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Inhibitory Activity of Green and Black Tea in a Free Radical-generating System Using 2-Amino-3-methylimidazo[4,5-*f*]quinoline as Substrate

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Green tea and black tea inhibit colon carcinogenesis in rats exposed to the cooked meat mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). In the present investigation, green tea, black tea and (−)-epigallocatechin gallate (EGCG) were shown to block the production of oxygen free radicals derived from IQ in the presence of NADPH-cytochrome P450 reductase. In kinetic studies using IQ as the substrate and DMPO as a free radical spin trap, EGCG increased the Km of the reaction without altering Vmax, suggesting competitive enzyme inhibition (Ki=9.96 *μM*). 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 (Ki=9.7 *μM*). These results suggest that the inhibitory activities of green tea and black tea in electron spin resonance assays using IQ as the substrate for the reductase are related to an indirect effect on the enzyme rather than via direct scavenging of the free radicals. The possible implications of these findings are discussed in the context of pathways involved in the activation and detoxification of IQ in the colon.

Key words: Green tea — Black tea — IQ — Free radical scavenging — NADPH-cytochrome P450 reductase.

Recent studies have demonstrated a cancer chemopreventive effect of tea or constituents of tea in experimental animals.[1–4] Extracts of green tea inhibited lung and forestomach tumorigenesis in mice given benzo[*a*]pyrene or *N*-nitrosodiethylamine.[1] Normal or decaffeinated green and black teas protected against ultraviolet light-induced skin carcinogenesis in mice initiated with 7,12-dimethylbenz(*a*)anthracene, and inhibited esophageal tumorigenesis in rats given *N*-nitrosomethylbenzylamine.[2–4]

Catechins and other polyphenols appear to play an important role in the protection afforded by tea.[5–7] Tea extracts and tea polyphenols exhibit antimutagenic activity *in vitro*, either via anti-oxidant mechanisms or by direct inhibition of cytochrome P450-dependent mixed-function oxidases.[8–10] In the rat, green and black teas also induce cytochrome P4501A2,[11,12] the isozyme of cytochrome P450 which activates heterocyclic amine mutagens. The latter are produced in meat and fish under normal cooking conditions,[13] and several exhibit carcinogenic activity in rodents and monkeys.[14,15] For example, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) produces tumors at several sites in the F344 rat, including the liver, small intestine and colon.[14]

We recently reported that green tea and black tea protect against IQ-induced colon carcinogenesis in the F344 rat.[12] Both teas caused a slight induction of hepatic cytochrome P4501A2, which theoretically could favor activation of IQ. However, the activated metabolites of IQ, and the enzymes producing them, are potential targets for inhibition by various constituents of tea. Tea is known to contain several compounds which scavenge free radicals,[5,9] and because IQ-derived free radicals can be produced in the presence of NADPH-cytochrome P450 reductase,[16] we sought to examine the possible role of free radical scavenging in the protective mechanism of tea against IQ-induced colon carcinogenesis.

MATERIALS AND METHODS

Chemicals IQ was purchased from Toronto Research Chemicals (Ontario, Canada). NADPH-cytochrome P450 reductase was obtained from Gentest Corp. (Woburn, MA). Cytochrome *c*, NADPH, DTPA, DMPO, (−)-epigallocatechin-3-gallate (EGCG) and several other tea standards were purchased from Sigma Chemical Co. (St. Louis, MO). Green tea (Sencha midoriiro) and black tea (English Breakfast tea, Choice Wing Brand) were obtained locally. Immediately before each experiment, teas were brewed for 5 min at a concentration of 1.25% (w/v), as described earlier.[12]

Electron spin resonance (ESR) studies DMPO spin adducts generated *in vitro* from IQ by NADPH-cytochrome P450 reductase were studied as described previously.[16] Unless stated otherwise, assay conditions were as follows (0.5 ml total reaction volume): 0.5 *μM* NADPH cytochrome P450 reductase, 150 *μM* NADPH, 1 m*M* IQ, 45 mM DMPO, 500 *μM* DTPA, and tea extract or purified tea standard (≤0.2 ml), in 10 m*M* sodium phosphate buffer, pH 6.5. Reactions typically were conducted for 1 min at room temperature using a Varian

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Model 3–9 ESR spectrometer (Palo Alto, CA) with the following instrument settings: 3400G, 100 Hz, gain 1.25 $\times 10^3$, 10 dB power, f=9.565. In subsequent experiments, kinetic assays were conducted using a range of IQ concentrations in the presence and absence of EGCG, and reaction rates were calculated following double-integration of the spectra.[16]

Enzyme studies The kinetics of NADPH-cytochrome P450 reductase were studied in the presence and absence of 10 μM EGCG using the assay conditions described previously.[17, 18]

HPLC studies Freshly brewed tea was passed through a 0.45 μm filter and injected onto a Waters C_{18}-NovaPak radial-compression cartridge (4 μm, 10 cm$\times$8.0 mm diameter). The major fractions absorbing at 273 nm were collected and dried under vacuum. The HPLC system, gradient programmer, and software were described in detail elsewhere.[19] The following mobile phase was used: 7% acetonitrile in 50 mM potassium phosphate buffer, pH 7.4 at 0 min, increased linearly to 10% acetonitrile at 12 min and 15% acetonitrile at 20 min, held isocratic until 27 min, increased to 40% acetonitrile at 42 min, held isocratic until 47 min, reduced to 0% acetonitrile at 48 min, and finally returned to the starting conditions by 60 min. The flow rate was 1 ml/min. Fractions were identified by co-elution with the authentic standard, and by comparison of the absorbance spectrum (200–800 nm) of each fraction with that of the standard, using a Perkin Elmer Lamda 2 UV/VIS spectrophotometer and associated PECSS software.

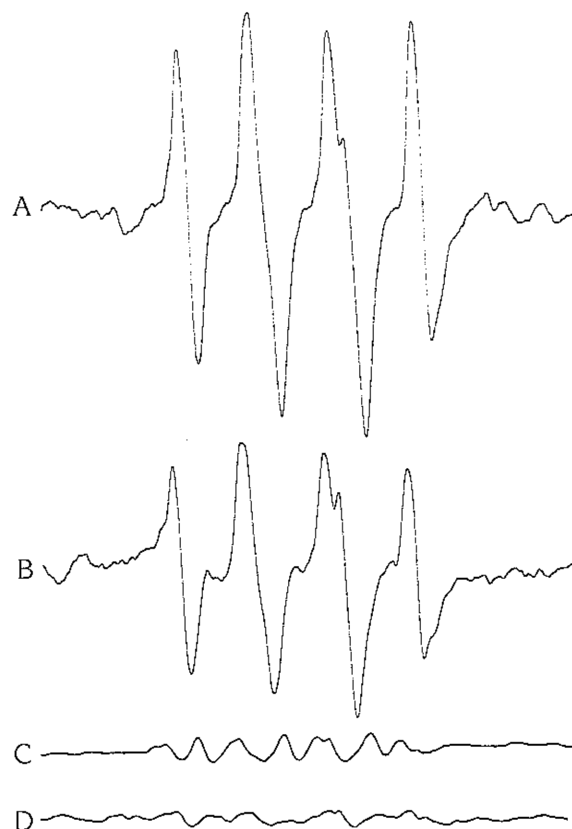


Fig. 1. ESR spectrum of the DMPO spin adduct of $O_2^{\bullet -}$ generated from IQ by NADPH-cytochrome P450 reductase. A, the reaction mixture (0.5 ml) contained 150 μM NADPH, 0.5 μM NADPH-cytochrome P450 reductase, 1 mM IQ, 0.5 mM DTPA and 45 mM DMPO in 10 mM sodium phosphate buffer, pH 6.5. B–D, the spectra obtained in the same reaction as 'A' but with the addition of (B) 50 μl of black tea or (C) 50 μl of green tea, or (D) minus IQ. For brew times and tea concentrations, see the text. Reactions were conducted for 1 min at room temperature and the spectra were analyzed by double-integration of the peak heights. The signal intensity in 'A' represents full-scale, using the instrument settings described in "Materials and Methods." In subsequent experiments, the inhibitory effects of green tea, black tea, or EGCG were compared to the uninhibited spectrum and expressed as "relative ESR signal intensity."

RESULTS

Sato *et al.*[16] used an NADPH-cytochrome P450 reductase assay system to generate free radicals from various heterocyclic amines *in vitro*, and by using DMPO as a spin trap the major reactive species was identified as superoxide radical ($O_2^{\bullet -}$). Fig. 1A shows a typical spectrum for DMPO-OOH(-$O_2^{\bullet -}$) in the present studies, with IQ as the substrate for the reaction. In accordance with the findings of Sato *et al.*,[16] the reaction was inhibited by inclusion of superoxide dismutase in the assay, but was unaffected by catalase (results not presented). Figs. 1B and 1C show the spectra under the same assay conditions as for Fig. 1A, but with the inclusion of 50 μl of black tea or green tea, respectively. No ESR signal was observed in the absence of IQ (Fig. 1D), or when DMPO, NADPH or the reductase was excluded from the reaction (not shown).

Different volumes of freshly brewed tea were tested in the NADPH-cytochrome P450 reductase assay system using IQ as the substrate (Fig. 2). Tea brewed for 5 min at a concentration of 1.25% (w/v) and tested using volumes $\leq$0.2 ml provided dose-related inhibition, green tea being considerably more effective than black tea. By interpolation from Fig. 2, the approximate volume of green tea and black tea required for 50% inhibition was 0.01 ml *versus* 0.13 ml, respectively.

To identify the inhibitory component(s) in green tea, fractions collected after HPLC separation were tested in the NADPH-cytochrome P450 reductase assay using IQ as the substrate and DMPO as a spin trap (Fig. 3). Each fraction was assayed in the amount corresponding to that

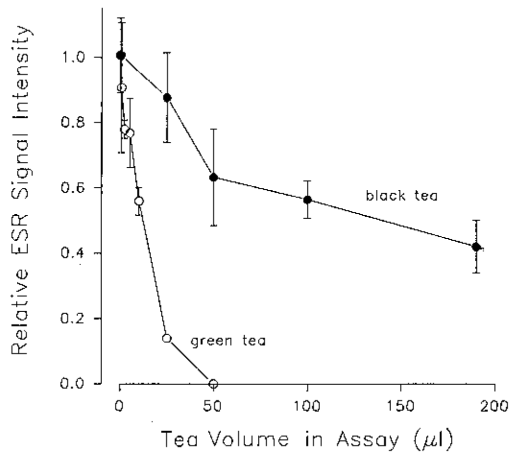


Fig. 2. Inhibition of the IQ/NADPH-cytochrome P450 reductase system by green tea and black tea. Teas were brewed for 5 min at a concentration of 1.25% (w/v) and various volumes were tested in the assay system described in the legend to Fig. 1. Data points and bars represent means±SD from testing triplicates for each volume of tea in the assay.

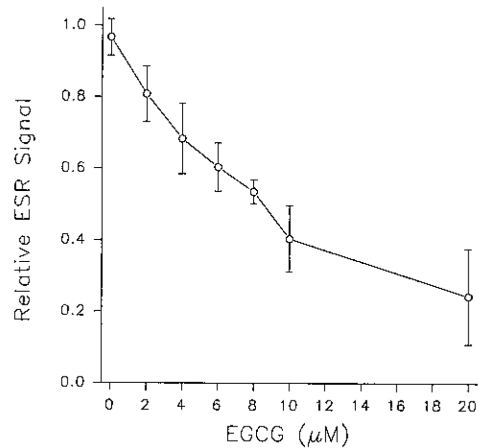
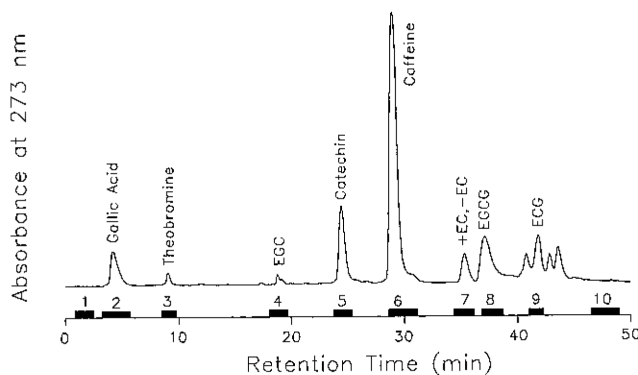


Fig. 4. Dose-response inhibition by EGCG in the IQ/NADPH-cytochrome P450 reductase assay. Data are given as mean±SD from the testing of triplicates for each concentration of EGCG.



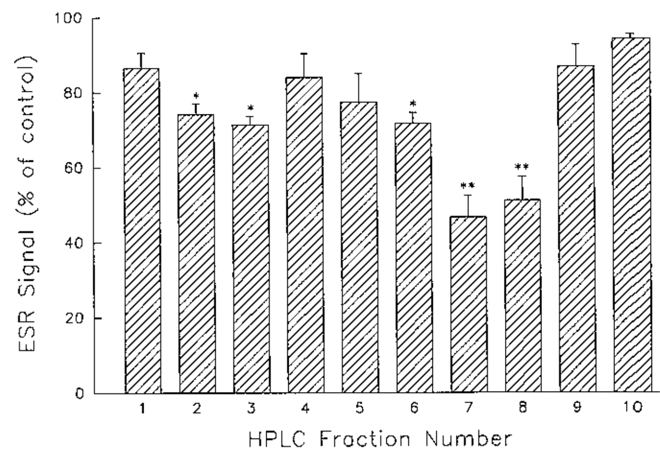


Fig. 3. Green tea components were separated by HPLC and the major peaks absorbing at 273 nm were tested for inhibitory activity in the IQ/NADPH-cytochrome P450 assay using DMPO as a spin trap. Eight peaks plus two "baseline" fractions were collected (see solid bars numbered under the HPLC trace). Fractions 2–9 were identified by co-elution with authentic standards: EGCG, epigallocatechin gallate; EGC, epigallocatechin; ECG, epicatechin gallate; −EC, (−)-epicatechin; +EC, (+)-epicatechin. Fractions were tested in the ESR assay (hatched bars, lower figure) in an amount equivalent to that obtained from 0.01 ml of green tea; data are given as mean±SD from the testing of triplicates, and inhibition was identified as significant using Student's t test (* $P<0.05$, ** $P<0.01$).

obtained from 0.01 ml of green tea; under these conditions, two fractions exhibited highly significant inhibitory activity, namely, fraction 7 (containing (+) and (−) epicatechins) and fraction 8 (containing EGCG). The latter compound was selected for further study of the dose-response for inhibition. Concentrations of EGCG in the range of 2–20 μM produced dose-related inhibition of the ESR signal, giving approximately 80% inhibition at the highest concentration tested (Fig. 4).

To provide some insight into the kinetics of inhibition, the assay conditions were modified to give concentrations of IQ in the range of 6.25–100 μM, as described by Sato *et al.*[16] When tested in the presence of 5 μM EGCG, the Km of the reaction was increased and the Vmax was

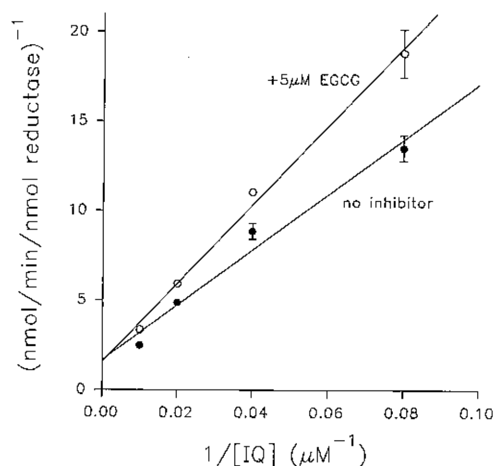


Fig. 5. Lineweaver-Burk plot of the rate of DMPO spin adduct formation from $O_2^{\bullet -}$ by the IQ/NADPH-cytochrome P450 reductase system. The reactions were conducted in triplicate in the presence and absence of 5 *μM* EGCG and concentrations of IQ in the range of 6.25 to 100 *μM*; all other components of the assay were as described in the legend to Fig. 1A. The method for calculating concentrations of the DMPO spin adduct was described elsewhere.[16] Ki=9.7 *μM*.

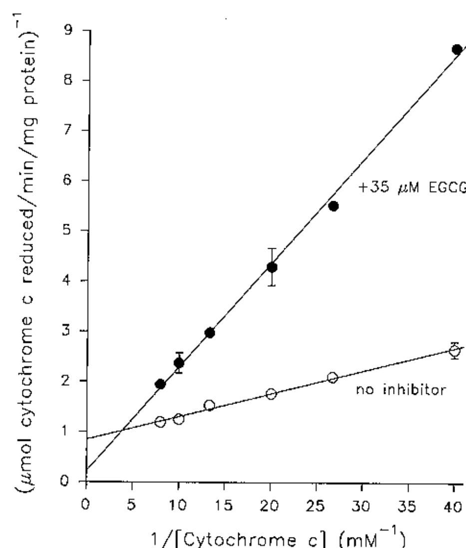


Fig. 6. Lineweaver-Burk plot of NADPH-cytochrome *c* reductase activity in the presence and absence of 35 *μM* EGCG. Assays were conducted as described previously.[17, 18] Results are given as mean±SD from the testing of triplicates at each point. Data were well fitted by a model of reversible competitive inhibition with Ki=9.96 *μM*.

unaffected, indicating competitive inhibition (Fig. 5). The Ki was determined to be 9.7 *μM* using the formula: slope increase=(1+[I]/Ki).[20]

Since EGCG might act by inhibiting NADPH-cytochrome P450 reductase, the redox pathway from IQ (via $O_2^{\bullet -}$) to DMPO, or a combination of the two, further experiments were conducted on the early steps in the pathway. Thus, the kinetics of NADPH-cytochrome P450 reductase were studied spectrophotometrically using cytochrome *c* as the substrate (Fig. 6). The data were well fitted by a model of reversible competitive inhibition with Ki=9.96 *μM*.

DISCUSSION

In accordance with previous work by Sato *et al.*,[16] the present study showed that NADPH-cytochrome P450 reductase can generate free radicals from IQ, leading to formation of reactive oxygen species. The possibility that these reactive species might contribute to the genotoxic and carcinogenic activity of IQ in the colon has not been thoroughly investigated, but deserves further attention. In contrast to liver, the colon plays a minor role in the initial activation of IQ via *N*-hydroxylation since the primary enzyme catalyzing this reaction, cytochrome P4501A2, is present at low levels in the colonic mucosa. However, several other enzymes contribute to the metabolism of IQ in extrahepatic tissues, including *β*-glucuronidase, sulfotransferase, acetyltransferase, and PGH-synthase.[17, 21–23] These enzymes can generate the presumed "ultimate carcinogen" of IQ, an aryl nitrenium ion that is a highly reactive electrophile.[21, 22, 24–26] However, a "leaky" reductase would set up an alternative pathway in which electrons from NADPH redox cycle through IQ to form $O_2^{\bullet -}$ instead of being passed to cytochrome P450 to form the electrophile. A study of 8-hydroxyguanosine and other DNA adducts associated with reactive oxygen species[27, 28] might clarify whether free radical mechanisms contribute significantly to IQ-induced colon carcinogenesis in the rat.

In the present study, both green tea and black tea inhibited the free radical reaction catalyzed by NADPH-cytochrome P450 reductase using IQ as the substrate, but the green tea was significantly more effective. Since polyphenols undergo oxidative polymerization to theaflavins and other oligomers during the fermentation of green tea into black tea,[9] a possible explanation for the greater inhibition associated with green tea might be the higher levels of catechins, such as EGCG.[5] Indeed, the percentage of the total inhibitory activity of green tea which can be explained solely by EGCG in this study (HPLC fraction 8) was 35–40%, and approximately the same level of inhibition was associated with epicatechins (fraction 7).

The ability of green and black tea, and of individual catechins, to block the redox reaction involving IQ provides one mechanistic explanation for the protection afforded by tea in the colon.[12] Previous work demonstrated free radical scavenging by various tea constituents,[5,9] but we cannot separate such a mechanism from inhibitory effects on NADPH-cytochrome P450 reductase in the present study. In fact, the Ki for EGCG was essentially identical in the ESR assay using IQ as the substrate and in the enzyme assay using cytochrome *c* as the substrate, and the kinetics indicated that ECGC acts as a reversible competitive enzyme inhibitor. Similar results were reported for another tea constituent, (+)-catechin,[8] and for chlorophyllin[18]; it is presently unclear whether these inhibitors simply "syphon" electrons from the reductase or interfere in the docking of NADPH-cytochrome P450 reductase with the cytochrome.

Inhibition of NADPH-cytochrome P450 reductase would serve as an upstream, indirect mechanism for attenuating the activity of cytochrome P450. Consistent with this hypothesis, the marked antimutagenic activity of green tea against several heterocyclic amines, polycyclic aromatic hydrocarbons, and nitrosamines was associated with inhibition of cytochrome P450-dependent activation of the promutagens.[10] Other mechanisms of inhibition by tea have also been examined, including direct interaction with the carcinogen, scavenging of electrophiles, and induction of phase I and phase II enzymes.[8,10-12,29] Taken together, these studies provide evidence that tea protects via multiple mechanisms, but further work is required to clarify which one(s) is most important in protecting against IQ in the colon.

In summary, the present investigation has shown that green tea, black tea, and EGCG inhibit the formation of free radicals derived from IQ in the presence of NADPH-cytochrome P450 reductase. Initially, this was assumed to be associated with the anti-oxidant components of tea scavenging IQ-derived free radicals. However, kinetic studies supported an indirect inhibitory effect on the enzyme NADPH-cytochrome P450 reductase. Such a mechanism could serve to reduce the damage associated with oxygen free radicals generated in the presence of IQ, and to lower the overall metabolism of IQ via indirect inhibition of cytochrome P450.

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