Comparison of White Tea, Green Tea, Epigallocatechin-3-Gallate, and Caffeine as Inhibitors of PhIP-Induced Colonic Aberrant Crypts

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Abstract: There is growing interest in the possible health benefits of tea. We reported previously on the inhibition by white tea of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)‐induced colonic aberrant crypt foci (ACF) in the rat (4). To distinguish between blocking and suppressing effects, and thus provide mechanistic insights into prevention during the initiation versus post-initiation phases of carcinogenesis, white tea, and green tea were administered at 2% (w/v) as the sole source of drinking fluid either 2 wk before and 2 wk during PhIP dosing (100 mg/kg, every other day by oral gavage), or starting 1 wk after the carcinogen and continued until the study was terminated at 16 wk. In the former protocol, each tea produced marginal inhibition of colonic ACF, despite evidence for changes in several hepatic enzymes involved in heterocyclic amine metabolism. Post-initiation, however, the data were as follows (ACF/colon, mean ± SE): PhIP/water 12.2 ± 1.5; PhIP/white tea 5.9 ± 0.9 (**P < 0.01); PhIP/caffeine 5.9 ± 1.5 (**P < 0.01); PhIP/EGCG 3.5 ± 0.8 (**P < 0.001); PhIP/green tea 8.9 ± 1.2 (P = 0.22, not significant). In the latter study, apoptosis was determined using in situ oligo ligation and cleaved caspase-3 assays, whereas cell proliferation was assessed via bromodeoxyuridine (BrdU) incorporation. No consistent changes were seen in apoptosis assays, but BrdU labeling was as follows (percent of cells positive/colonic crypt, mean ± SE): PhIP/water 10.4 ± 0.6; PhIP/white tea 8.6 ± 0.2 (*P < 0.05); PhIP/EGCG 6.0 ± 0.8 (**P < 0.01); PhIP/caffeine 8.75 ± 0.45 (*P < 0.05); PhIP/green tea 9.5 ± 0.4 (P > 0.05, not significant). The data imply that white tea, caffeine, and EGCG may be most effective post-initiation, via the inhibition of cell proliferation in the colon and through the suppression of early lesions.

Introduction

There is growing interest in the possible health benefits of tea (1). In animal studies, tea and its individual constituents have been reported to inhibit cancers of the skin, lung, esophagus, stomach, liver, small intestine, pancreas, colon, prostate, bladder, and mammary gland. However, the extent to which this protection might translate to the human situation remains an open question (reviewed in ref 1).

During commercial production, leaves of *Camellia sinensis* undergo different degrees of processing, giving rise to various types of tea. White and green teas are the least processed types of tea and contain the highest levels of epigallocatechin-3-gallate (EGCG) and other monomeric catechins, whereas the more highly processed oolong and black teas have high levels of complex polyphenols called theaflavins and thearubigins (1,2). The content of these various tea polyphenols can vary considerably according to growing season, cultivar, storage, and brewing conditions.

We reported previously on the potent antimutagenic activity of white tea compared with green tea against 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and related heterocyclic amines in the Salmonella assay (2). In vivo, white tea and green tea were equally effective at inhibiting small intestine polyps in *Apc*min mice, and tumor suppression was augmented by co-administration of white tea with sulfonac, a non-steroidal anti-inflammatory agent (3). However, it was unclear whether white tea and green tea were equally protective in the large intestine, due to the small number of polyps that developed in the colon of *Apc*min mice.

To address this question, we conducted a side-by-side comparison of the blocking and suppressing effects of white and green tea in the rat colon. We report here that white tea

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Figure 1. Blocking and suppressing protocols in the rat colon. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP, 100 mg/kg) or vehicle alone (phosphate-buffered saline:DMSO (1:1), pH 4.5) was given by oral gavage, every other day for 2 wk. In the blocking protocol (A), rats received water, 2% white tea (w/v), or 2% green tea (w/v) for 2 wk before and 2 wk during PhIP treatment, and drinking water (no tea) for the remainder of the experiment. Arrow, three animals in each group were sacrificed prior to PhIP dosing to study changes in drug metabolizing enzymes. In the suppressing protocol (B), 2% white tea, 2% green tea, EGCG (0.5 mg/ml), or caffeine (0.5 mg/ml) were administered as sole source of drinking fluid, starting 1 wk after the last dose of PhIP and continued until the study was terminated at 16 wk. Aberrant crypt foci (ACF), bromodeoxyuridine (BrdU) incorporation, and apoptosis indices were scored at 16 wk.

Materials and Methods

Teas

Mutan white tea and Dragonwell green tea, referred to hereafter as “white tea” and “green tea,” were provided by Stash Tea Co. (Portland, OR) and stored air-tight at 4°C. Each tea was prepared fresh every 1–2 days, by brewing loose leaves in just-boiled water for 2 min at a concentration of 2% (w/v). We previously reported that most of the antimutagens were eluted within 2–3 min, and animals found the tea more palatable compared with longer brew times (2,3). Under these conditions, white tea contained 0.5 mg/ml EGCG and 0.5 mg/ml caffeine, whereas green tea contained 0.35 mg/ml EGCG and 0.45 mg/ml of caffeine, as determined by high performance liquid chromatography (HPLC). These levels did not vary markedly during the course of the investigation, based on HPLC analyses performed at least once per week throughout the experiment, using a single batch of each tea to avoid possible variability between batches. Concentrations of other tea constituents were as reported before (3).

Animals

Male F344 rats, obtained at 5–6 wk of age from NCI, were housed 2/cage and given AIN-93G diet (Dyets, Bethlehem, PA) ad libitum. PhIP (Toronto Res, Ontario, Canada) was dissolved in phosphate-buffered saline:DMSO (1:1), pH 4.5 and administered by oral gavage every other day for 2 wk (100 mg/kg body wt). Controls received vehicle alone. In the blocking protocol, tea was provided as sole source of fluid for 2 wk before and 2 wk during PhIP treatment (Fig. 1A). In the suppressing protocol (Fig. 1B), white tea, green tea, EGCG, or caffeine were started 1 wk after the last dose of PhIP, and continued until week 16. Solutions of EGCG (purity ~95% by HPLC, TEAVIGO™, DSM Nutritional Products, Basel, Switzerland) and caffeine (Sigma Chemical Co., St. Louis, MO) were made fresh every 1–2 days in water, at concentrations corresponding to those in brewed white tea, i.e., 0.5 mg EGCG and 0.50 mg caffeine per ml.

Scoring of Colonic Aberrant Crypts

The blocking and suppressing experiments were terminated after 16 wk, and rats were examined for colonic ACF using the methodology reported before (4–8). In brief, each colon was opened longitudinally, flushed with ice-cold PBS, fixed in buffered formalin, stained with 0.2% methylene blue, and examined under the light microscope by individuals.
blinded to the treatment group. A record was kept of the number of total aberrant crypts (AC) and ACF per colon, as well as small vs. large ACF containing <4 or ≥4 aberrant crypts per focus, respectively (9–11).

Changes in Drug Metabolizing Enzymes

Assays were performed as reported before (4,5) with the following substrates: ethoxyresorufin O-deethylase (EROD) with 7-ethoxyresorufin, methoxyresorufin O-demethylestase (MROD) with methoxyresorufin, UDP-glucuronosyltransferase (UDPGT) and sulfotransferase (SULT) with p-nitrophenol, and glutathione S-transferase (GST) with 1-chloro-2,4-dinitrobenzene. Enzyme activities were normalized to the corresponding microsomal or cytoplasmic protein content and expressed as “fold-induction” (mean ± SE), with controls assigned an arbitrary value of 1.0.

Scoring of Cell Proliferation and Apoptosis Indices

At the end of the suppression study (Fig. 1B), 5–6 rats in each group were selected at random and injected i.p. with bromodeoxyuridine (BrdU, 20 mg/kg body wt), 1 h before sacrifice. Immunostaining used the BrdU in situ detection kit by incubating sections in 3% H2O2 in Dako TBST for 10 min, followed by 20 min cooling at room temperature. After washing with water, endogenous peroxidases were blocked by incubating sections in 3% H2O2 in Dako TBST for 10 min, followed by TBST alone. Sections were blocked with Dako serum-free protein for 10 min, and then covered with polyclonal rabbit anti-cleaved caspase-3, which specifically detects the endogenous large fragment of cleaved caspase-3 resulting from cleavage adjacent to Asp175 (Cell Signaling Technology, Inc., Danvers, MA). Sections were developed with Nova Red and counterstained with hematoxylin.

Labeling indices were determined as reported (8), based on the number of positive cells/total cells per crypt x 100, scored separately for basal, central, and apical regions within the colonic crypt column, using 50 well-distinguished crypts in the distal and proximal regions of the colon. Only complete, well-orientated longitudinally sectioned crypts with lumen at the top and muscularis mucosae at the base were evaluated.

Statistics

Data (mean ± SE) were evaluated using non-parametric tests (Kruskal-Wallis test, followed by Wilcoxon test), comparing each test agent to the control. Exact P-values were generated using Npar1way in SAS version 9.1.3 (SAS Institute, Inc., Cary, NC). In the figures, significant p-values are shown as follows: * P < 0.05, ** P < 0.01, *** P < 0.001.

Results

Previous studies of white, green and black teas showed that inhibition of ACF was correlated with changes in enzymes associated with heterocyclic amine metabolism and excretion (4,7). Therefore, we examined a select group of phase 1 and phase 2 drug metabolizing enzymes following treatment with white tea or green tea.

Hepatic EROD and MROD were assayed as indicators of cytochrome P4501A1 (CYP1A1) and CYP1A2, respectively, which catalyze ring- and N-hydroxylation of heterocyclic amines. Both teas induced EROD and MROD activities significantly (P < 0.05), compared with water controls (Fig. 2). Among the phase 2 enzymes examined, UDPGT and GST were induced by white tea significantly (P < 0.05), and green tea also augmented UDPGT activity (P < 0.05), whereas no alteration of SULT was seen with either tea treatment.

In the ACF blocking study (Fig. 3, left panel), white tea reduced the number of AC/colon (P < 0.05) and green tea lowered the number of small ACF containing <4 AC/focus (P < 0.05), but no other significant differences were seen versus controls. In the suppression protocol (Fig. 3, right panel), white tea and caffeine lowered the number of AC/colon and small ACF/colon significantly (P < 0.01), and EGCG inhibited both these parameters as well as AC/colon (P < 0.001). Green tea partially reduced the number of small ACF/colon (P < 0.05). We interpret these data as indicating slightly greater protection by white tea than green tea, especially post-initiation for ACF/colon.
Figure 3. Blocking and suppression of PhIP-induced ACF by white tea and green tea in the rat. Results for the blocking study are in the left panel (A), and for suppression in the right panel (B). Data (mean ± SE) are presented for AC, ACF, small ACF (<4 AC/focus), and large ACF (≥4 AC/focus) quantified at 16 wk; *P < 0.05, **P < 0.01, ***P < 0.001. For the concentration of each test agent, see Fig. 1 legend.

As reported (8), BrdU-positive cells typically localize to the lower third of the crypt column (Fig. 4A), whereas cells at the top of the colonic crypts stain positive using the in situ oligo ligation assay (arrows, Fig. 4B). Quantification of the corresponding labeling indices provided a measure of cell proliferation and apoptosis in each treatment group (Figs. 4C, D). In PhIP controls, 10–11% of the cells/crypt stained positive for BrdU incorporation (Fig. 4C), compared with 9.5%, 8.3%, 6.0%, and 8.6% in groups given PhIP followed by green tea, white tea (P < 0.05), EGCG (P < 0.01), or caffeine (P < 0.05), respectively. No significant differences were seen in the in situ oligo ligation assay, except a slight increase in rats given PhIP followed by EGCG (P < 0.05, Fig. 4D). A follow-up study of cleaved caspase-3 revealed no significant differences among the various treatment groups (Fig. 5). We further examined the cleaved caspase-3 data for possible changes within each compartment of the colonic crypt (lower, middle, upper third), but detected no significant differences (data not shown).

Discussion

We reported previously that white tea was more potent than green tea at inhibiting PhIP-induced mutagenicity in the Salmonella assay (2), but both teas were equally effective at suppressing intestinal polyps in the Apc<sup>min</sup> mouse (3). The present study represents the first side-by-side comparison of white tea and green tea as modulators of PhIP-induced ACF, cell proliferation/apoptosis, and drug metabolizing enzymes.

Drug metabolizing enzymes induced after treatment with white tea and green tea included EROD, MROD, and UDPGT, and white tea also augmented GST (but not SULT) activities. These data do not provide a complete picture of all
of the enzymes involved in PhIP metabolism, and in particular those that may be important in the tissue at risk for cancer (i.e., the colon). Nonetheless, based on prior work (4,7) we anticipated that white tea might inhibit PhIP-induced ACF more effectively than green tea in the blocking study.

Unexpectedly, the blocking activity of both teas towards PhIP-induced ACF was marginal at 16 wk, whereas white tea and the corresponding concentrations of caffeine and EGCG suppressed PhIP-induced ACF significantly when administered post-initiation. These results imply that the major inhibitory effects of white tea in the colon occur post-initiation, even when administered before, during and after PhIP exposure, as in our prior study (4). This is supported by evidence for reduced cell proliferation in the colonic crypts after post-initiation treatment, with a similar order of inhibition as in the ACF study, namely EGCG > caffeine = white tea > green tea (compare Fig. 4C with Fig. 3, right panel). Although EGCG augmented the apoptosis index according to the in situ oligo ligation assay, we interpret this finding with caution, because an alternative and more specific apoptosis assay, cleaved caspase-3, revealed no significant increase by any of the test agents, including EGCG (Fig. 5).

Tea catechins synergistically inhibited TNF-α release in cultured cells (12), but our results suggest that antagonistic effects might occur between tea constituents in vivo. This could explain the rather surprising observation that EGCG alone was more effective than white tea, which contained the same concentration of EGCG as well as several other catechins and caffeine. Caffeine is an interesting compound because it is present in tea and several other beverages consumed by humans, and in rats treated simultaneously with caffeine and heterocyclic amines there was evidence for co-carcinogenicity in the colon but protection in the mammary gland (13,14). Further studies are warranted on the protocol-dependent and organ-specific effects of caffeine and other constituents in tea, including possible assessment of apoptosis and cell proliferation indices directly in the early biomarkers. This is feasible only for large, well-defined ACF, and is technically more time demanding than scoring labeling indices in randomly selected regions of the colon, which includes non-involved areas as well as possible early lesions. Changes at later times might be more pertinent, such as the inhibition of frank tumors and alterations in molecular targets such as β-catenin and Apc (3,15–17).

In summary, we evaluated two different protocols for white tea and green tea against PhIP-induced ACF in the rat. Both teas induced phase 1 and phase 2 enzymes associated with PhIP metabolism and excretion, but had only marginal blocking effects towards ACF scored at 16 wk. In contrast, white tea, caffeine, and EGCG each suppressed PhIP-induced ACF significantly, in accordance with the relative order of inhibition of cell proliferation in the colonic crypts. Further studies appear to be warranted on the chemopreventive effects of EGCG and caffeine post-initiation, including possible antagonistic effects in vivo. This should be done in the context of physiologically relevant concentrations and the most likely inhibitory mechanisms (1). Overall, the study outcome provides interesting mechanistic leads for
the timing of preventive intervention during multi-step colon carcinogenesis.

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