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Green tea extract markedly lowers the lymphatic absorption and increases the biliary secretion of ^{14}C-benzo[*a*]pyrene in rats☆

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Abstract

Previously, we have shown that green tea extract (GTE) lowers the intestinal absorption of lipids and lipophilic compounds in rats. This study was conducted to investigate whether GTE inhibits the intestinal absorption and biliary secretion of benzo[*a*]pyrene (BaP), an extremely lipophilic potent carcinogen, present in foods as a contaminant. Male rats with lymph or bile duct cannula were infused at 3.0 ml/h for 8 h via a duodenal catheter with lipid emulsion containing ^{14}C-BaP with or without GTE in PBS buffer. Lymph and bile were collected hourly for 8 h. The ^{14}C-radioactivities in lymph, bile and intestine were determined and expressed as % dose infused. Results showed that GTE drastically lowered the lymphatic absorption of ^{14}C-BaP (7.6±3.2% in GTE-infused vs. 14.4±2.7% dose/8 h in control rats), with a significantly higher amount of ^{14}C-radioactivity present in the small intestinal lumen and cecum in rats infused with GTE. GTE also markedly increased the hourly rate (3.9±0.1% dose/h in GTE-infused vs. 3.0±0.1% dose/h in control rats) and the total biliary secretion of ^{14}C-BaP (31.5±0.8% dose/8 h in GTE-infused vs. 24.3±0.4% dose/8 h in control rats). The findings provide first direct evidence that GTE has a profound inhibitory effect on the intestinal absorption of BaP and promotes the excretion of absorbed BaP via the biliary route. Further studies are warranted to investigate whether green tea could be recommended as a dietary means of ameliorating the toxicity and carcinogenic effect of BaP.

Keywords: Absorption; Benzo[*a*]pyrene; Bile; Green tea; Lipids; Lymph; Rats; α-Tocopherol

1. Introduction

In recent years, considerable attention has been directed toward the potential use of nutrients and bioactive food components to mitigate the toxicity of persistent organic pollutants (POP) such as polycyclic hydrocarbons (PAH) and to reduce the risks for diseases and disorders associated with exposure to POP [1].

Benzo[*a*]pyrene (BaP) is a PAH consisting of five fused benzene rings. It is ubiquitously present in the environment and considered a potent carcinogen. Human exposure to this compound is mainly through the ingestion of foods such as grilled foods, dairy products and sea foods [2–4]. Although cigarette smoke is another major source of human exposure to BaP, diet contributes most, up to 97% of its daily exposure [5]. The daily intake of BaP, via diet alone, has been estimated to be 125 ng/days [6]. Despite the fact that BaP enters the human body mainly via the intestinal route, little is known about the precise mechanism of its absorption. Furthermore, it is unknown whether any food components can modulate its entry via the intestinal route. Because of its extreme lipophilic properties, BaP is thought to be absorbed largely via chylomicrons into the lymphatics [7–10].

Green tea is a worldwide popular beverage, processed from the dried raw leaves of the tea plant *Camellia sinensis*. Green tea is a rich source of catechins that represent up to one third of the dry weight of green tea extract (GTE). The major catechins are (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate, (−)-epigallocatechin and (−)-epicatechin. Previously, we and other investigators have shown that green tea and its catechins strongly inhibit the intestinal digestion and absorption and increase the fecal excretion of lipids and lipophilic compounds [11–19]. Ample evidence indicates that GTE and its catechins interfere with the luminal emulsification, hydrolysis, micellar solubilization and subsequent uptake of lipids by the enterocyte, thereby inhibiting their absorption. The inhibitory effect of GTE is more marked on extremely lipophilic compounds [15–18].

Consistent with these observations, Morita et al. [20] first reported that powdered green tea (matcha), when fed to male rats along with rice bran oil contaminated with PAH such as polychlorinated biphenyls, polychlorinated dibenzofurans and polychlorinated dibenzo-*p*-dioxins, significantly increased the fecal excretion and reduced the liver contents of the PAH. Also, a study with bile duct-cannulated rats [21] showed that dietary quercetin increased the recovery of ^{14}C-label in the bile after a single oral dose of [4-^{14}C]-BaP (^{14}C-BaP). These findings suggest that green tea may inhibit the

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intestinal absorption and lower the body burden of these POP. At present, however, no direct evidence is available indicating that green tea or tea flavonoids (catechins) inhibit the intestinal absorption and/or increase the biliary secretion of PAH including BaP.

The present study, using a rat model with mesenteric lymph- and bile-duct cannula, was conducted to examine whether GTE affects the intestinal absorption and biliary secretion of BaP under *in vivo* conditions. The results reported here provide the first direct evidence that GTE drastically lowers the lymphatic absorption and increases the biliary secretion of BaP.

2. Materials and methods

2.1. Experiment 1

2.1.1. Animals and diet

Ten male Sprague-Dawley rats (Harlan Sprague Dawley, Japan SLC, Shizuoka, Japan) initially weighing 290–300 g were placed individually in stainless-steel wire-bottomed cages in an environmentally controlled room maintained at 22°C, with a 12:12-h light/dark cycle (the light period from 0330 to 1530 hours). All animal care and experimental procedures were approved by the Changwon National University Institutional Animal Care and Use Committee. The rats were given free access to deionized water and fed a diet formulated by Dyets according to the AIN-93 recommendations [22,23], except that tocopherol-stripped soybean oil and dried egg white were used as the fat and protein sources, respectively.

2.1.2. Mesenteric lymph duct cannulation

Rats weighing ~350 g were starved overnight for 16 h and anesthetized with isoflurane (2.0% isoflurane in 2.0 L O_2/min). The duodenum and the mesenteric lymph duct were cannulated as previously described [24,25]. Briefly, polyethylene tubing (SV 31, Dural Plastics & Engineering, Auburn, Australia) was inserted into the superior mesenteric lymph duct for lymph collection, and a silicone catheter (Silastic Medical Grade Tubing, Dow Corning, Midland, MI, USA) was inserted into the duodenum approximately 2 cm below the pylorus for infusion of lipid emulsion and saline solutions as described below. After the abdominal incision was closed by suture, rats were placed in restraining cages and housed in a recovery chamber at 30°C. During the 20-h postoperative recovery period, a maintenance solution [277.0 mmol/L glucose in phosphate buffered saline (PBS) containing 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/h through the intraduodenal catheter by an infusion pump (NE-1600, New Era Pump Systems, New York, USA).

2.1.3. Measurement of ^{14}C-BaP absorption

After the postoperative recovery, each rat was infused at 3.0 ml/h via the duodenal catheter with a lipid emulsion with or without GTE. The lipid emulsion consisted of 27.4 kBq ^{14}C-BaP (specific activity, 3.8 GBq/mmol; Dupont-New England Nuclear, Boston, MA, USA), 3.96 μmol BaP, 20.7 μmol cholesterol (99%; Sigma-Aldrich St. Louis, MO, USA), 451.8 μmol triolein (95%; Sigma-Aldrich), 3.1 μmol all-rac-α-tocopherol (97%; Sigma-Aldrich) and 396.0 μmol Na-taurocholate with or without 76.1 mg GTE powder in 24 ml PBS. The GTE powder was kindly provided by Indena (Seattle, WA, USA). GTE was analyzed for catechin and caffeine content by HPLC, as described previously [15]. The GTE contained 29.2% catechins and 5.6% caffeine (wt/wt). The distributions (%) of the catechins were 47.7 EGCG, 31.2 epigallocatechin, 13.4 epicatechin gallate and 7.6 epicatechin. The amount of GTE (76.1 mg) added to the emulsion contained 22.2 mg total catechins, which was equivalent to two to three cups per day of green tea in humans, as estimated on the basis of daily energy intake [15]. The amount of triolein was approximately 29% of the daily fat intake of a rat consuming 20.0 g/day of an AIN-93G diet. The amount of α-tocopherol was set at levels that approximate their daily intakes in humans.

During the infusion of the lipid emulsions, lymph was collected hourly for 8 h into conical tubes containing 25 mM disodium EDTA as an anticoagulant. From the lymph collected at the hourly intervals, 100 μl was mixed with scintillation fluid (Ready Safe, Beckman Coulter, Brea, CA, USA) and the ^{14}C-radioactivity therein was determined by scintillation spectrometry (Wallac 1414, Perkin Elmer, Waltham, MA, USA). ^{14}C-Radioactivity appearing in the hourly lymph samples was expressed as percentage of the total ^{14}C-BaP infused.

2.1.4. Measurement of ^{14}C-radioactivity in the small intestinal lumen, mucosa and cecum

At the end of the 8-h lymph collection, rats were anesthetized with isoflurane and killed by cervical dislocation. The small intestine and the cecum were removed separately and chilled immediately on ice, as described previously [16]. Briefly, the luminal content of the small intestine was collected into a plastic tube by washing with ice-cold PBS (pH 6.4) containing 16.5 mM sodium taurocholate. The intestine and cecum were cut opened afterwards and lipids were extracted according to the method of Folch et al. [26]. ^{14}C-Radioactivities were determined from aliquots of the luminal washings and the intestinal and cecal lipid extracts. The recoveries of ^{14}C-radioactivity in the intestinal lumen, mucosa and cecum were expressed as percentage of the dose infused.

2.1.5. Lymph lipid analyses

For fatty acid analysis, total lipids were extracted from a 100-μl lymph. Fatty acids were analyzed by gas chromatography [27]. An internal standard (17:0) was added to each tube during lipid extraction. 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, Santa Clara, CA, USA). Nu-Chek-Prep fatty acid standards were used for analysis. For α-tocopherol analysis, a 100-μl lymph was extracted with acetone with a slight modification of the procedure [28]. As an internal standard, α-tocopherol acetate was added. α-Tocopherol and α-tocopherol acetate were separated with a Beckman HPLC instrument with System Gold software (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, California, USA). One hundred percent methanol was used as the mobile phase at 2 ml/min. Detection was monitored at 292 nm.

2.2. Experiment 2

2.2.1. Bile duct cannulation

The protocols for diet formulation, animal care, surgical procedure and lipid emulsion preparation were the same as described for Experiment 1, except that the common bile duct was cannulated in rats weighing ~300 g (Harlan Sprague Dawley, Japan SLC). Cannulation of the bile duct was performed, as described in our previous study [24]. After the postoperative recovery period, the rats were infused intraduodenally for 8 h with a lipid emulsion prepared as in Experiment 1. Bile was collected hourly via the bile-duct cannula for 8 h into ice-chilled conical tubes containing 10 μg of *n*-propyl gallate as an antioxidant under subdued light. From the hourly bile samples (100 μl), ^{14}C-radioactivity was determined, as described above, and expressed as percentage of the total ^{14}C-BaP infused.

2.2.2. Statistics

All statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software, La Jolla, CA, USA). For data on ^{14}C-radioactivities appearing in lymph and bile, repeated measures ANOVA with a post hoc Bonferroni multiple-comparison test was used to compare group means and time-dependent changes within groups. The recoveries of ^{14}C-radioactivity in the intestinal lumen, mucosa and cecum were compared using the Student's *t* test. Differences were considered significant at $P<.05$. Data were expressed as means±S.D.

3. Results

3.1. Lymph flow

In response to the infusion of lipid emulsion, lymph flow was steadily increased with time and reached its peak in both groups at 5–6 h. The flow rates were 1.8±0.4 ml/h in GTE-infused rats and 1.9±0.2 ml/h in their respective controls. The presence of GTE in the lipid emulsion did not affect the rates of lymph flow or the total volume of lymph collected for 8 h (Table 1).

Table 1
Cumulative lymphatic absorption and biliary secretion of ^{14}C-BaP and other lipids in rats infused with GTE

Lipids	Control	GTE
Lymph		
Lymph volume, ml/8 h	15.0±1.8	14.5±2.9
^{14}C-BaP, % dose/8 h	14.4±2.7*	7.6±3.2
% dose/h	1.8±0.3*	0.9±0.4
α-Tocopherol, % dose/8 h	44.4±4.6*	20.8±12.6
nmol/8 h	1391.3±143.9*	652.5±394.6
Oleic acid, μmol/8 h	857.6±14.1	969.6±86.6
Total fatty acid, μmol/8 h	1107.7±16.9	1230.6±139.9
Bile		
Bile volume, ml/ 8 h	7.3±0.3	7.4±0.2
^{14}C-BaP, % dose/8 h	24.3±0.4	31.5±0.8*
% dose/h	3.0±0.1	3.9±0.1*

Values are shown as mean±S.D., $n=5$. Asterisks (*) denote significant differences at $P<.05$.

Table 2
Hourly rates (% dose) of the lymphatic absorption of ^{14}C-BaP in rats infused with a lipid emulsion with or without (control) containing GTE for 8 h

Time (h)	Control	GTE
1	0.25±0.18	0.20±0.08
2	1.54±0.95	0.79±0.46
3	2.37±0.48	0.86±0.55*
4	3.83±0.64	1.52±0.86*
5	2.72±0.39	1.72±0.89*
6	1.46±0.55	1.09±0.43
7	1.11±0.41	0.77±0.26
8	1.06±0.67	0.61±0.31

Values are shown as mean±S.D., n=5. Asterisks (*) denote significant differences at P<.05.

3.2. Lymphatic absorption of ^{14}C-BaP

The hourly rates of ^{14}C-BaP absorption peaked at 4–5 h in both GTE-infused and control rats. During this period, the rate of ^{14}C-BaP absorption in GTE-infused rats declined sharply to 37–60% of the control levels (Table 2). The average rates of ^{14}C-BaP absorption over the 8-h period were 0.9±0.4% dose/h in GTE-infused and 1.8±0.3% dose/h in control rats. Consequently, the cumulative absorption of ^{14}C-BaP decreased significantly in GTE-infused rats at 4 h and, thereafter, compared with controls (Fig. 1). The total absorption of ^{14}C-BaP was decreased by GTE to 53% of the control level.

3.3. ^{14}C-Radioactivity remaining in the intestinal lumen, mucosa and cecum

The ^{14}C-radioactivity remaining in the small intestinal lumen was significantly higher in rats infused with GTE (27.5±4.5% dose) relative to controls (14.7±2.8% dose) (Table 3). The ^{14}C-radioactivity remaining in the cecal content was also significantly increased in the GTE-infused rats (3.2±0.8% dose) compared with the controls (1.0±0.2%). No difference was noted in the mucosal ^{14}C-radioactivity between groups (Table 3).

Table 3
Recovery (% dose) of ^{14}C-radioactivity in the intestine of rats after an 8-h infusion of a lipid emulsion containing ^{14}C-BaP with or without GTE

Fractions	% Recovery	
	Control	GTE
Small intestinal lumen	14.7±2.8	27.5±4.5*
Small intestinal mucosa	14.3±3.2	15.7±2.7
Cecal content	1.0±0.2	3.2±0.8*

Values are shown as mean±S.D., n=5. Asterisks (*) denote significant differences at P<.05.

3.4. Lymphatic outputs of lipids

The average rates of α-tocopherol outputs in GTE-infused rats and control groups were 81.6±49.3 and 173.9±18.0 nmol/h, respectively, with a significant difference between groups. Consequently, GTE significantly lowered the cumulative absorption of α-tocopherol to 46.8% of the control level (P<.05, Table 1). GTE did not affect the lymphatic output of oleic acid, which was infused as triolein as a marker of exogenous fat source. Also, GTE did not influence the outputs of other fatty acids of endogenous origin such as 16:0, 18:0, 18:2, 18:3, 20:4 and 22:6 (data not shown).

3.5. Biliary secretion of ^{14}C-BaP

With the infusion of lipid emulsion, bile flow increased significantly with time in both groups. The average rates of bile flow were 0.93±0.03 ml/h in GTE-infused rats and 0.91±0.04 ml/h in controls with no significant difference between groups. Also, GTE did not affect the total volume of bile secreted for 8 h (Table 1). However, the biliary secretion of ^{14}C-BaP was markedly increased by GTE; the average rates of ^{14}C-BaP secretion over the 8-h period were 3.9±0.1% dose/h in GTE-infused and 3.0±0.1% dose/h in control rats. The total amounts of ^{14}C-BaP secreted into the bile for 8 h were 31.5±0.8% dose/h in GTE-infused and 24.3±0.4% dose/h in control rats, with a significant difference between groups (Fig. 2).

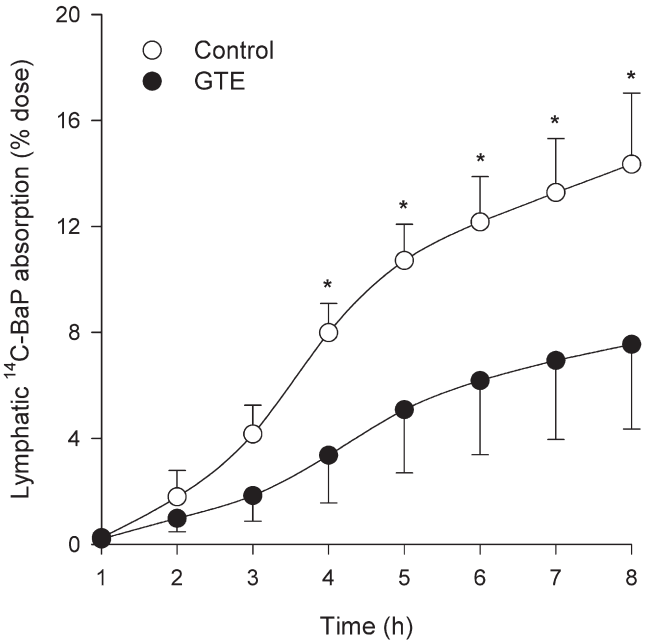

Fig. 1. Cumulative lymphatic absorption (% dose) of ^{14}C-BaP in rats infused with a lipid emulsion containing GTE. Values are means±S.D., n=5. Asterisks (*) denote significant differences at P<.05.

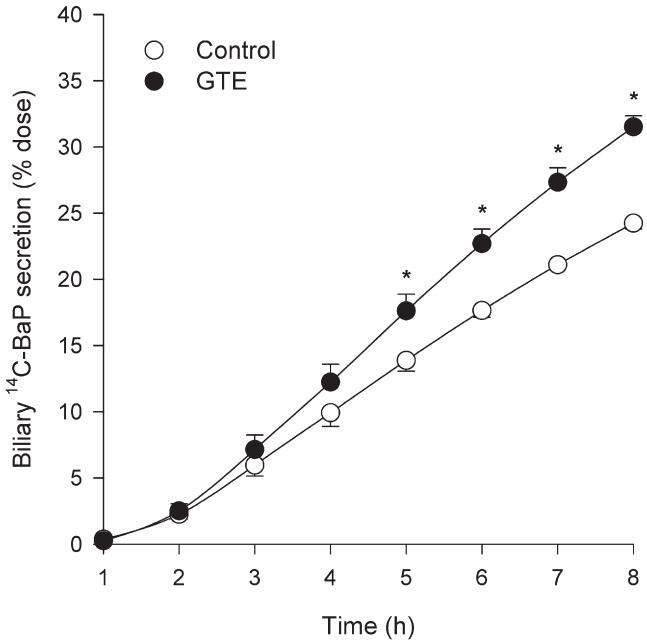

Fig. 2. Cumulative biliary secretion (% dose) of ^{14}C-BaP in rats infused with a lipid emulsion containing GTE. Values are means±S.D., n=5. Asterisks (*) denote significant differences at P<.05.

4. Discussion

The present study using rats with mesenteric lymph- and bile-duct cannula provides convincing evidence that GTE, at a dose equivalent to two to three servings of green tea per day in humans, drastically decreases the intestinal absorption and increases the biliary secretion of ^{14}C-BaP. The intestinal absorption of ^{14}C-BaP was decreased to approximately 50% of the control level, whereas the biliary secretion of enterally infused ^{14}C-BaP was increased by about 30%, by GTE. This was further evidenced by the significantly higher amounts of the ^{14}C-tracer present in the intestinal lumen and cecum of the rats infused with GTE. At present, little is known about the possible mechanisms underlying such effects of green tea. Evidence indicates that due to its extreme lipophilic property, BaP is solubilized in dietary (or luminal) lipids and absorbed through the intestine following the transport pathways for lipids. Studies demonstrated that the intestinal absorption of BaP is influenced by the presence of dietary fat [8,9] and the intraluminal conditions regulating lipolysis [9], micellarization [7–10] and transfer of lipids across the unstirred water layer [7,31] to the enterocyte. Once taken up by the enterocyte, it is packaged into chylomicrons and transported via the lymphatics into the circulation [7–9].

Ample evidence indicates that GTE and its catechins [16] inhibit the intestinal absorption of lipids [18]. The inhibitory effect of GTE has been attributed to its interference with the emulsification and hydrolysis in the intestinal lumen [16,29,30] and micellar solubilization of lipids [14,19], critical to their transfer to and subsequent uptake by the enterocyte. The extent of the inhibition by GTE or its catechins is greater with the lipids of extreme hydrophobicity such as α-tocopherol that are not readily emulsified or solubilized in mixed micelles, whereas GTE or its catechins have little or only a moderate inhibitory effect on less hydrophobic lipids such as fatty acids, hydrolytic products of triglyceride [16,17]. These observations are consistent with the drastic decrease in lymphatic ^{14}C-BaP absorption by GTE, whereas the lymphatic output of fatty acid (oleic acid as administered in triolein) remained unaffected by GTE. The cumulative lymphatic outputs of both ^{14}C-BaP and α-tocopherol were lowered by GTE to about 50% of their control levels. The hourly absorptive pattern for ^{14}C-BaP closely followed that for α-tocopherol.

BaP, subsequent to its uptake by the entrocyte, is extensively metabolized into highly soluble metabolites by Phase 1 cytochrome P450 enzymes (CYP1A1, CYP1A2 and CYP1B1) and Phase 2 enzymes (sulfotransferases, UDP-glucuronosyltransferases and glutathione *S*-transferases) [8,9,31,32]. A recent study [33] showed that the absence of intestinal P450 enzyme activity increased the tissue burden of orally administered BaP in mice, suggesting a critical role of intestinal BaP metabolism in protecting against systemic BaP exposure. Unlike intact BaP, the polar BaP metabolites formed in the enterocyte may be transported out of the cell into the intestinal lumen or delivered to the liver via the portal route. Studies using Caco-2 cells showed that the enterocyte expresses ATP-binding cassette (ABC) efflux proteins including breast cancer resistance protein (BCRP) that is involved in transporting BaP Phase 2 metabolites toward the apical direction [31,34]. Evidence from *in vitro* studies suggests that flavonoids such as quercetin induce the expression of Phase 1 enzymes [35] and also increase the apical efflux of BaP sulfates via BCRP [36]. Thus, it is possible that green tea flavonoids (catechins) may decrease the intestinal absorption by enhancing the efflux of BaP metabolites. At present, however, no evidence exists that GTE or its catechins regulate the efflux of BaP metabolites into the intestinal lumen involving ABC transporters including BCRP and other transporters such as P-glycoproteins and multidrug-resistant proteins. The soluble metabolites of BaP produced in the enterocyte can also be transported directly to the liver via the portal vein [7,8]. Although the lymphatic pathway via chylomicrons represents a major route for BaP absorption, an earlier study [37] using a ligated rat jejunal loop showed that a significant proportion (~40%) of the BaP dose was recovered in the portal blood largely as soluble metabolites. This suggests that the portal route may be a quantitatively important pathway for BaP absorption, depending on the extent of BaP metabolism in the enterocyte. In contrast to the lymphatic route that initially bypasses the liver and hence the hepatic first-pass metabolism, 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. Our study here using rats with bile-duct cannula but with intact mesenteric lymph duct showed that approximately 24% of the ^{14}C-BaP dose was secreted into the bile over the 8-h period without GTE infusion, whereas with GTE, the biliary secretion of the radiolabel was increased to 32%. The ^{14}C-radioactivity released into the bile may represent a combined pool of ^{14}C-labeled BaP metabolites derived from the metabolism of ^{14}C-BaP in both the intestine and the liver which takes up BaP carried by chylomicron remnants and other lipoproteins [40]. In this regard, it is worthy to note that irrespective of GTE, the total amount secreted into the bile was greater than that absorbed via the mesenteric lymph during the 8-h period. This finding is in agreement with the observation [38] that significantly greater amounts of the ^{3}H-radioactivity was found in the bile than in lymph following a single dose of ^{3}H-BaP in rats with both lymph- and bile-duct cannula. These observations further support the notion [33] that, in addition to the lymphatic route of absorption, the portal delivery of BaP (as metabolites) from the intestine to the liver may represent another important route for absorption and elimination of orally ingested BaP. Thus, the marked increase in biliary BaP secretion, as observed with GTE, may substantially reduce the body burden and carcinogenic potential/toxicity of BaP. At present, how green tea or tea catechins alter biliary BaP secretion is unknown. Green tea or catechins may facilitate the biliary excretion of BaP by increasing the activities of intestinal and hepatic Phase 1 and Phase 2 enzymes, as suggested previously [39]. Since the biliary output of BaP metabolites increases as the degree of BaP hydroxylation [40] and glucuronidation/sulfation increases [41], further studies are warranted to examine whether green tea or catechins induce the expression of these enzymes and/or efflux transporters in both the liver and intestine.

In summary, the present study using rats with lymph- and bile-duct cannula provides the first direct evidence that GTE, at a moderate level (equivalent to two to three cups per day in humans), effectively lowers the intestinal absorption of BaP and increases its biliary secretion. The inhibition of BaP absorption by GTE may be attributable to its ability to interfere with luminal lipolysis and micellar solubilization as previously demonstrated. Coupled with the drastic inhibition of intestinal BaP absorption, the marked increase in biliary BaP secretion by GTE suggests that green tea may be used as a dietary means of mitigating the toxicity and carcinogenic potential of BaP. At present, it is unknown whether GTE catechins or other components may directly interact with BaP in the intestinal lumen forming complexes unavailable for absorption. Further studies are needed to elucidate the mechanisms underlying the effects of green tea and examine whether green tea intake reduces the body burden and hence toxic effects of other PAH in humans.

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