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ARTICLE

Anticancer and Anti-inflammatory Effects of Cysteine Metabolites of the Green Tea Polyphenol, (−)-Epigallocatechin-3-gallate

Joshua D. Lambert,*,† Shengmin Sang,‡ Jungil Hong,§ and Chung S. Yang‖

†Department of Food Science, The Pennsylvania State University, 332 Food Science Building, University Park, Pennsylvania 16802, ‡Center for Excellence in Post-Harvest Technologies, North Carolina A&T State University, Kannapolis, North Carolina 28081, §Division of Food Science, College of Natural Science, Seoul Women's University, Seoul, Korea 139-774, and ‖Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854

(−)-Epigallocatechin-3-gallate (EGCG) has been shown to have cancer preventive activity in vitro and in vivo. We have previously shown that EGCG can undergo conjugation to cysteine to form 2′-cysteinyl-EGCG and 2″-cysteinyl-EGCG. Studies of thiol-conjugated metabolites of methamphetamine indicate that such metabolites are not detoxified but retain biological activity. Here, we examined the growth inhibitory, pro-oxidant, and anti-inflammatory activities of the cysteine metabolites of EGCG. Both compounds dose-dependently inhibited the growth of colon cancer and intestinal cell lines. Both metabolites prevented aberrant arachidonic acid release and nitric oxide production by lipopolysaccharide-stimulated RAW264.7 cells. Under cell culture conditions, 2″-cysteinyl-EGCG produced H_2O_2 at a faster rate than EGCG. The results of the present study show that cysteine conjugates of EGCG retain the growth inhibitory, anti-inflammatory, and pro-oxidant activities of EGCG in vitro and may play a role in disease prevention in vivo. These results remain to be confirmed in vivo.

KEYWORDS: Green tea; (−)-epigallocatechin-3-gallate; oxidative stress; phase II metabolism

INTRODUCTION

(−)-Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol in green tea (*Camellia sinensis*, Theaceae) and has been extensively studied for its chemopreventive effects in in vitro and animal models of carcinogenesis (reviewed in refs *1*, *2*). On the basis of studies in cancer cell lines and cell-free systems, a number of potential mechanisms of action have been suggested, including inhibition of growth factor signaling, enhancement of antioxidant activity and phase II metabolism, and inhibition of key cellular enzymes (*3*, *4*). More recently there have been reports demonstrating the potential of EGCG to generate oxidative stress in vitro: a more limited number of studies have shown that EGCG can also induce oxidative stress in vivo (*5*, *6*).

Under cell culture conditions, EGCG undergoes auto-oxidation, resulting in the formation of dimeric and oligomeric polyphenol oxidation products and hydrogen peroxide (*7*). Hong et al., have reported that incubation of EGCG in McCoy's 5 medium at 37 °C results in the formation of an approximately 0.5 mol equiv of hydrogen peroxide (*8*). These in vitro oxidative effects have been shown to underlie at least some of the biological activity of EGCG. For example, treatment of KYSE150 human esophageal cancer cells with EGCG resulted in decreased levels of epidermal growth factor receptor phosphorylation and protein levels (*5*). Inclusion of superoxide dismutase, which stabilizes EGCG and prevents is pro-oxidative effects, blocked these effects on epidermal growth factor. Elbling et al., have reported that treatment of RAW264.7 murine macrophage cells and HL-60 human promyelocytic leukemia cells with EGCG results in the formation of hydrogen peroxide and induction of DNA damage (*9*).

EGCG undergoes extensive biotransformation in vivo resulting in the formation of glucuronidated, sulfated, and methylated metabolites (*10*, *11*). In addition, EGCG undergoes ring-fission metabolism catalyzed by colonic microflora to form the valerolactone-containing metabolites: (−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone (M4) and (−)-5-(3′,5′-dihydroxyphenyl)-γ-valerolactone (*12*, *13*). More recently, we have demonstrated that oral or intraperitoneal administration of EGCG to mice results in the formation of two cysteine metabolites: 2′-cysteinyl-EGCG and 2″-cysteinyl-EGCG (*14*, *15*). It is hypothesized that these metabolites form after the oxidation of EGCG to a quinone, which results in the activation of the 2′- or 2″-carbons of the B- and D-rings, respectively. Subsequently, a Michael-type reaction occurs between these activated carbons and the thiol group of cysteine. Chemical synthesis of cysteine and other thiolconjugates of EGCG, and studies in cell culture, seem to support this proposed mechanism (*14*, *16*).

It is generally accepted that phase II metabolism leads to the decreased bioavailability and inactivation of biologically active molecules (*17*). This appears to be the case for methylated metabolites of EGCG, which have significantly reduced activity to induce tumor cell death, inhibit catechol-*O*-methyltransferase activity, and inhibit DNA methyltransferase (*18*−*20*). Similarly, the microbial metabolite M4 has significantly reduced growth

*To whom correspondence should be addressed. Phone: (732) 445-3400 ext 306. Fax: (814)865-5223. E-mail: jdl134@psu.edu.

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inhibitory activity than EGCG (*21*). Previous reports with 3,4-methylenedioxymethamphetamine (MDMA), however, have demonstrated that conjugation of catechol metabolites to glutathione or *N*-acetylcysteine results in the formation of a highly neurotoxic species that is able to redox cycle and may contribute to the neurotoxic effects of MDMA observed in vivo (*22*, *23*).

Since we have demonstrated that structurally analogous metabolites of EGCG are formed following oral administration of high doses of EGCG, we sought to determine whether these EGCG-cysteine metabolites have the potential to contribute to the pool of biologically active EGCG metabolites. In the present report, we describe the in vitro anticancer, anti-inflammatory, and pro-oxidant effects of the two major EGCG-cysteine metabolites, 2′-cysteinyl-EGCG and 2″-cysteinyl-EGCG.

MATERIALS AND METHODS

Chemicals. EGCG (99% pure) was provided by Mitsui Norin Co. Ltd. (Tokyo, Japan). EGCG cysteine conjugates were prepared as previously described (*24*). Stock solutions (100 mM) of EGCG and EGCG cysteine conjugates were prepared in DMSO. [5,6,8,9,11,12,14,15-^{3}H](N) arachidonic acid was purchased from NEN Life Science (Boston, MA). All other reagents were of the highest grade available.

Cell Culture. All cell lines were purchased from ATCC (Manassas, VA). HT-29 and HCT-116 human colon cancer cells were maintained in log-phase growth in McCoy's 5A medium. RAW264.7 murine macrophages were maintained in log-phase growth in Dulbecco's Modified Eagle's Medium. INT-407 immortalized human intestinal epithelial cells and IEC-6 immortalized rat intestinal epithelial cells were maintained in glutamine-free Basal Medium Eagle (containing Earle's salts). All medium was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

Growth Inhibition. To determine the growth inhibitory activity of EGCG, cysteine conjugates cell lines were plated in 96-well plates (10×10^3 cells per well) and allowed to attach for 24 h. The medium was replaced with fresh, serum-free medium containing 0−40 μM of 2′-cysteinyl-EGCG or 2″-cysteinyl-EGCG. Cells were incubated for 48 h at 37 °C. The medium was removed, the cells were washed once with fresh serum-complete medium to remove residual test compound, and growth inhibition was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (*25*).

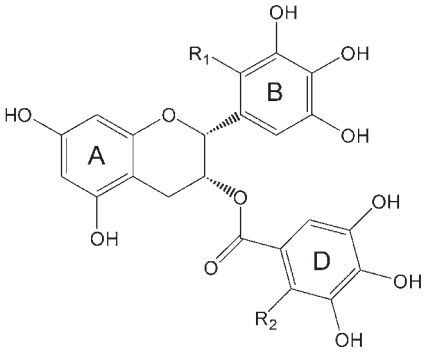
Inhibition of Arachidonic Acid Release and NO Production. The ability of EGCG-cysteine conjugates to inhibit the release of arachidonic acid and production of NO by lipopolysaccharide (LPS)-stimulated murine macrophages was determined using previously described methods (*26*). In brief, to determine arachidonic acid release, RAW264.7 cells were incubated overnight with 0.1 μCi/mL [5,6,8,9,11,12,14,15-^{3}H](N) arachidonic acid to allow membrane incorporation. Cells were then washed with PBS containing 0.1% bovine serum albumin. Cells were then stimulated with 2 μg/mL LPS for 1 h, the cells were washed, and fresh medium containing EGCG-cysteine conjugates (0 or 20 μM) was added. Following 18 h incubation, radioactivity in the medium was determined by scintillation counting. To determine inhibition of NO formation, cells were stimulated and treated with test compounds as above. NO levels were determined by measuring nitrite production spectrophotometrically using the Greiss reagent (*27*).

H_2O_2 Determination. EGCG or 2″-cysteinyl-EGCG (50 μM) was incubated in McCoy's 5A at 37 °C under 5% CO_2 atmosphere. At different time points, samples were collected and the concentration of H_2O_2 in the medium was analyzed using an Amplex Red hydrogen peroxide assay kit (Molecular Probes, Eugene, OR) with slight modifications (*28*).

Statistical Analysis. All data are expressed as the mean of 3−18 determinations (depending on the experiment). Error bars represent the standard deviation. Effects of EGCG-cysteine conjugates on NO production and arachidonic acid release compared to LPS-stimulated control cells were tested using the Student's *t* test. Differences in the rate of H_2O_2 production as a function of time were tested by two-way ANOVA with Bonferroni Post-test. Significance was achieved at $p < 0.05$.

RESULTS AND DISCUSSION

EGCG has been shown to have growth inhibitory and pro-apoptotic activity against a number of human cancer cell lines (reviewed in refs *3*, *4*). These effects have been shown to occur via a number of mechanisms including induction of oxidative stress. In vivo, EGCG has been shown undergo extensive phase II metabolism resulting in the formation of methylated, glucuronidated, and sulfated metabolites, as well as cysteine conjugates (*10*, *24*). Previous studies with glutathione conjugates of MDMA have shown that these metabolites retain the neurotoxic effects of the parent compounds and have the ability to undergo redox cycling and induce oxidative stress (*22*). Here, we investigated the in vitro anticancer, anti-inflammatory, and pro-oxidative effects of analogous metabolites of EGCG: 2′-cysteinyl-EGCG and 2″-cysteinyl-EGCG (**Figure 1**).



	R_1	R_2
EGCG	H	H
EGCG-2′-cys	cysteine	H
EGCG-2″-cys	H	cysteine

Figure 1. Structure of EGCG, 2′-cysteinyl-EGCG, and 2″-cysteinyl-EGCG.

The growth inhibitory effects of both 2′-cysteinyl-EGCG (**Figure 2A**) and 2″-cysteinyl-EGCG (**Figure 2B**) against HT-29 and HCT-116 human colon cancer cells, as well as INT-407A and IEC-6 immortalized intestinal cells, were determined using the MTT assay. Both compounds dose-dependently inhibited the growth of all cell lines tested. 2″-Cysteinyl-EGCG was significantly more potent than 2′-cysteinyl-EGCG, resulting in approximately 80% growth inhibition of HCT-116, IEC-6, and INT-407A cells at 40 μM. HT-29 cells were the least sensitive to the growth inhibitory effects of either 2″-cysteinyl-EGCG or 2′-cysteinyl-EGCG (less than 20% growth inhibition at 40 μM).

These results are in contrast to those from studies by us and others on the growth inhibitory activity of other EGCG metabolites. For example, M4 had significantly less growth inhibitory activity than EGCG (*5*, *21*). These results suggest that an intact A-, C-, and D-ring system is necessary for growth inhibitory activity. Likewise, the major methylated metabolites of EGCG, 4′-*O*-methyl-EGCG and 4′,4″-di-*O*-methyl-EGCG, have much less growth inhibitory activity than EGCG, indicating that the intact trihydroxy ring structures are also critical for growth inhibitory activity (*18*).

Both 2′-cysteinyl-EGCG and 2″-cysteinyl-EGCG demonstrated significant anti-inflammation related activity in the LPS-stimulated RAW264.7 murine macrophage cell line model. LPS stimulation increased arachidonic acid release and NO production by 1.8- and 6.7-fold, respectively, compared to control (**Figure 3A**,**B**). Both EGCG-cysteine conjugates (20 μM) reduced the aberrant arachidonic acid release and NO production to unstimulated levels.

By contrast, we have previously reported that EGCG had no significant effect on LPS-mediated NO production and arachidonic acid release by RAW264.7 cells (*29*). M4 and M6 have differential effects on NO production by LPS-stimulated macrophages. M4,

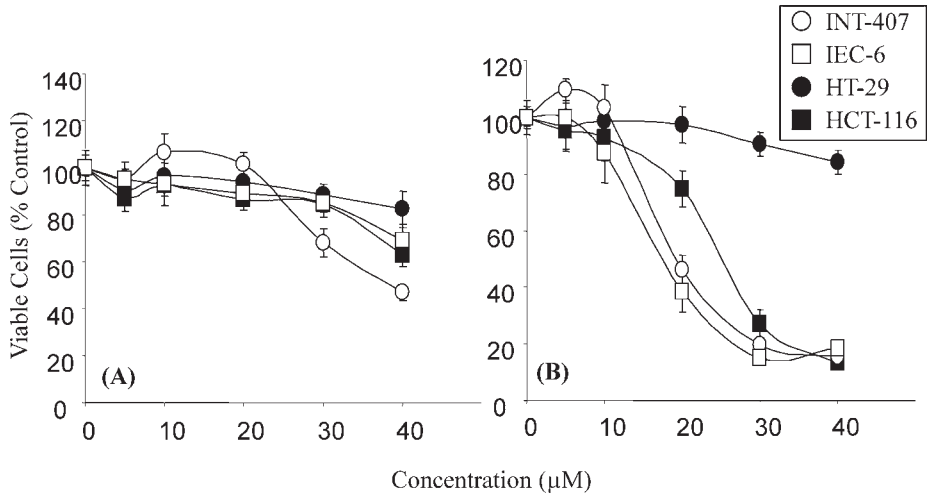


Figure 2. Growth inhibitory effects of 2′-cysteinyl-EGCG (**A**) and 2′′-cysteinyl-EGCG (**B**) against HT-29, HCT-116, IEC-6, and INT-407A cells. Cells were treated for 48 h, and growth inhibition was determined by the MTT assay. Each point represents $n = 12-18$. Error bars represent the standard deviation.

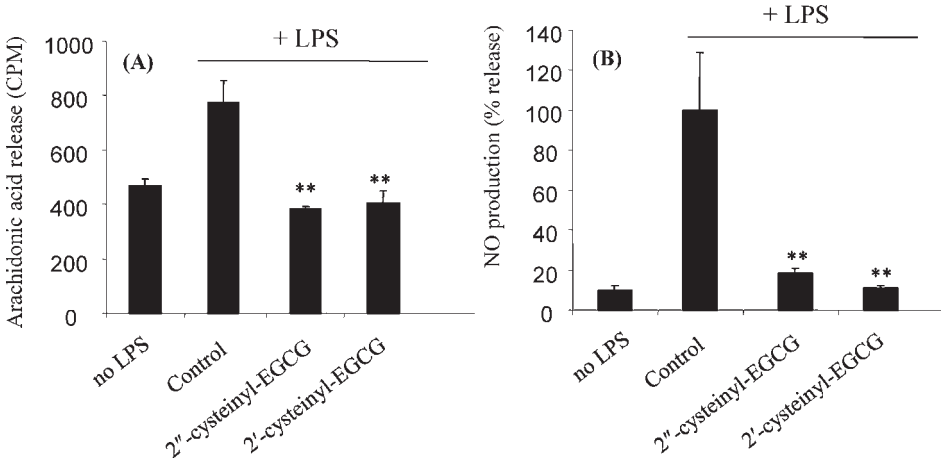


Figure 3. Effect of 2′-cysteinyl-EGCG and 2′′-cysteinyl-EGCG on NO production (**A**) and aberrant arachidonic acid release (**B**) by LPS-stimulated RAW264.7 murine macrophage cells. Data represent the mean of $n = 3$. Error bars represent the standard deviation. ** = $p < 0.01$ compared to LPS-stimulated control.

which has an intact gallate ring, was shown to inhibit the production of NO by activated RAW264.7 cells, whereas M6 was not (*21*). Overall our new findings with 2′-cysteinyl-EGCG and 2′′-cysteinyl-EGCG demonstrate the importance of an intact trihydroxy ring structure for the inhibition of NO production.

Previous studies with EGCG and EGCG-glucuronides have demonstrated that glucuronidation of EGCG on the B- or D-ring has no effect on the ability of the compound to prevention aberrant arachidonic acid release by HT-29 cells, however, glucuronidation of EGCG on the 7-OH position on the A-ring significantly reduced the inhibitory activity of EGCG (*30*). These results suggest that the A-ring is more important for this effect than the B- or the D-ring.

We and others have reported that EGCG is unstable under cell culture conditions and forms oligomers and H_2O_2. The ability of 2′′-cysteinyl-EGCG to produce H_2O_2 was compared to that of EGCG (**Figure 4**). Incubation of equimolar concentrations of EGCG and 2′′-cysteinyl-EGCG (50 μM) produced similar concentrations of H_2O_2 after 24 h incubation. The kinetics of H_2O_2 formation, however, were accelerated for 2′′-cysteinyl-EGCG compared to EGCG. The time to half-maximal H_2O_2 level were 15 and 60 min for 2′′-cysteinyl-EGCG and EGCG, respectively. These results are similar to those reported by Monks, et al. on the pro-oxidant activity of glutathione-conjugated catechol metabolites of MDMA (*22*). Those authors suggest that the neurotoxic

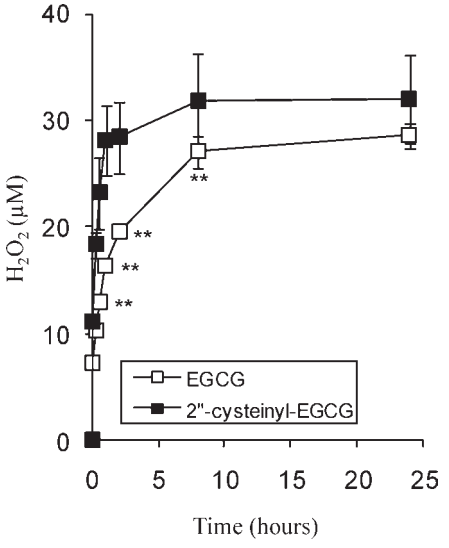


Figure 4. H_2O_2 generation by EGCG and 2′′-cysteinyl-EGCG under cell culture conditions. EGCG or 2′′-cysteinyl-EGCG (50 μM) were incubated in serum-free cell culture medium at 37 °C under 5% CO_2 atmosphere. Each point represents the mean of $n = 6$. Error bars represent that standard deviation. ** = $p < 0.01$.

effects of MDMA are at least in part due to the enhanced redox cycling activity of these glutathione-conjugated metabolites, and that in the case of MDMA, glutathione conjugation represents a maladaptive metabolic response.

In summary, the present results demonstrate that, in contrast to other phase II metabolites of EGCG, 2′-cysteinyl-EGCG and 2′′-cysteinyl-EGCG retain the growth inhibitory and anti-inflammatory capacity of EGCG in vitro and are more pro-oxidative. Whether these effects are observed in vivo, and by extension whether these metabolites contribute to the cancer preventive effects of EGCG, remains to be determined.

ABBREVIATIONS USED

EGCG, (−)-epigallocatechin-3-gallate; LPS, lipopolysaccharide; M4, (−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone; MDMA, 3,4-methylenedioxymethamphetamine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

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