

## Piperine Enhances the Bioavailability of the Tea Polyphenol (–)-Epigallocatechin-3-gallate in Mice<sup>1</sup>

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**ABSTRACT** (–)-Epigallocatechin-3-gallate (EGCG), from green tea (*Camellia sinensis*), has demonstrated chemopreventive activity in animal models of carcinogenesis. Previously, we reported the bioavailability of EGCG in rats (1.6%) and mice (26.5%). Here, we report that cotreatment with a second dietary component, piperine (from black pepper), enhanced the bioavailability of EGCG in mice. Intra-gastric coadministration of 163.8  $\mu\text{mol/kg}$  EGCG and 70.2  $\mu\text{mol/kg}$  piperine to male CF-1 mice increased the plasma  $C_{\text{max}}$  and area under the curve (AUC) by 1.3-fold compared to mice treated with EGCG only. Piperine appeared to increase EGCG bioavailability by inhibiting glucuronidation and gastrointestinal transit. Piperine (100  $\mu\text{mol/L}$ ) inhibited EGCG glucuronidation in mouse small intestine (by 40%) but not in hepatic microsomes. Piperine (20  $\mu\text{mol/L}$ ) also inhibited production of EGCG-3''-glucuronide in human HT-29 colon adenocarcinoma cells. Small intestinal EGCG levels in CF-1 mice following treatment with EGCG alone had a  $C_{\text{max}} = 37.50 \pm 22.50$  nmol/g at 60 min that then decreased to  $5.14 \pm 1.65$  nmol/g at 90 min; however, cotreatment with piperine resulted in a  $C_{\text{max}} = 31.60 \pm 15.08$  nmol/g at 90 min, and levels were maintained above 20 nmol/g until 180 min. This resulted in a significant increase in the small intestine EGCG AUC ( $4621.80 \pm 1958.72$  vs.  $1686.50 \pm 757.07$  (nmol/g·min)). EGCG appearance in the colon and the feces of piperine-cotreated mice was slower than in mice treated with EGCG alone. The present study demonstrates the modulation of the EGCG bioavailability by a second dietary component and illustrates a mechanism for interactions between dietary chemicals. *J. Nutr.* 134: 1948–1952, 2004.

**KEY WORDS:** • epigallocatechin-3-gallate • piperine • green tea • bioavailability • mice

Tea (*Camellia sinensis*) is a beverage with a worldwide popularity second only to that of water. Studies with animal models showed that green tea has preventive activity against cancer of the oral cavity, esophagus, stomach, intestine, colon, liver, lung, prostate, skin, and other sites (1). Epigallocatechin-3-gallate (EGCG)<sup>3</sup> is the major catechin component of green tea and may be a major active constituent (Fig. 1). Studies with human cancer cell lines have shown that EGCG possesses a number of activities related to cancer prevention, such as inhibition of activator protein 1 and nuclear factor  $\kappa\text{B}$  transactivation and epidermal growth factor receptor signaling. It is not known, however, whether these actions occur in animals or humans because of the limited bioavailability of EGCG following oral administration (1,2).

Previously, we reported that the absolute bioavailability of EGCG in CF-1 mice and Sprague-Dawley rats is 26.5 and 1.6%, respectively (3,4). In these studies, the glucuronidated, sulfated, and aglycone forms of EGCG were quantified as total

EGCG. EGCG undergoes methylation, glucuronidation, and sulfation in vivo; we showed that EGCG is largely present as the glucuronide in the plasma of treated mice (1,5,6). Modulation of the factors affecting EGCG bioavailability might increase plasma and tissue levels of this compound as well as its cancer preventive activity.

Piperine (Fig. 1), an alkaloid derived from black pepper (*Piper spp.*), has been reported to inhibit glucuronidation activity in rats and guinea pigs (7,8). Singh et al. (7) reported that piperine inhibited rat hepatocyte-mediated glucuronidation of 3-hydroxybenzo[a]pyrene with an  $\text{IC}_{50}$  of 50  $\mu\text{mol/L}$ . It was reported that coadministration of piperine and curcumin to humans and rats enhanced the bioavailability of curcumin by 2000% and 154%, respectively (9). The authors suggested that this increase was due to inhibition of the glucuronidation of curcumin.

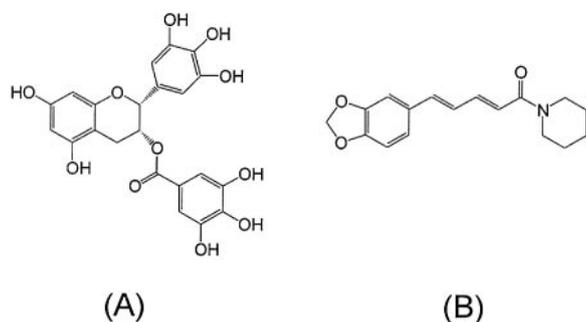
Because black pepper (world production was 47.6 million kg in 1999) and green tea are widely consumed dietary components, it is predictable that coexposure to piperine and EGCG will occur in dietary situations. We hypothesized that piperine may represent a potential dietary modulator of the bioavailability of EGCG by virtue of its ability to inhibit glucuronidation. Herein, we report the results of a study to determine whether coadministration of piperine affects the bioavailability of EGCG in mice.

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<sup>3</sup> Abbreviations used: AUC, area under the curve; CEAS, coulloch electrochemical detection; EGCG, epigallocatechin-3-gallate; GI, gastrointestinal tract; MRP, multidrug resistance related protein; PGP,  $\rho$ -glycoprotein.



**FIGURE 1** Structure of (A) epigallocatechin-3-gallate and (B) piperine.

## MATERIALS AND METHODS

**Chemicals.** EGCG (100% pure) was provided by Tokyo Food Techno.  $\beta$ -D-Glucuronidase (G-7896, EC 3.2.1.31, from *Escherichia coli* with 9 MU/g solid), sulfatase (S-9754, EC 3.1.6.1, from abalone entrails with 0.23 MU/g solid), and piperine were purchased from Sigma Chemical. All other reagents were of the highest grade commercially available. Dosing solutions of EGCG and piperine were prepared in 0.154 mol/L NaCl. For analytical purposes, a standard stock solution of EGCG, epigallocatechin, epicatechin, and epicatechin-3-gallate (10 mg/L each) was prepared in 11.4 mmol/L ascorbic acid–0.13 mmol/L EDTA (pH 3.8) and stored at  $-80^{\circ}\text{C}$ .

**Mice.** Male CF-1 mice (30–35 g) were purchased from Charles River Laboratories and allowed to acclimate for at least 1 week prior to the start of the experiment. The mice were housed 10 per cage and maintained in air-conditioned quarters with a room temperature of  $20 \pm 2^{\circ}\text{C}$ , relative humidity of  $50 \pm 10\%$ , and an alternating 12-h light/dark cycle. Mice were fed Purina Rodent Chow #5001 (Research Diets) and water and were allowed to eat and drink ad libitum. Mice were deprived of food for 12 h prior to the experiment.

**Microsomal glucuronidation activity.** The ability of piperine (0–500  $\mu\text{mol/L}$ ) to inhibit EGCG glucuronidation was determined using mouse hepatic microsomes prepared as previously described (10). Small intestinal microsomes were prepared in a similar manner with the following modifications. After mice were killed, the small intestine (between the pyloric sphincter and the cecum) was removed and the contents were rinsed with 10 mL of ice-cold Tris-HCl (2 mmol/L, pH 7.4, containing 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 150 mmol/L KCl). The intestine was cut open longitudinally and placed on a wetted glass plate, and the enterocytes were gently scraped from the intestine using a metal spatula. The enterocytes were collected and homogenized in a manner analogous to liver samples. Microsomal glucuronidation assays using EGCG (50  $\mu\text{mol/L}$ ) as the substrate were performed using the method of Lu et al. (6). The inhibitory activity of piperine is expressed as a percentage of glucuronidation in the vehicle-treated control reactions.

**Cell culture and treatment.** HT-29 human colon cancer cells (American Type Tissue Culture) were maintained at subconfluence in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 kU/L penicillin, and 0.1 g/L streptomycin at  $37^{\circ}\text{C}$  in 95% humidity and 5%  $\text{CO}_2$ .

Uptake studies using 10  $\mu\text{mol/L}$  EGCG were performed as previously described (11). To determine the effect of piperine on EGCG metabolism and uptake, cells were cotreated with 20  $\mu\text{mol/L}$  piperine and 10  $\mu\text{mol/L}$  EGCG. Cytosolic levels of EGCG and its metabolites were determined by HPLC and normalized to cytosolic protein concentrations.

**Treatment of mice and sample collection.** EGCG (163.8  $\mu\text{mol/kg}$ , i.g.) or EGCG plus piperine (163.8 and 70.2  $\mu\text{mol/kg}$ , i.g.) was administered to mice (6 per group). Mice were then killed at 60, 90, 180, and 300 min posttreatment, blood was collected via cardiac puncture, and plasma was isolated by centrifugation at  $500 \times g$  for 15 min. Plasma was combined with 0.1 vol of ascorbate preservative (1.14 mol/L ascorbic acid, 1.3 mmol/L EDTA) and stored at  $-80^{\circ}\text{C}$

for later analysis. Feces were collected from the interior of the colon following dissection. The small intestine and colon were collected, washed in 0.154 mol/L NaCl, and frozen at  $-80^{\circ}\text{C}$  for later analysis.

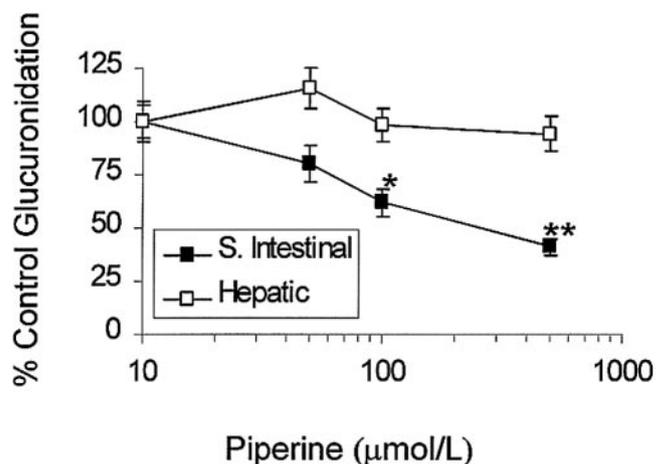
**Quantification of EGCG and metabolites.** Plasma levels of EGCG and its metabolites were analyzed as previously reported (4). Fecal samples were diluted 1:10 in 100 g/L ascorbate preservative and sonicated. A 20- $\mu\text{L}$  aliquot was then hydrolyzed with  $\beta$ -glucuronidase/sulfatase as described previously (3). Following hydrolysis, the sample was extracted twice with ethyl acetate. The organic phase was dried under vacuum, resuspended in 10% aqueous acetonitrile, and analyzed by HPLC-electrochemical detection (ECD). Tissue samples were homogenized in 114.0 mmol/L ascorbic acid and processed as previously described (3). Duplicate samples of feces, tissues, and plasma were prepared without sulfatase/ $\beta$ -glucuronidase treatment to determine the unconjugated fraction of EGCG and its metabolites. Samples were analyzed by HPLC-ECD.

**Sample analysis.** EGCG levels were analyzed using an HPLC system consisting of 2 ESA Model 580 dual-piston pumps, a Waters Model 717plus refrigerated autosampler, and an ESA 5500 coulchem electrode array system (CEAS). The potentials of the CEAS were set at  $-100$ , 100, 300, and 500 mV. Separation was achieved using previously described methods (12). The exposure (area under the curve, AUC) and maximum concentrations ( $C_{\text{max}}$ ) of EGCG were determined using Microsoft Excel. Values are means  $\pm$  SE. Differences were determined using Student's *t* test or ANOVA with Tukey's test, as appropriate, and were considered significant at  $P < 0.05$ .

## RESULTS

**Piperine inhibits small intestinal but not hepatic glucuronidation.** Mouse small intestinal and hepatic microsomes were both capable of rapidly glucuronidating EGCG on the B-ring and D-ring (data not shown). Piperine (50–500  $\mu\text{mol/L}$ ) dose-dependently inhibited the glucuronidation reaction with small intestinal microsomes, but not with hepatic microsomes (Fig. 2). At 100 and 500  $\mu\text{mol/L}$ , piperine inhibited small intestinal glucuronidation of EGCG by 40 and 60%, respectively. Hepatic glucuronidation of EGCG was not affected at these concentrations.

**Piperine affects glucuronidation but not accumulation of EGCG by HT-29 cells.** Incubation of HT-29 human colon cancer cells with 10  $\mu\text{mol/L}$  EGCG resulted in intracellular levels of  $90.4 \pm 5.4$ ,  $15.6 \pm 2.4$ , and  $35.8 \pm 4.3$  nmol/mg



**FIGURE 2** Piperine-mediated inhibition of mouse hepatic and small intestinal microsomes-mediated glucuronidation of EGCG. Each point represents the mean  $\pm$  SE,  $n = 3$ . Asterisks indicate different from control incubations performed in the absence of piperine: \* $P < 0.05$ , \*\* $P < 0.01$ .

protein of EGCG, EGCG-3''-glucuronide, and 4''-O-methyl-EGCG, respectively (Fig. 3). Cotreatment with 20  $\mu\text{mol/L}$  piperine reduced the concentration of EGCG-3''-glucuronide to  $8.1 \pm 2.1$  nmol/mg, but increased the concentration of 4''-O-methyl-EGCG to  $48.8 \pm 6.7$  nmol/mg (Fig. 3). The concentration of EGCG was unchanged. The total intracellular concentration of EGCG and its metabolites was unaffected by cotreatment with piperine ( $141.8 \pm 12.1$  vs.  $146.4 \pm 15.8$  nmol/mg, for EGCG plus piperine and EGCG alone, respectively).

**Piperine increases the plasma EGCG concentration.** Co-administration of piperine (70.2  $\mu\text{mol/kg}$ , i.g.) and EGCG (163.8  $\mu\text{mol/kg}$ , i.g.) substantially increased the plasma levels of EGCG in male CF-1 mice compared to those treated only with EGCG (Fig. 4A). The  $C_{\text{max}}$  of total EGCG following treatment with EGCG and piperine was 1.1-fold higher compared to treatment with EGCG only. Unconjugated EGCG was present at much lower levels in both treatment groups but was still substantially increased by cotreatment with piperine. Cotreatment with piperine increased the  $\text{AUC}_{60-300\text{min}}$  by 1.2- and 1.3-fold for total EGCG and unconjugated EGCG, respectively (Table 1). The ratio of unconjugated to total EGCG in mice treated with EGCG plus piperine ( $0.18 \pm 0.03$ ) did not differ from that in mice treated with EGCG alone ( $0.19 \pm 0.05$ ).

**Piperine affects gastrointestinal levels of EGCG in CF-1 mice.** Following coadministration of piperine and EGCG to male CF-1 mice, the levels of EGCG in the small intestine reached a maximum of  $43.3 \pm 20.0$  nmol/g tissue at 90 min and remained above 20 nmol/g until 180 min (Fig. 4B). In contrast, mice treated with EGCG alone had a maximal small intestinal level of  $44.2 \pm 26.7$  nmol/g at 60 min after dosing, after which EGCG levels declined rapidly and were only  $5.1 \pm 1.6$  nmol/g at 90 min after dosing (Fig. 4B). Piperine increased the small intestine EGCG AUC by 1.7-fold compared to intestinal tissue from mice treated with EGCG alone (Table 1).

The levels of EGCG in the colon following piperine coadministration were lower than that in mice treated with EGCG alone at the earliest time points but rose to equivalent levels by 300 min (Fig. 4C). The colon levels of EGCG in mice fed EGCG alone were substantially higher than that in mice cotreated with piperine (Table 1). Consistent with our previous results (3), EGCG was exclusively in the unconjugated

form in both the small intestine and the colon (data not shown).

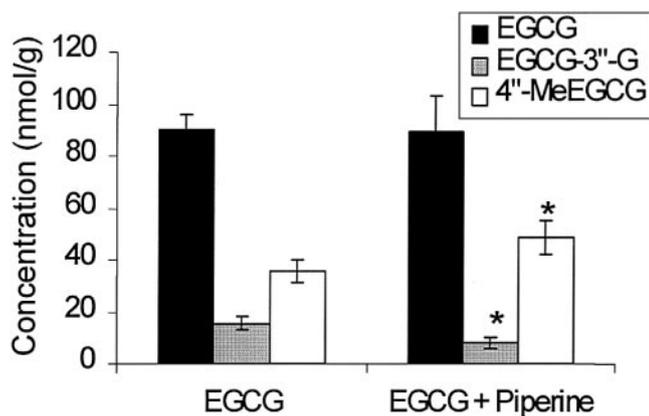
**Piperine decreases excretion of EGCG in the feces.** Cotreatment of mice with piperine and EGCG decreased the appearance of EGCG in the feces relative to mice treated with EGCG alone (Fig. 4D). At 60 min, the concentrations of EGCG in the feces were  $0.04 \pm 0.015$  and  $0.30 \pm 0.22$   $\mu\text{mol/g}$  in mice treated with EGCG plus piperine and EGCG alone, respectively. At 5 h, the levels of EGCG in the feces of piperine-cotreated mice ( $0.83 \pm 0.22$   $\mu\text{mol/g}$ ) still was less than in mice treated with EGCG alone ( $1.76 \pm 0.50$   $\mu\text{mol/g}$ ).

## DISCUSSION

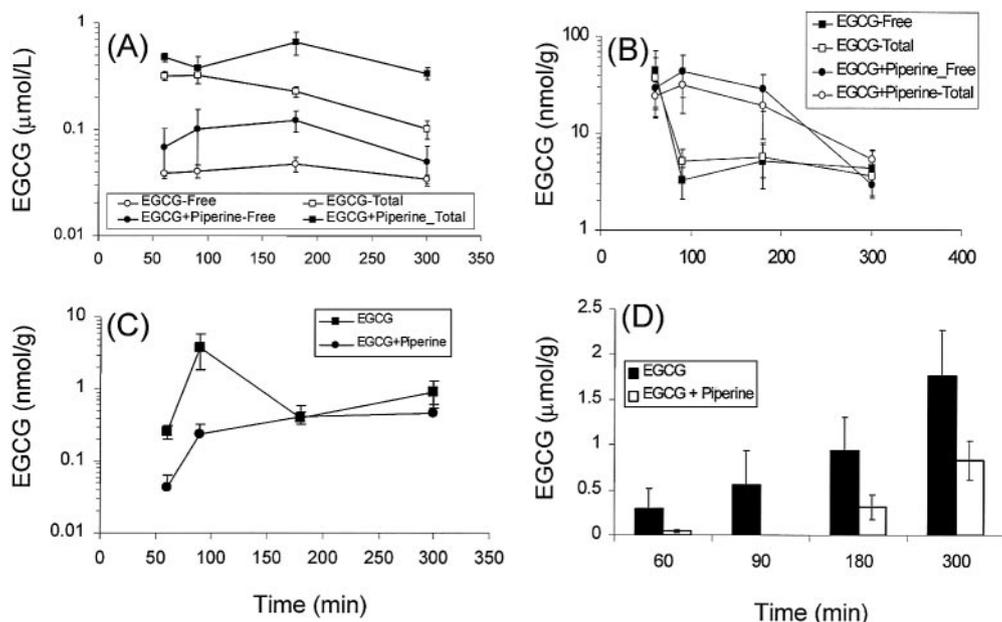
In the present study, we sought to determine whether the black pepper alkaloid, piperine, could serve as a potential dietary modulator of the bioavailability of the green tea catechin, EGCG, in mice. Previously, piperine was shown to inhibit the glucuronidation of 3-hydroxybenzo[a]pyrene by small intestinal and hepatic microsomes (7,13). Moreover, coadministration of piperine and curcumin substantially enhanced the bioavailability of curcumin in humans and rats compared to treatment with curcumin only (9). Given that EGCG is subject to glucuronidation, we hypothesized that piperine could inhibit this glucuronidation and therefore increase EGCG bioavailability in mice.

In the current study piperine dose-dependently inhibited glucuronidation of EGCG by small intestinal but not hepatic microsomes (Fig. 2). This difference in sensitivity may be the result of differential expression of UGT isoforms in these 2 tissues. Piperine also inhibited EGCG glucuronidation in HT-29 human colon cancer cells. In this model, cotreatment of cells with EGCG and piperine did not increase the total cytosolic level of EGCG and its metabolites. It did, however, decrease the level of EGCG-3''-glucuronide and concomitantly increase the level of 4''-O-methyl-EGCG compared to cells treated only with EGCG.

Following cotreatment of male CF-1 mice with EGCG (163.8  $\mu\text{mol/kg}$ , i.g.) and piperine (70.2  $\mu\text{mol/kg}$ , i.g.), plasma levels of total and unconjugated EGCG increased by 1.1- and 1.4-fold, respectively, compared to mice treated with EGCG alone. Piperine also substantially increased the AUC of both total and unconjugated EGCG by 1.2- and 1.3-fold, respectively. We predicted that if piperine inhibited glucuronidation in vivo, it would increase not only the total amount of EGCG in the plasma, but also the ratio of unconjugated to total EGCG. We found, however, that cotreatment with piperine did not affect this ratio. Selective inhibition of small intestinal glucuronidation by piperine could increase the absorption of EGCG into the portal circulation, but may not affect the overall profile of EGCG glucuronidation since the compound could be subsequently glucuronidated by the liver. The results of our inhibition studies with mouse hepatic and small intestinal microsomes support this hypothesis. An additional potential confounder for the hypothesis that piperine increased EGCG bioavailability by inhibiting glucuronidation is the observed compensation by methylation in the HT-29 cells. These results suggest that any inhibition of glucuronidation is compensated for by methylation, which would negate any increase in EGCG bioavailability. However, we previously reported that the capacity for glucuronidation of EGCG is greater than the capacity for its methylation in the mouse small intestine (5,6). Based on this, we suggest that even if methylation of EGCG compensates for some of the piperine-mediated inhibition of glucuronidation, the increased amount of free EGCG may exceed methylation capacity and result in



**FIGURE 3** Intracellular accumulation of EGCG, EGCG-3''-glucuronide, or 4''-O-methyl-EGCG by HT-29 human colon cancer cells treated with EGCG (10  $\mu\text{mol/L}$ ) or EGCG (10  $\mu\text{mol/L}$ ) plus piperine (20  $\mu\text{mol/L}$ ). Bars represent the means  $\pm$  SE,  $n = 3$ . \*Different from EGCG alone,  $P < 0.05$ .



**FIGURE 4** Plasma (A), small intestinal (B), colonic (C), and fecal (D) levels of EGCG following intragastric administration of either EGCG (163.8 μmol/kg) or EGCG plus piperine (70.2 μmol/kg) to male CF-1 mice. Each point represents the mean ± SE, n = 6.

a net increase in EGCG. The extent to which methylation compensates for glucuronidation following piperine treatment remains to be determined in vivo.

The lack of change in the ratio of total to unconjugated EGCG suggests that other factors might also play a role in the piperine-mediated increase in EGCG bioavailability. Piperine was shown to substantially inhibit gastric emptying and gastrointestinal transit in mice and rats (13). Inhibition of gastrointestinal transit was demonstrated to increase the bioavailability of many drugs (14,15).

We hypothesized that piperine, if it increased EGCG plasma levels by inhibiting gastrointestinal transit, should also increase both the levels and the residence time of EGCG in the small intestine, while simultaneously delaying the arrival of EGCG in the colon and excretion in the feces. Consistent with this hypothesis, we found that following piperine treatment, the levels of EGCG in the small intestine remained higher for a longer period of time compared to mice treated only with EGCG. The small intestine EGCG AUC was in-

creased by 1.7-fold by cotreatment with piperine. Appearance of EGCG in the colon of mice cotreated with piperine was somewhat delayed and levels remained lower than in mice treated only with EGCG. Finally, EGCG appeared in the feces of mice cotreated with piperine at a slower rate than in mice treated only with EGCG. These changes in the gastrointestinal levels and kinetics of EGCG support the hypothesis that piperine increased plasma levels at least in part by delaying transit through the gastrointestinal (GI) tract. Alternatively, the changes in fecal and colon levels of EGCG may reflect an increase in the net overall absorption of EGCG from the upper gastrointestinal tract, thereby decreasing the pool reaching the colon and feces for absorption or excretion. Further studies are needed for clearer delineation.

Piperine was also reported to inhibit digoxin and cyclosporine A transport by *p*-glycoprotein (PGP) in Caco-2 cells; this action might also be hypothesized to affect EGCG bioavailability (16). However, we previously showed that EGCG is a substrate for the multidrug resistance related protein (MRP)-1

**TABLE 1**

*Effect of piperine on AUC and C<sub>max</sub> of EGCG in the plasma and tissues of male CF-1 mice after intragastric administration of EGCG or EGCG plus piperine<sup>1</sup>*

	C <sub>max</sub>		AUC <sub>60-300</sub>	
	EGCG	EGCG + Piperine	EGCG	EGCG + Piperine
	μmol/L		μmol/L · min	
Plasma				
Unconjugated	0.05 ± 0.01	0.12 ± 0.04 <sup>a</sup>	9.95 ± 1.09	22.67 ± 4.18 <sup>a</sup>
Total	0.32 ± 0.05	0.66 ± 0.16 <sup>a</sup>	53.82 ± 7.95	118.71 ± 24.99 <sup>a</sup>
	nmol/g		nmol/g · min	
S. intestine, total <sup>2</sup>	37.50 ± 22.50	31.60 ± 15.68	1686.50 ± 757.07	4621.80 ± 1958.72 <sup>a</sup>
Colon, total <sup>2</sup>	3.76 ± 1.93	0.47 ± 0.14 <sup>a</sup>	325.10 ± 109.58	85.20 ± 25.44 <sup>a</sup>

<sup>1</sup> Values are means ± SE, n = 6. <sup>a</sup> Different from the EGCG only group, Student's *t* test, *P* < 0.01.

<sup>2</sup> All unconjugated.

and MRP-2 but not for PGP (11). It is likely, therefore, that this activity of piperine is unrelated to its ability to enhance EGCG bioavailability.

In conclusion, the present results demonstrate that piperine, a component of the widely consumed spice black pepper, can increase the bioavailability of EGCG, a component of green tea. Mechanistically, it appears that piperine inhibits small intestinal glucuronidation of EGCG, which may result in increased absorption, and that piperine may also slow the GI transit of EGCG, thus increasing residence time in the intestine and allowing for greater absorption. More in-depth mechanistic studies are required to fully establish the relative importance of each of these mechanisms. The increase in plasma bioavailability for EGCG may improve its cancer preventive activity *in vivo*. Moreover, the effect of a second dietary component on the bioavailability of EGCG suggests that variations in dietary habits may impact the outcome of epidemiological studies of the health effects of green tea. Such effects should be further studied.

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