Published in final edited form as:
Proc Soc Exp Biol Med. 1999 April ; 220(4): 239–243.

Cancer Chemopreventive Mechanisms of Tea Against Heterocyclic Amine Mutagens from Cooked Meat (44373)

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Abstract

Cooking meat and fish under normal conditions produces heterocyclic amine mutagens, several of which have been shown to induce colon tumors in experimental animals. In our search for natural dietary components that might protect against these mutagens, it was found that green tea and black tea inhibit the formation of heterocyclic amine-induced colonic aberrant crypt foci (ACF) in the rat. Since ACF are considered to be putative preneoplastic lesions, we examined the inhibitory mechanisms of tea against the heterocyclic amines. In the initial studies using the Salmonella mutagenicity assay, green tea and black tea inhibited according to the concentration of tea leaves during brewing and the time of brewing; a 2–3-min brew of 5% green tea (w/v) was sufficient for >90% antimutagenic activity. *N*-hydroxylated heterocyclic amines, which are direct-acting mutagens in Salmonella, were inhibited by complete tea beverage and by individual components of tea, such as epigallocatechin-3-gallate (EGCG). Inhibition did not involve enhanced mutagen degradation, and EGCG and other catechins complexed only weakly with the mutagens, suggesting electrophile scavenging as an alternative mechanism. Enzymes that contribute to the metabolic activation of heterocyclic amines, namely microsomal NADPH-cytochrome P450 reductase and *N*,*O*-acetyltransferase, were inhibited by tea *in vitro.* Studies *in vivo* established that tea also induces cytochromes P450 and Phase II enzymes in a manner consistent with the rapid metabolism and excretion of heterocyclic amines. Collectively, the results indicate that tea possesses anticarcinogenic activity in the colon, and this most likely involves multiple inhibitory mechanisms.

This minireview describes the cancer chemopreventive properties of green tea and black tea against cooked meat heterocyclic amine mutagens, with emphasis on recent findings from the authors' own laboratory (1–4). The impetus for these studies came from earlier work in rats and mice, which demonstrated that tea, or individual constituents of tea, inhibit forestomach, lung, skin, and esophageal tumorigenesis induced by ultraviolet B light, polycyclic aromatic hydrocarbons, or *N*-nitrosamines (5–7). To determine whether tea also might protect in the colon, we conducted studies in the male F344 rat using a colon carcinogen from cooked meat as the initiating agent, namely 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). An intermediate biomarker called the aberrant crypt focus (ACF) was used as an end point in these experiments. ACF are putative preneoplastic lesions that have been detected in the human and rodent colon (8,9); they contain molecular changes found commonly in colon tumors (10–12), and they provide a quick screening tool for detecting potential inhibitors of colon cancer (13–16).

Because green tea and black tea protected significantly against IQ-induced ACF (1), we sought to clarify the inhibitory mechanisms of individual tea constituents against the heterocyclic

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amines, as well as provide information on the effects of tea concentration and brew time on the inhibitory activity (1–4). The findings from these studies will be reviewed briefly in the following sections.

Inhibition of ACF by Green Tea and Black Tea

In the first experiment (1), male F344 rats were exposed for a total of 8 weeks to green tea (2% w/v), black tea (1% w/v), or drinking water (control), and on alternating days in experiment weeks 3 and 4, the animals were given IQ by oral gavage (50 mg/kg body wt). In these initial studies, green tea and black tea were prepared according to the conditions preferred by one of us (RHD) for the daily consumption of each beverage, with a maximum brew time of 5 min. Compared with controls given carcinogen alone, green tea and black tea reduced both the number of total aberrant crypts and ACF per colon, the former tea being particularly effective ($P < 0.05$).

To expand upon these studies, a second experiment was undertaken in which green tea (2% w/v) and black tea (2% w/v) were given by one of three exposure protocols, namely a) for 4 weeks in the initiation phase (2 weeks before and 2 weeks during IQ treatment); b) for 11 weeks in the post-initiation phase, starting 1 week after the final dose of carcinogen; or c) continuously for 16 weeks. None of the vehicle controls given drinking water, black tea, or green tea had ACF after 16 weeks (not shown). In the groups treated with carcinogen, both green tea and black tea reduced the total number of rats bearing ACF (Table I).

Green tea was exceptionally effective against IQ-induced ACF when administered during the initiation phase ($P < 0.01$), to the extent that 12/15 rats had no ACF at 16 weeks, and each of the remaining three rats had a single small ACF containing only one or two aberrant crypts per focus. The total number of animals bearing ACF also was reduced when green tea was given continuously for 16 weeks, such that only 8/15 rats had ACF (Table I). The latter result is consistent with results from the 8-week study, and in both cases inhibition involved loss (or reversal) of the larger ACF so that only small foci with fewer than four aberrant crypts remained (1). In the 16-week study, one rat had a single ACF with more than four aberrant crypts following continuous green tea exposure, and the majority of animals had no ACF or only small ACF with one or two aberrant crypts. Because green tea contains several catechins with potent antioxidant activities (17), and antioxidants can be effective suppressing agents (18), it was surprising that green tea did not protect during the postinitiation phase; indeed, there was an increase in ACF, but this proved not to be statistically significant.

Black tea protected to some extent using all three exposure protocols, such that only 5/15 rats had ACF in the continuous- and initiation-exposure protocols, and 8/14 animals had ACF following postinitiation exposure to 2% black tea (Table I). The average number of ACF for all animals in a group was reduced significantly ($P < 0.05$), but when corrected for the number of rats bearing ACF, only the initiation exposure gave statistically significant inhibition for black tea (Table I, final column).

Mechanism Studies

Since green tea and black tea both inhibited during the initiation phase, further experiments were conducted to clarify the inhibitory mechanisms of tea against the heterocyclic amines. The following mechanism studies were undertaken *in vitro* and *in vivo:* a) Western blotting and cytochrome P450 enzyme assays of liver microsomes from rats given tea; b) high-performance liquid chromatography (HPLC) analyses of the urinary metabolites of IQ; c) spectrophotometric and electron paramagnetic resonance (EPR) studies of the scavenging activities of tea toward various reactive intermediates of IQ (electrophiles and free radicals generated by cytochromes P450 and NADPH-cytochrome P450 reductase); and d) Salmonella

mutagenicity assays and spectrophotometric studies of molecular complex formation and mutagen degradation. Each of the mechanisms will be described briefly.

Enzyme Induction

Western blotting and enzyme assays using 7-ethoxyresorufin and methoxyresorufin as substrates showed that green tea and black tea caused induction of hepatic CYP1A in rats given either beverage for 2–8 weeks (1). The induction was less marked than that produced by indole-3-carbinol, a positive control, but a clear increase in CYP1A2 was detected in rats given black tea and green tea, and the latter tea also produced a slight elevation of CYP1A1. Green tea also induced UDP-glucuronosyl transferase (19), a Phase II detoxification enzyme that facilitates the excretion of heterocyclic amines in the rat. The urinary metabolite profiles of IQ were altered by tea, such that the levels of parent compound and IQ-sulfamate were decreased significantly, and a concomitant increase occurred in the amounts of IQ-5-*O*-glucuronide and IQ-5-*O*-sulfate (1). These findings suggested that green tea and black tea augment the metabolism of heterocyclic amines *in vivo*, leading to the more rapid excretion of detoxification products.

Enzyme Inhibition and Free Radical Scavenging

The inhibitory activity of tea was studied further in a free radical-generating system, using IQ as a substrate (2). Green tea and black tea were shown to block the production of oxygen free radicals derived from IQ in the presence of rat liver microsomes or purified NADPH-cytochrome P450 reductase. Green tea was significantly more effective than black tea in this *in vitro* assay system, and separation of the tea by HPLC revealed that most of the quenching activity resided in the fractions containing catechins. Some catechins, such as epigallocatechin gallate (EGCG), were effective quenching agents, whereas others with known antioxidant activities were much less effective (e.g., epicatechin gallate, ECG). In kinetic studies using IQ as the substrate and DMPO as a free radical spin trap, EGCG increased the K_m of the reaction without altering V_{max}, suggesting competitive enzyme inhibition (K_i = 9.96 μ*M*). This was confirmed in spectrophotometric studies using cytochrome *c* as the substrate, in which EGCG acted as a competitive inhibitor of NADPH-cytochrome P450 reductase (K_i = 9.7 μ*M*). These results suggested that the inhibitory activities of green tea and black tea in EPR assays using IQ as the substrate for the reductase are related to an indirect effect on the enzyme, although direct scavenging of free radicals also might occur due to the presence of EGCG and other catechins (2). Inhibition of NADPH-cytochrome P450 reductase alters the activity of cytochromes P450; thus, subsequent mechanism studies to be described below avoided the use of microsomal activation systems.

Degradation of Mutagens

The Salmonella mutagenicity assay was used to test individual constituents of tea as inhibitors of 2-hydroxyamino-3-methylimidazo[4,5-*f*]quinoline (*N*-hydroxy-IQ), a direct-acting metabolite of IQ (3). Green tea, black tea, and several fractions of tea obtained by HPLC inhibited the mutagenic activity of *N*-hydroxy-IQ in a concentration-dependent manner (4). The testing of pure compounds at doses relevant to their levels in tea identified EGCG and epigallocatechin (EGC) as the primary antimutagens. Spectrophotometric assays were used to monitor the fate of the mutagen *in vitro*. Under aqueous conditions, *N*-hydroxy-IQ converts spontaneously to the electrophilic ‘ultimate carcinogen’ (an aryl nitrenium ion), which can interact covalently with DNA or undergo time-dependent degradation to inactive breakdown products. Green tea and black tea, and their constituent catechins and theaflavins, failed to enhance the rate of conversion of *N*-hydroxy-IQ *in vitro*, in contrast to the results obtained with chlorophyllin (a positive control), which rapidly degraded the mutagen.

Molecular Complex Formation

Chlorophyllin is known to form molecular complexes with IQ and other heterocyclic amines and to reduce their bioavailability *in vitro* and *in vivo* (20–22). Spectral titration studies were undertaken to investigate this mechanism for tea. Briefly, the absorption spectrum of the mutagen alone was obtained using a double-beam spectrophotometer, and sequential additions of tea were made thereafter to both cuvettes, the spectra being recorded after each addition. Complete tea and individual constituents of tea, such as EGCG, quenched the spectrum of *N*-hydroxy-IQ in a manner consistent with complex formation, and in some cases an isosbestic point indicative of a 1:1 complex was detected. However, binding constants obtained from the Benesi-Hildebrand plots were only on the order of 10^3 M^{-1}, suggesting that the interactions were weak and that mechanisms other than complex formation most likely prevail both *in vitro* and *in vivo.*

Electrophile Scavenging

The above experiments indicated that tea did not complex strongly with *N*-hydroxy-IQ or alter its rate of degradation *in vitro,* suggesting that the antimutagenic activity observed in the Salmonella assay results from a direct effect on the ultimate carcinogen, such as electrophile scavenging. However, one other possibility is that constituents of tea might inhibit the enzyme *N,O*-acetyltransferase, which is present in Salmonella strain TA98. This enzyme rapidly converts the *N*-hydroxylated metabolites of heterocyclic amines to the ultimate carcinogen, and a polymorphism in humans gives rise to “fast acetylators,” which are at increased risk following exposure to heterocyclic amines (23). Comparison of the results in Salmonella strain TA98 and a second strain that lacks the enzyme (TA98/1,8-DNP_6) indicated that the antimutagenic activity of EGCG was dependent, at least in part, on a functional *O*-acetyltransferase. However, the major component of the inhibitory activity appeared to involve direct effects on the ultimate carcinogen, possibly electrophile scavenging or enhanced rates of degradation (2,3). Further experiments to resolve these mechanisms, such as detection of a covalent interaction product between the mutagen and EGCG, are in progress.

Multiple Mechanisms of Inhibition

Based on the results from our laboratory (1–4) and elsewhere (19,24–28), it can be concluded that the catechins and perhaps other components in green tea and black tea most likely protect against heterocyclic amines *via* multiple mechanisms of inhibition (Table II). These include: i) inhibition of NADPH-cytochrome P450 reductase; ii) inhibition of *N,O*-acetyltransferase; iii) induction of CYP1A2 and UDP-glucuronosyl transferase (leading to increased metabolism of IQ and rapid elimination of detoxification products in the urine); and iv) electrophile scavenging/degradation. Which of the mechanisms (i)–(iv) is most important for protection at the time of carcinogen exposure remains to be determined. The antioxidant properties of tea also might be important during the postinitiation phase of carcinogenesis, but other mechanisms of suppression need to be evaluated.

Brew Time and Tea Concentration

Finally, a recent study from our laboratory considered how the preparation of tea might influence inhibitory activity (4). The concentrations of tea leaves used in brewing and the time for infusion of the tea leaves were chosen to reflect the various conditions that might be encountered commonly among tea drinkers. Inhibitory activity was monitored in the Salmonella mutagenicity assay using *N*-hydroxy-IQ in the absence of an exogenous metabolizing system. Green tea and black tea brewed at concentrations of 1.25%, 2.5%, and 5% (w/v) inhibited the mutagenic activity of *N*-hydroxy-IQ in a concentration-dependent manner, the former tea being more effective. Most of the antimutagens were released from the

tea leaves within 1–2 min of brewing. Fractionation of tea by HPLC showed that various catechins, including EGCG, EGG, and EGG were present in the tea extracts brewed for 1–2 min and accounted for most of the antimutagenic activity. Other components of tea, such as caffeine and tannins, were released in larger quantities after prolonged brewing (3, 5, or 10 min), but these provided no additional inhibitory activity. Caffeine has been widely studied for its pharmacological activities, and tannins contribute bitterness to tea; thus, purely from the perspective of antimutagenesis and cancer chemoprevention, brewing tea at higher concentrations but for only 1–2 min might provide the best protection against heterocyclic amines. Whether this is true for other classes of carcinogen, or applies in the context of other chronic conditions such as atherosclerosis and aging, is the subject of much current investigation.

Acknowledgements

This paper discusses the results from work that was supported in part by NIH grant CA65525.

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Table I

Inhibitory Effects of Green Tea and Black Tea Against IQ-Induced Aberrant Crypt Foci Using Initiation, Post-Initiation, and Continuous Exposure Protocols

Treatment group	Number with ACF/total number rats	Tea exposure protocol	ACF per colon (mean ± SD)	ACF/ACF-bearing animal (mean ± SD)
IQ	10/14	none	1.7 ± 1.73	2.40 ±1.58
	8/15	continuous	1.6 ± 1.88	3.00 ±1.51
IQ+GT	3/15	initiation	0.2 ± 0.41**	1.00 ± 0.00*
	15/15	post-initiation	4.5 ± 2.72	4.47 ± 2.72
	5/15	continuous	0.7 ±1.39*	2.20 ± 1.64
IQ+BT	5/15	initiation	0.5 ± 0.92*	1.60 ± 0.89*
	8/14	post-initiation	0.9 ± 0.95*	1.50 ± 0.76

Note. IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; GT, green tea (2% w/v); BT, black tea (2% w/v); $P < 0.05$* or 0.01** by Students *t* test versus controls given IQ alone.

Table II

Summary of Possible Inhibitory Mechanisms of Green Tea and Black Tea Against Cooked-Meat Heterocyclic Amines

Mechanism	Green tea	Black tea
(1) Enzyme induction	Increase in CYP1A2 and CYP1A1; increase in UDP-glucuronosyl transferase	Slight increase in CYP1A2 and UDP-glucuronosyl transferase
(2) Enzyme inhibition	Inhibition of NADPH-cytochrome P450 reductase and acetyltransferases	Slight inhibition of NADPH-cytochrome P450 reductase
(3) Complex formation	Weak	Weak
(4) Mutagen degradation	No effect on *N*-hydroxylated metabolites	No effect on *N*-hydroxylatred metabolites
(5) Electrophile scavenging	Inhibition of aryl nitrenium	Inhibition of aryl nitrenium
(6) Free radical scavenging	Likely contribution of catechins	Possible contribution of catechins, theaflavins, thearubigins??