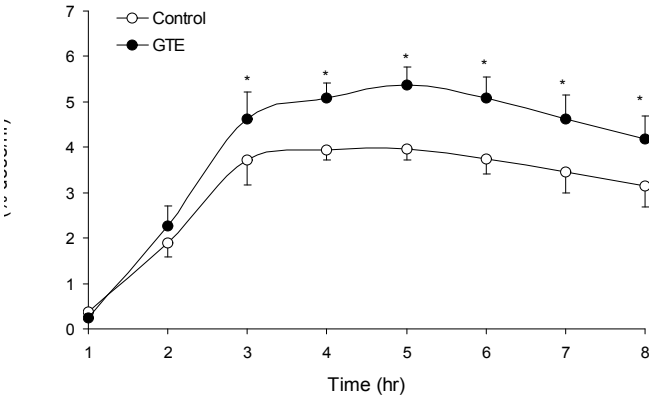

Fig. 2. Hourly rates of the biliary excretion of ^{14}C-benzopyrene (^{14}C-BAP) in rats infused with a lipid emulsion without (control) or with green tea extract (GTE). Values are means±SD, n=5. Asterisks (*) denote significant differences at $p<0.05$.

Table 3. Cumulative biliary secretion of ^{14}C-benzo[a]pyrene in rats infused with a lipid emulsion without (control) or with green tea extract (GTE) for 8 hr[1)] (% dose/8 hr)

Time	Control	GTE
1 hr	0.37±0.09	0.25±0.09
2 hr	2.26±0.35	2.52±0.53
3 hr	5.97±0.83	7.15±1.09
4 hr	9.92±1.02	12.23±1.36*
5 hr	13.88±0.82	17.61±1.27*
6 hr	17.64±0.50	22.69±1.10*
7 hr	21.10±0.15	27.33±1.10*
8 hr	24.24±0.43	31.51±0.83*

[1)]Mean±SD, n=5. Asterisks (*) denote significant differences at $p<0.05$.

level.

Biliary outputs of α-tocopherol, cholesterol, phospholipid, and fatty acids

Intraduodenal infusion of GTE did not affect the biliary secretion of α-tocopherol. GTE infusion significantly increased the biliary output of α-tocopherol with time but did not affect the hourly rates or the total amount of the biliary α-tocopherol secretion, compared to controls (Table 2). The biliary output of phospholipid, which was not included in the emulsion and available endogenously, was significantly increased with time but also did not differ between the groups. GTE did not affect the biliary secretion of oleic acid, which was the sole fatty acid in the form of triolein in the lipid emulsion and a marker of exogenous fat source (Table 2). Also, GTE did not influence the biliary secretion of fatty acids of endogenous origin such as 16:0, 18:0, 18:2, 18:3, 20:4 and 22:6 (data not shown).

DISCUSSION

This study, using an *in vivo* animal model with bile-duct cannulation, provides new evidence that GTE, at the dose equivalent to 2～3 servings/day of green tea in humans, markedly enhances the biliary secretion of ^{14}C-BAP. However, GTE, under the same conditions, does not affect the biliary outputs of α-tocopherol, cholesterol, phospholipid, and fatty acids secreted into the bile. Our findings here indicate that enteral infusion of GTE enhances the biliary secretion of a potent carcinogenic BAP but does not affect other biliary lipids and lipid-soluble vitamin.

Our recent study demonstrated that GTE, at the same GTE dose used in this current study, significantly lowers the intestinal absorption of ^{14}C-BAP (16). Also, the unabsorbed ^{14}C labeled BAP remaining in the small intestinal lumen and cecal contents was higher in rats infused with GTE. Taken together, these recent and current findings indicate that green tea may be used as an effective dietary means of reducing the body's absorption of BAP, possibly lowering any carcinogenic effects. At present, however, the mechanism by which GTE enhances the biliary secretion of BAP with a simultaneous decrease in its intestinal absorption is yet to be determined. Available evidence suggests that uptake of BAP by the enterocyte is mediated in part by specific transport enzymes on the brush border membrane. The brush border's epithelial cells may play a central role in decreasing the intestinal absorption of BAP because these epithelial cells are equipped with various phase 1 (*e.g.*, CYP isoenzymes) and phase 2 (*e.g.*, sulfotransferases), by which BAP is metabolized (32). The major metabolites of BAP generated by these enzymes are shown to be mostly BAP-1-sulfate and BAP-3-sulfate, which are more polar and water soluble than BAP itself, resulting in preferential backward transport (33-35). In addition, when BAP is present in the cell, ABC-transporters are expressed at the luminal side of the cells. Studies with human intestinal Caco-2 cells showed that BAP induces its own metabolism by up-regulating these

BAP-metabolizing enzymes and that its primary metabolites, BAP-1-sulfate and BAP-3-sulfate are preferentially transported out of the cells toward the luminal region, mediated via ABC-transporters (34). Furthermore, Ebert et al. (36) also demonstrated that flavonoids induce ABC-transporter activity on the Caco-2 cell's apical membrane and enhance the efflux of BAP-3-sulfate toward the lumen. This study clearly indicates that the efflux transporter for BAP is inducible by the presence of BAP and is further activated by flavonoids such as green tea catechins, thereby resulting in the decrease in BAP absorption, as observed in our recent finding (16). It is also possible that BAP, when changed to its metabolite(s), may escape from the lymphatics but be delivered directly to the liver via the portal venous route, allowing efficient biliary excretion. In the present study, we clearly observed that following the 4-week dietary treatment, GTE infusion markedly enhances the biliary secretion of ^{14}C-BAP, which was infused intraduodenally. When ^{3}H-BAP was infused luminally using bile duct cannulated rats with a lymph duct diversion, total radiolabel recovered for 24 hr was about 20%, with 79% of the recovered radiolabel was found in bile (37). This study indicates that the liver has the capacity to metabolize and transport BAP. Our current study also showed that when rats were infused enterally with a lipid emulsion having ^{14}C-BAP and GTE, the biliary secretion of ^{14}C-BAP was significantly higher in rats infused with GTE, indicating that GTE is an effective means not only in lowering the intestinal absorption of BAP but also in enhancing its biliary secretion. This also indicates that the portal transport pathway may facilitate the secretion of intestinally-derived BAP metabolites via the bile into the intestinal lumen and ultimately increase its elimination via feces. This is also in line with our most recent finding (unpublished data). In this study, in order to investigate if GTE would affect the tissue distribution and deposition of ^{14}C-BAP, rats were injected i.p. with 3.96 μmol BAP in corn oil and continued to receive a daily prepared GTE solution via their drinking water for 2 week, which is about 6～7 cups per day for humans. We found that the ^{14}C-labeled BAP remained significantly lower in most of the tissues of rats given GTE at 4 week, especially at the liver, heart, spleen, epididymal fat and brain. This study clearly indicates that GTE, when given via drinking water is effective in mitigating BAP burden from the body.

In summary, the present study provides evidence that GTE, when enterally administered, effectively enhances the biliary secretion of BAP but does not affect α-tocopherol, cholesterol and fatty acid (fat) in rats fed GTE added diet for 4 week. The increase of the biliary BAP secretion by GTE infusion appears to be associated with preferential portal transport pathway to the lymphatics, which may facilitate the secretion of intestinally-derived BAP metabolites via the bile. Currently, a study is planned to investigate whether an enteral infusion of GTE would affect the biliary secretion of BAP and other lipids in rats with or without its dietary treatment. Also, attention is being directed to assess the efficacy of GTE in reducing the intestinal absorption and biliary secretion of other extremely lipophilic compounds such as dioxins.

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