ENZYMEOLOGY OF METHYLATION OF TEA CATECHINS AND INHIBITION OF CATECHOL-O-METHYLTRANSFERASE BY (−)-EPIGALLOLATECHIN GALLATE

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ABSTRACT:

(−)-Epigallocatechin gallate (EGCG) and (−)-epigallocatechin (EGC) are the major polyphenolic constituents in green tea. In this study, we characterized the enzymology of cytosolic catechol-O-methyltransferase (COMT)-catalyzed methylation of EGCG and EGC in humans, mice, and rats. At 1 μM, EGCG was readily methylated by liver cytosolic COMT to 4′-O-methyl-EGCG and then to 4′,4″-di-O-methyl-EGCG; EGC was methylated to 4′-O-methyl-EGC. The Km and Vmax values for EGCG methylation were higher than those for EGC; for example, with human liver cytosol, the Km were 4.0 versus 0.16 μM and Vmax were 1.28 versus 0.16 nmol/mg/min. Rat liver cytosol had higher COMT activity than that of humans or mice. The small intestine had lower specific activity than the liver in the methylation of EGCG and EGC.

Glucuronidation on the B-ring or the O-ring of EGCG greatly inhibited the methylation on the same ring, but glucuronidation on the A-ring of EGCG or EGC did not affect their methylation. Using EGCG and 3,4-dihydroxy-L-phenylalanine as substrates, EGCG, 4′-O-methyl-EGCG, and 4′,4″-di-O-methyl-EGCG were all potent inhibitors (IC50 < 0.2 μM) of COMT. The COMT-inhibiting activity of (−)-EGCG-3″-O-glucuronide was similar to EGCG, but (−)-EGCG-4″-O-glucuronide was less potent. The present work provides basic information on the methylation of EGCG and suggests that EGCG may inhibit COMT-catalyzed methylation of endogenous and exogenous compounds.

Green tea has been suggested to have beneficial effects against many diseases including cancer, cardiovascular disease, and Parkinson’s disease (Yang and Landau, 2000; Yang et al., 2002). The active constituents and mechanisms involved, however, are not known. (−)-Epigallocatechin gallate (EGCG) and (−)-epigallocatechin (EGC) are considered to be the major effective constituents of green tea. The bioavailability and biotransformation of tea catechins are not well understood, even though several studies on this topic have been published (Chen et al., 1997; Yang et al., 1998; Li et al., 2001; Meng et al., 2001, 2002). After oral absorption, tea catechins undergo extensive methylation, glucuronidation, and sulfation (Li et al., 2001; Meng et al., 2001). After oral administration of green tea, 4′-O-methyl-EGC (4′-MeEGC) and 4′,4″-di-O-methyl-EGCG (4′,4″-DiMeEGCG) were detected in the blood and urine samples of humans, mice, and rats (Meng et al., 2001, 2002). The biliary excretion of EGCG methylation products varied with the change of EGCG dosage (Kida et al., 2000; Kohri et al., 2001), suggesting that the methylation of EGCG is dose-dependent.

Studies on the methylation of 30 structurally diverse compounds by human and rat recombinant soluble COMT showed that human and rat soluble COMT had similar substrate selectivity (Lautala et al., 1999). Incubations of catechins with rat liver homogenate and S-adenosylmethionine (SAM) produced 4′-O-methyl-(−)-EGC (4′-MeEGC), 4′-O-methyl-(−)-EGCG (4′-MeEGCG), and 4′-O-methyl-(−)-epicatechin (Okushio et al., 1999). Although the enzymatic methylation of EGCG and EGC has been reported (Lautala et al., 1999; Zhu et al., 2000, 2001), most of these studies used rather high concentrations of EGCG (10–500 μM), which were far above the levels achievable in the plasma and tissues after ingesting green tea. The kinetic parameters of EGCG methylation by human liver and the contribution of methylation to the biotransformation of EGCG remain unknown.

COMT is present in both soluble and membrane-bound forms in mammals, with the soluble form predominant in most tissues. COMT catalyzes the O-methylation of various catecholic compounds. The general function of COMT is to eliminate the potentially active or toxic catechol structures of endogenous and exogenous compounds. Methylation decreases the hydrophilicity of catecholic compounds; further sulfation/glucuronidation of the methylated product is usually needed for the effective elimination of the methylation product from the body. COMT has been found in all mammalian tissues investigated, with the highest activity in the liver, then the kidney and gastrointestinal tract. The COMT activity in erythrocytes varies from species to species, high in rats and low in humans (Nissinen et al., 1992; Keranen et al., 1994).
3,4-Dihydroxy-l-phenylalanine (l-DOPA), catecholamines (dopamine, norepinephrine, epinephrine) and catecholestrogens are physiological substrates of COMT. Certain dietary and medicinal products, such as flavonoids, carbidopa, and dihydroxyphenylserine, are also COMT substrates (Mannisto and Kaakkola, 1999; Lautala et al., 2001). Inhibition of COMT may significantly affect the metabolism of these compounds. Flavonoids are known to inhibit COMT (Gugler and Dengler, 1973). EGCG has been suggested to exhibit a flattening effect through inhibition of COMT, which augments and prolongs norepinephrine-regulated thermogenesis (Dulloo et al., 2000). The inhibition of COMT by EGCG remains to be further characterized.

The present study aims to elucidate the enzymology of methylation of EGCG and EGC by COMT. The species difference among humans, rats, and mice in the methylation of catechins, the effect of glucuronidation on catechin methylation, and the inhibition of COMT activity by EGCG and its metabolites are also investigated.

Materials and Methods

Chemicals and Reagents. EGCG was a gift from Unilever-Bestfoods (Englewood Cliffs, NJ). EGC and (−)-epicatechin gallate (ECG) were purified; their structures were identified by NMR and mass spectrum in our laboratory (Hu et al., 1999). EGC and ECG were purchased from Sigma-Aldrich (St. Louis, MO). The protein assay kit was obtained from Bio-Rad Labs (Heracles, CA). Other reagents and HPLC-grade solvents were purchased from VWR Scientific Products (West Chester, PA). Pooled human liver cytosol samples were obtained from BD Biosciences (Woburn, MA) in a frozen well preserved state.

Treatment of Animals and Preparation of Cytosol. Eight-week-old female CF-1 mice and male Sprague-Dawley rats were purchased from Jackson Laboratory (Bar Harbor, ME). All mice and rats were fed Purina Laboratory Chow 5001 (Purina, St. Louis, MO) diet and allowed one week acclimation. Afterwards, eight mice and five rats were sacrificed. Liver and intestines were promptly removed, washed with ice-cold saline, and samples were pooled for preparation of microsomes and cytosol by differential ultracentrifugation (Hong et al., 1989). The protein content was determined according to the instruction of the Bio-Rad protein assay kit.

Methylation of Catechins and Their Glucuronides by Cytosolic COMT. The methylation of substrates by cytosolic COMT was conducted at conditions similar to our previous studies (Meng et al., 2001). Incubation mixture contained 0.1 mg hepatic or intestinal cytosolic protein, different concentrations of catechins or their glucuronides, 10 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 1.2 mM MgCl2, and 0.05–0.2 mM SAM in a total volume of 0.1 ml. To determine the IC50 of COMT inhibition, 0.1 mg rat hepatic cytosolic protein, 10 μM EGC, 0.05 mM SAM, and different concentrations (0.05–25.6 μM) of COMT inhibitors were used in a 10-min incubation. For studies on the mechanism of inhibition of COMT, 0.1 mg rat hepatic cytosolic protein, 0.2 mM SAM and different concentrations (10, 20, 40, 60, 100, and 160 μM) of EGC were used as substrates in a 5-min incubation; alternatively, 1.0 mM EGC and different concentrations of SAM (12.5, 25, 50, 100, 200, and 400 μM) were used. The reaction was started by the addition of SAM and stopped by adding 0.1 ml of ice-cold methanol containing 1% ascorbic acid after incubation at 37°C for the stated length of time. After centrifugation, 20 μl of the supernatant was used for the analysis of methylation products with HPLC or LC/MS/MS.

Methylation of l-DOPA by Porcine Liver COMT. Similar conditions with slight modifications were used for enzymatic methylation of l-DOPA.

The reaction mixture consisted of 100 μM l-DOPA, 25 units porcine liver COMT, 60 mM SAM, 1.2 mM MgCl2, and 1.0 mM dihydrothiol in a final volume of 100 μl of 10 mM Tris-HCl buffer (pH 7.4). The reaction was stopped by adding 20 μl of perchorlic acid after incubation for 30 min at 37°C. After vortexing and centrifugation, 50 μl of aqueous phase was injected onto HPLC to analyze the conversion of l-DOPA to 3-MeDOPA.

Glucuronidation of Methylated Catechins in Liver Microsomes. The reaction mixture consisted of 0.2 mg of mouse liver microsomal protein, different concentrations of methylated catechins, 1 mM UDPGA, 0.15 mM ascorbic acid, 2 mM MgCl2, 0.02% Triton-X-100, 1 mM saccharic acid-l,4-lactone, and 40 mM Tris-HCl buffer (pH 7.5) in a final volume of 100 μl. After incubation for 30 min at 37°C, the reaction was stopped by 100 μl of ice-cold methanol containing 1% ascorbic acid. After centrifugation at 10,000 g for 10 min, 160 μl of the supernatant was vortexed with 200 μl of methylene chloride to remove Triton-X-100 and lipids. After centrifugation, the supernatant (10 μl) was analyzed by LC/MS/MS.

HPLC Analysis of Methylated Catechins and 3-MeDOPA. Our previous HPLC method (Meng et al., 2001) was used with modifications. The eluent started with 4% buffer B and 96% buffer A from 0 to 7 min; then the linear binary gradient was changed by increasing buffer B to 22% at 25 min, 38% at 31 min, 41% at 37 min, and 98% at 38 min. It was maintained at 98% buffer B from 38 to 42 min and changed back to 4% at 43 min. The total running time was 54 min. A coulochem electrode array system with potential settings of −100, 100, 300, and 500 mV in the four channels was used to analyze catechins and their metabolites simultaneously. EGCG, EGC, 4′-MeEGC, and their glucuronides were detected in channel 2, whereas 4′,4″-DiMeEGC, 4′-MeEGC, 3′-MeEGC, and their glucuronides could only be detected in channel 3 and 4 (Fig. 1). The detection limits (S/N = 3) of catechins and methylated catechins were 5 to 10 ng/ml. 3-MeDOPA was detected with retention times of 7.5 min in channel 3. The detection limit was also 5 to 10 ng/ml. The formations of 4′-MeEGGC, 4′,4″-DiMeEGGC, 4′-MeEGC, 3′-MeEGC, and 3-MeDOPA were quantified with standard curves of these compounds.

LC/MS/MS Analysis of Methylated Catechins. The HPLC conditions were similar to previously described (Li et al., 2001) with modifications. The LC/MS/MS system consisted of a Finnigan SpectraSystem separation module equipped with a AS3000 refrigerated autosampler, a P4000 gradient pump, and a UV6000LP UV/VIs detector, followed by a Finnigan LCQmax mass detector (San Jose, CA) fitted with an electrospray ionization source. A 3-μm Supelcosil LS C18 column (75 mm × 2.1 mm i.d.; Supelco, Bellefonte, PA) was used, and the flow rate was maintained at 0.2 ml/min. The linear gradient was 100% A (0–3 min), 90% A to 67% A (3–17 min), 67% A to 10% A (17–30 min), and 100% A (30–40 min). Solvent A was methanol/water (5:495, v/v), and Solvent B was methanol/water (450:50, v/v). The LCQmax ion trap mass detector was operated in negative ion polarity mode, and the acquisition time was set for 40 min. EGCG or EGC-7-Gluc was used for the tune-up selection of optimal settings for the MS detector to detect methylated catechins or methylation products of catechin glucuronides, respectively. The deprotonated molecules exhibiting the same molecular mass as the target catechin conjugates were selected and dissociated with 30% relative collision energy to form product ions. If a deprotonated aglycone ion of tea catechins or their metabolites appeared in the product-ion mass spectrum, the assigned molecule was confirmed as a certain methylation product or glucuronide of catechins. The chemical identity of the methylated catechins was determined by comparing their retention time and fragment patterns with those of the standards. The deprotonated aglycone ions for EGCG, EGC, 4′-MeEGC, 4″-MeEGC, and 4′,4″-DiMeEGGC were at m/z 457, 305, 319, 471, and 485, respectively.

Data Analyses. The kinetic parameters (Km and Vmax) of methylation were calculated with GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). The values obtained represent the best-fit values ± standard error. Two-way analysis of variance was performed with Microsoft Excel software (Microsoft, Redmond, WA) for evaluating statistical differences between different groups of data. Differences were considered significant when P < 0.05.

Results

Time Course of Methylation of EGCG, 4′-MeEGCG, and EGC by Rat Liver Cytosolic COMT. The possible sites of methylation...
and glucuronidation of EGCG and EGC, based on our present and previous studies, are summarized in Fig. 2. In the presence of SAM, EGCG was readily methylated to 4′-MeEGCG by rat liver cytosolic COMT (Fig. 3A). The level of 4′-MeEGCG reached a maximum within 5 min and then started to decrease. The production of 4′,4″-DiMeEGCG was low before 2.5 min and increased markedly after 5 min. This result suggests a sequential methylation of EGCG to 4′-MeEGCG and then to 4′,4″-DiMeEGCG. The results in Fig. 3B indicated the second step is slower than the first step. The methylation of EGC was linear within 5 min at an initial rate similar to that of EGCG (Fig. 3C). At 10 min, the level of 4′-MeEGC reached a plateau, but the level of 4′-MeEGCG decreased due to its conversion to 4′,4″-DiMeEGCG. Upon incubating EGCG, EGC, or EGCG-4′-Gluc with rat liver cytosol in the presence of SAM, the disappearance of the substrate corresponded to the formation of the methylated product. Figure 3D showed that EGCG-4′-Gluc was also methylated, although at a slightly slower rate than EGCG.

**Concentration-dependent Methylation of EGCG and EGC in Humans, Rats, and Mice.** Methylation of EGCG and EGC resulted in the formation of 4′-MeEGCG and 4′-MeEGC as the major products in short incubation (2 or 5 min), respectively, and the product formation approximately followed Michaelis-Menten kinetics (Fig. 4). The apparent kinetic parameters of methylation of EGCG and EGC by the liver cytosolic COMT of humans, mice, and rats are summarized in Table 1. In all three species, the $K_m$ values of EGCG for COMT were much lower than EGC and the catalytic efficiencies ($V_{max}/K_m$) for the methylation of EGCG were 3- to 9-fold higher than that for EGC. Rat liver had the highest $V_{max}$ values in methylating EGCG and EGC, whereas human liver had the highest $V_{max}/K_m$ values for the methylation of EGCG and EGC.

**Effect of Substrate Concentration on the Product Profile of EGCG Methylation.** Previous in vivo studies suggest that the product profile of EGCG methylation is dose-dependent (Kida et al., 2000; Kohri et al., 2001; Meng et al., 2002). To further investigate this phenomena, concentration-dependent methylation of EGCG and 4′-MeEGCG by rat liver cytosol was studied using a 20-min incubation. At low concentrations ($<1.0 \mu M$) of substrate, methylation of 4′-MeEGCG and EGC proceeded at the same rate, but at high concentrations ($>3.0 \mu M$) of substrate, EGCG was the preferred substrate (Fig. 5A). Methylation of EGCG formed 4′-MeEGCG and 4′,4″-DiMeEGCG, and they were plotted separately as percentages of their sum in Fig. 5B. 4′,4″-DiMeEGCG was the predominant product in incubations with low concentrations ($<1.0 \mu M$) of EGCG, whereas 4′-MeEGCG was the predominant product in incubations with high concentrations ($>3.0 \mu M$) of EGCG (Fig. 5B).

**Methylation of EGCG and EGC by Small Intestinal Cytosolic COMT.** The methylation of EGCG by small intestinal cytosolic COMT (Fig. 6A) was approximately 10-fold slower than that by the liver cytosolic COMT (Fig. 5B). In contrast to the very low capacity
of EGCG methylation (Fig. 6A), the activities of EGCG methylation by small intestinal cytosolic COMT were much higher (Fig. 6B). Rats had much higher intestinal COMT activities than mice with either EGCG or EGC as the substrate.

**Effect of Glucuronidation on Methylation of EGC and EGCG.**
EGC-7-Gluc was readily methylated by rat liver COMT (data not shown). When EGC-7-Gluc and EGCG (each at 10 µM) were coincubated with rat liver cytosol, the methylation of EGC-7-Gluc or EGCG was each inhibited by 50%, indicating that glucuronidation of EGC on the A-ring has no significant effect on its interaction with COMT. After incubation of rat liver cytosol with 10 µM of EGC-3′-Gluc or EGC-7-Gluc, the peak height of methylated EGC-3′-Gluc was only 1% that of methylated EGC-7-Gluc (analyzed by LC/MS/MS), suggesting that methylation of the B-ring is inhibited by the glucuronidation at the 3′-position.

Similarly, the methylation of EGCG was not affected by glucuronidation at the A-ring, but significantly hindered by glucuronidation at the B-ring. For example, after incubation of EGCG-7-Gluc with rat liver cytosol and SAM for 20 min, large peaks of both mono- and di-methylated EGCG-7-Gluc were detected (by LC/MS/MS). Methylation of EGCG-3′-Gluc, however, resulted in the formation of a mono-methylated product as the major product and only a very small amount of a di-methylated product (data not shown). The mono-methylated product of EGCG-3′-Gluc (Fig. 7A) had the same product-ion spectrum as the glucuronide of 4′-MeEGCG (Fig. 7B), suggesting that the methylation of EGCG-3′-Gluc is on the 4′-position of the d-ring. With 4′-position glucuronidation in EGCG-4′-Gluc, the only catechol site for COMT is on the B-ring. Two clear peaks of methylated EGCG-4′-Gluc with retention times of 24.4 (P1) and 24.9 (P2) min were observed (Fig. 7C). The product-ion m/z 485 (Fig. 7, D and E) indicated the formation of di-methylated products, and P1 and P2 were tentatively identified as 3′,4′-di-O-methyl-EGCG-4′-Gluc and 3′,5′-di-O-methyl-EGCG-4′-Gluc. The 3′- and 5′-positions are symmetrical.

**Inhibition of COMT Activity by Catechins and Their Derivatives.** In view of the low \( K_m \) value of EGCG methylation, we investigated possible inhibitory effects of EGCG on the activities of cytosolic COMT with EGC as a substrate. The hepatic activities of COMT of rats and mice were inhibited by EGCG (Fig. 8A), with a similar IC\( _{50} \) of \(~0.15\) µM. Rat liver cytosol was used in all of the subsequent studies on the inhibition of COMT due to the abundance of enzyme.

To elucidate the structural basis for the inhibition of COMT by EGCG, the inhibitory activities of EGCG, EGCG metabolites, and EGCG analogs were investigated using EGCG (10 µM) as a substrate. All the methylated EGCG and EGCG glucuronides in Fig. 2 showed COMT-inhibitory activities (Table 2). EGCG-7-Gluc was less effective than EGCG, and EGCG-4′-Gluc was only one tenth as effective. EGC, which has one less hydroxyl group than EGCG on the B-ring, also potently inhibited COMT; whereas pyrogallol, which only contains the 3,4,5-trihydroxyl moiety of EGCG, was much less potent.

When L-DOPA was used as a substrate, EGCG and its derivatives showed similar potency as they inhibited the methylation of EGC (Table 2). The inhibitory activities of the four major catechins followed the order of EGCG > EGC > EGC > (-)-epicatechin. Methylation and glucuronidation of EGC on the B-ring greatly decreased their COMT-inhibiting activities.

**Mechanism of Inhibition of COMT Activity by EGCG and Methylated EGCG.** The inhibition of EGCG methylation by EGCG was uncompetitive with respect to SAM (Fig. 8B), suggesting that there is no direct interaction between EGCG and the SAM-binding site on the COMT. With a saturating concentration of SAM (200 µM), the \( V_{\text{max}} \) (pmol/mg/min) and \( K_m \) (µM) values for the methylation of EGCG by rat liver cytosol were 2752 ± 76 and 12.8 ± 1.5, respectively; EGCG displayed a mixed-type inhibition of COMT activity with respect to EGC (Fig. 8C); with the increase of EGCG concentrations, the \( V_{\text{max}} \) values for EGC methylation decreased, whereas the corresponding \( K_m \) values increased. In the presence of different concentrations of 4′-MeEGCG (Fig. 8D) and 4′,4′-DiMeEGCG (Fig. 8E), the \( V_{\text{max}} \) values for EGC methylation decreased in a concentration-dependent manner, whereas the corresponding \( K_m \) values were unchanged, indicating a noncompetitive mechanism of enzyme inhibition with respect to EGC. 4′-MeEGCG appeared to be more potent than 4′,4′-DiMeEGCG in inhibiting COMT activity (Fig. 8, D and E). The Lineweaver-Burk plot (Fig. 8F) indicated that 4′,4′-DiMeEGCG also inhibited the methylation of L-DOPA in a noncompetitive manner.

**Discussion**
Our current studies demonstrate the rapid O-methylation of EGCG and EGC by liver cytosolic COMT and the potent inhibition of cytosolic COMT by EGCG and its metabolites. The much lower \( K_m \) and higher \( V_{\text{max}}/K_m \) values of EGCG methylation than EGC methylation (Table 1) suggest that EGCG is a better substrate for COMT than EGC. The O-methylation at the 4′-position of EGCG was rapid, suggesting that the B-ring of catechins is a favorable site for the COMT. With EGCG, the methylation on the 4′-position was strongly favored over the 4″-position, suggesting a higher affinity of the d-ring than the B-ring to COMT. Zhu et al. (2000, 2001) reported that the methylation of EGCG by rat liver cytosolic COMT was considerably slower than that of EGC. This is probably due to the high concentrations of EGCG (10–100 µM) and long incubation time (20 min) used.

When longer incubation time (20 min) was used for the methylation of EGCG (Fig. 5), 4′,4′-DiMeEGCG was the major product at low EGCG concentrations (<1 µM). This is consistent with in vivo results that after oral administration of green tea, 4′,4′-DiMeEGCG was the major metabolite of EGCG detected in the urine samples of humans, mice, and rats (Meng et al., 2002). After an i.v. injection of 4 mg of EGCG, 4′,4′-DiMeEGCG was also the major metabolites excreted in the bile of rats (Kohri et al., 2001). At higher concentrations (>3 µM) of EGCG, 4′-MeEGCG was the predominant product (Fig. 5B). In vivo, when a high dose of EGCG (100 mg) was administered orally,
MeEGCG was the major methylation product of EGCG excreted in the bile of rats (Kida et al., 2000). In phase II metabolism in vivo, EGCG could be methylated first and then conjugated by glucuronidation or sulfation or be conjugated first and then methylated. The lower $K_m$ values (0.2–0.5 μM) for EGCG methylation (Table 1) than those for glucuronidation (50 μM) by human and rodent liver microsomes (Lu et al., 2003) suggest that with low concentrations of EGCG (e.g., after drinking green tea), methylation of EGCG would proceed to form 4′-MeEGCG and then 4′,4″-DiMeEGCG, and this indeed has been observed in vivo (Kohri et al., 2001; Meng et al., 2002). At very high doses of EGCG, glucuronidation may become more prominent because the $V_{max}$ (Lu et al., 2003) is higher than the methylation reaction. For example, EGCG-4′-Gluc is the sole glucuronide formed in the mouse small intestine and the major glucuronide formed in the mouse liver (Lu et al., 2003). This 4′-glucuronide can be methylated on the B-ring (Fig. 7, C-E). Therefore, different mono- and di-methylated products would be formed. This prediction is consistent with the observation that after 4′-MeEGCG was the major methylation product of EGCG excreted in the bile of rats (Kida et al., 2000).

In phase II metabolism in vivo, EGCG could be methylated first and then conjugated by glucuronidation or sulfation or be conjugated first and then methylated. The lower $K_m$ values (0.2–0.5 μM) for EGCG methylation (Table 1) than those for glucuronidation (≥30 μM) by human and rodent liver microsomes (Lu et al., 2003) suggest that with low concentrations of EGCG (e.g., after drinking green tea), methylation of EGCG would proceed to form 4′-MeEGCG and then 4′,4″-DiMeEGCG, and this indeed has been observed in vivo (Kohri et al., 2001; Meng et al., 2002). At very high doses of EGCG, glucuronidation may become more prominent because the $V_{max}$ (Lu et al., 2003) is higher than the methylation reaction. For example, EGCG-4′-Gluc is the sole glucuronide formed in the mouse small intestine and the major glucuronide formed in the mouse liver (Lu et al., 2003). This 4′-glucuronide can be methylated on the B-ring (Fig. 7, C-E). Therefore, different mono- and di-methylated products would be formed. This prediction is consistent with the observation that after

**TABLE 1**

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<th>Kinetic parameters of EGCG and EGC methylation by liver cytosolic COMT</th>
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<tr>
<td>EGCG</td>
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<td>$V_{max}$</td>
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<td>Rat liver</td>
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$V_{max}$, picomoles per milligram per minute; $K_m$, micromolar; $V_{max}/K_m$, microliter per milligram per minute.

**FIG. 4.** Concentration-dependent methylation of EGCG and EGC by liver cytosol from humans (○), mice (●), and rats (■).

Incubations were conducted for 2 min with EGCG (A) or 5 min with EGC (B) in the presence of 1 mg/ml of hepatic cytosolic proteins and 50 μM SAM. The formation of methylated EGCG and EGC was quantified with HPLC. Values represent mean of duplicate analysis.

**FIG. 5.** Methylation of EGCG and 4′-MeEGCG by rat liver cytosol.

Incubations were conducted for 20 min using 1 mg/ml of hepatic cytosolic proteins in the presence of 50 μM SAM and different concentrations of EGCG or 4′-MeEGCG. The formation of methylated products was quantified with HPLC. With EGCG, the sum of both products (4′-MeEGCG and 4′,4″-DiMeEGCG) was used in the plot (A). 4′-MeEGCG and 4′,4″-DiMeEGCG were also shown as percentage of the sum (B). Values represent mean of duplicate analysis.
administration of a large quantity of EGCG to mice, four mono-methylated and four di-methylated EGCG were detected in the urine after hydrolysis by H9252-glucuronidase and sulfatase; of which, three mono-methylated EGCG had similar peak heights (Meng et al., 2002). This metabolism profile is not expected if EGCG does not undergo conjugation first; in that case 4'-MeEGCG would have been the predominant metabolite produced.

Our studies show that EGCG potently inhibits the activities of COMT. The gallated catechins (EGCG and ECG) have 60-fold higher activities than nongallated catechins (EGC and (−)-epicatechin) at

![Graph A](image1)

**Fig. 6.** Methylation of EGCG and EGC by cytosol preparation from small intestine of rats (•) and mice (○).

Incubations were conducted for 20 min using 1 mg/ml of intestinal cytosolic proteins in the presence of 50 µM SAM and different concentrations of EGCG (A) or EGC (B). The formation of methylated EGCG and EGC was quantified with HPLC. Values represent mean of duplicate analysis.

![Graph B](image2)

**Fig. 7.** Product-ion mass spectrum of methylation products of EGCG glucuronides.

Incubations were conducted for 20 min using 1 mg/ml of rat hepatic cytosolic proteins in the presence of 50 µM SAM and 1 µM EGCG-3'-Gluc. The formation of EGCG metabolites was detected with LC/MS/MS. The spectrum of the product mono-methylated EGCG-3'-Gluc is shown in (A) with parent ion of m/z 647. The same spectrum was also observed with a mono-glucuronide of 4'-MeEGCG formed by incubation of 100 µM 4'-MeEGCG with 1 mM UDPGA in the presence of mouse hepatic microsomal protein (2 mg/ml) for 30 min (B). LC/MS/MS chromatogram of di-methylated EGCG-4'-Gluc with parent ion of m/z 661 (C); chromatogram obtained by monitoring the deprotonated product ion of m/z 485. Product-ion mass spectrum of di-methylated EGCG-4'-Gluc, P1 (D) and P2 (E).
inhibiting COMT activity, suggesting the importance of the d-ring for the inhibitory activity. When the 4'-position of d-ring is glucuronidated, inhibitory activity also decreases markedly. The 2- to 3-fold differences between trihydroxyl-catechins (EGCG and EGC) and dihydroxyl-catechins (ECG and (-)-epicatechin) suggest that the B-ring also contributes significantly to the COMT-inhibiting activity of EGCG. The noncompetitive inhibition of COMT by 4'-MeEGCG and 4',4''-DiMeEGCG suggests that they inhibit COMT by binding to sites other than the catechol binding site. It has been proposed that many SAM-dependent methyltransferases, including DNA methyltransferases and COMT, have a common catalytic domain structure (Cheng, 1995). It is possible that EGCG/methylated EGCG can bind to certain sites on the catalytic domain of DNA methyltransferase and inhibit its activity.

**TABLE 2**

Median inhibition concentration (IC$_{50}$) of the inhibition of methylation of EGC and L-DOPA by catechins and their derivatives

Studies on the inhibition of EGC methylation were conducted for 10 min using 1 mg/ml of liver cytosolic proteins in the presence of 10 μM EGC, 50 μM SAM and different concentrations of inhibitors. Studies on the inhibition of L-DOPA methylation were conducted for 30 min using 25 units of porcine liver COMT in the presence of 100 μM L-DOPA, 60 μM SAM and different concentrations of inhibitors. Values of IC$_{50}$ (μM) were calculated from duplicate determinations and represent the concentrations of inhibitor that inhibited the formation of 4'-MeEGC or 3-MeDOPA by 50%.

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<th>Inhibitor</th>
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<td>EGC</td>
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<td>EGC</td>
</tr>
<tr>
<td>EGCG</td>
<td>0.15</td>
<td>0.20</td>
<td>ECG</td>
</tr>
<tr>
<td>4'-MeEGCG</td>
<td>0.16</td>
<td>0.10</td>
<td>(-)-Epicatechin</td>
</tr>
<tr>
<td>4',4''-DiMeEGCG</td>
<td>0.20</td>
<td>0.30</td>
<td>N/D</td>
</tr>
<tr>
<td>4',3',4'-TriMeEGCG</td>
<td>0.15</td>
<td>N/D</td>
<td>3,5-Dinitrocatechol</td>
</tr>
<tr>
<td>EGCG-7-Gluc</td>
<td>0.30</td>
<td>0.50</td>
<td>EGCG-3'-Gluc</td>
</tr>
<tr>
<td>EGCG-3'-Gluc</td>
<td>0.11</td>
<td>N/D</td>
<td>EGC-7-Gluc</td>
</tr>
<tr>
<td>EGCG-3'-Gluc</td>
<td>0.15</td>
<td>N/D</td>
<td>3,5-Dinitrocatechol</td>
</tr>
<tr>
<td>EGCG-4'-Gluc</td>
<td>1.5</td>
<td>2.5</td>
<td>N/D</td>
</tr>
</tbody>
</table>

N/D, not determined.
its the methylation of \( \text{L-DOPA} \). The IC\(_{50} \) of 0.2 \( \mu \text{M} \) is lower than the peak human blood levels of EGCG after taking 800 mg of EGCG (\( \sim 1 \) \( \mu \text{M} \)). EGCG, as a potent COMT inhibitor, a mild irreversible inhibitor of dopa decarboxylase (Bertoldi et al., 2001), a neuroprotective agent may have beneficial effects in patients with Parkinson’s disease. The mouse liver tissue for the biosynthesis of EGC glucuronides was collected from an experiment conducted by Drs. Yaoping Lu and Checkoway et al., 2002.

The present study characterized the COMT-catalyzed methylation of EGCG and EGC in humans, mice, and rats. The methylation of EGCG is highly dose-dependent. Additionally, these catechins and their metabolites are potent inhibitors of COMT. Further studies on effects of tea catechins on the metabolism of catecholic hormones and their related disease are warranted. The potential interactions between EGCG and catecholic hormones or drugs should also be considered.

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References


