Inhibition of the Mutagenicity of Aromatic Amines by the Plant Flavonoid (+)-Catechin

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ABSTRACT

Addition of the plant phenolic flavonoid (+)-catechin to rat liver microsomes inhibited the mutagenicity of the aromatic amines 2-aminofluorene and 4-aminobiphenyl in the Ames test. Similarly, (+)-catechin decreased the mutagenicity of N-hydroxy-4-aminobiphenyl, the proximate carcinogen, but, in contrast, had no effect on the mutagenicity of other direct-acting carcinogens such as N-methyl-N'-nitro-N-nitrosoguanidine and 9-aminoacridine. In vitro addition of (+)-catechin gave rise to a dose-dependent inhibition of the cytochrome P-450-dependent benzphetamine N-demethylation and ethoxyresorufin O-deethylase activities. This was achieved by impairment of the electron flow from the reduced pyridine nucleotide to the cytochrome. However, administration of (+)-catechin to rats had no effect on the in vivo mixed-function oxidase activities. It is concluded that the (+)-catechin inhibits the mutagenicity of aromatic amines in the Ames test by interfering with their cytochrome P-450-dependent bioactivation and by direct interaction with the proximate carcinogen, but the former mechanism is unlikely to occur in vivo because the high doses of the flavonoid required are not achieved.

INTRODUCTION

It has been reported recently that naturally occurring plant phenolic flavonoids may act as anticarcinogenic agents against polycyclic aromatic hydrocarbon-induced neoplasia in animals. Ellagic acid administration to mice afforded protection against benzo(a)pyrene-induced pulmonary adenomas and following topical application against 3-methylcholanthrene- and 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis (1, 2). The mechanism of action may involve either (a) inhibition of the formation of the ultimate carcinogens (3) and/or (b) direct interaction of the phenol with the ultimate carcinogen (4). The mutagenicity of benzo(a)pyrene 7,8-diol-9,10-epoxide, the ultimate carcinogen and mutagen, was markedly inhibited by phenolic flavonoids (5).

(+)-Catechin ([+]-cyanidanol-3; 3',4',5,7-tetrahydroxyflavan-3-ol) is a plant phenolic flavonoid which is currently used as a hepatoprotective agent against acute viral hepatitis and hepatotoxic agents (6). Its mechanism of action is believed to involve its antioxidant and free-radical scavenging properties (7) and its stabilizing effects on cytoplasmic and other cell membranes (8). In contrast to ellagic acid, which may exhibit toxicity after i.p. administration (1), (+)-catechin is remarkably nontoxic and free from side effects (9).

The present study was therefore undertaken to investigate if (+)-catechin possesses antimutagenic properties and if flavonoids can also antagonize other groups of chemical carcinogens such as the aromatic amines.

MATERIALS AND METHODS

(+)-Catechin, N-aminoacridine, cytochrome c and all cofactors (Sigma Co., Poole, Dorset, U.K.), N-methyl-N'-nitro-N-nitrosoguanidine and 2-aminofluorene (Aldrich Chemicals Ltd., Gillingham, Dorset, U.K.), 4-aminobiphenyl (Phase Separations Ltd., Queensferry, Flintshire, U.K.), and resorufin and ethoxyresorufin (Molecular Probes, Inc., Junction City, OR) were all purchased. N-Hydroxyaminobiphenyl was obtained as described previously (10). Benzphetamine was a generous gift from Upjohn Co. Ltd., 27100 Le Vaudreuil Ville, Naivelle, France.

Male Wistar albino rats (150 to 200 g) were obtained from the Animal Breeding Unit, University of Surrey. Animals received single daily i.p. administrations of (+)-catechin (200 mg/kg), suspended in 0.9% NaCl solution (20 mg/ml), for 3 days, while control animals received the corresponding volume of the vehicle. All animals were killed 24 h after the last injection.

Hepatic postmitochondrial supernatant (9,000 x g supernatant, S9 fraction) and microsomal suspension (105,000 x g pellet resuspended) were prepared as described previously (11). Benzphetamine N-demethylation (12) was determined in the postmitochondrial supernatant and ethoxyresorufin O-deethylase (13) and NADPH cytochrome c reductase (14) in the microsomal suspension; protein was determined in both fractions (10). Binding to cytochrome P-450 was studied in washed microsomes using a Varian-Cary double-beam spectrophotometer equipped with automatic baseline correction (15).

The mutagenicity studies were conducted using the Ames test procedure (16) and using Salmonella typhimurium strains TA 1535, TA 1537, TA 1538, and TA 98. Double the usual amount of the S9 fraction was incorporated in the S9 mix, i.e., 100 µl per plate. All carcinogens and (+)-catechin were freshly dissolved in dimethyl sulfoxide so that less than 100 µl were added to each plate.

RESULTS

(+)-Catechin was nonmutagenic in all of the bacterial strains used in the present study and had no effect on their spontaneous reversion rates (data not shown). Addition of catechin into the activation system caused a concentration-dependent inhibition of the mutagenicity of the aromatic amines 2-aminofluorene and 4-aminobiphenyl (Chart 1). Under the same conditions, (+)-catechin had no effect on the mutagenicity of the direct-acting carcinogens 9-aminoacridine and N-methyl-N'-nitro-N-nitrosoguanidine (Table 1) but, in contrast, gave rise to a concentration-dependent decrease in the mutagenicity of N-hydroxy-4-aminobiphenyl (Chart 2).

Addition of (+)-catechin in vitro gave rise to a dose-dependent inhibition of the N-demethylation of benzphetamine and the O-deethylation of ethoxyresorufin (Chart 3, A and B), 50% of inhibition being achieved at a concentration of 1.4 µM. A more marked inhibition of NADPH-cytochrome c reductase was observed (Chart 3C), no activity having been detected at a (+)-catechin concentration of 20 mg/ml.
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i. 2

•o

2400 -

100 -

50 -

1.4 mM

2.8 mM

4.2 mM

Control

1.4 mM

2.8 mM

4.2 mM

20 40 60

4-Aminobiphenyl (ug/plate)

80 0 20 5 10 15

2-Aminofluorene (pg/plate)

Chart 1. Effect of (+)-catechin on the activation of 4-aminobiphenyl (A) and 2-aminofluorene (B) to mutagens. Each point represents the average of 3 plates, and the experiment was repeated twice with similar data. Salmonella typhimurium strain TA 1538 was used, and the spontaneous reversion rate [11 ± 2 (SD)] has been subtracted.

Table 1

Effect of (+)-catechin on the mutagenicity of direct-acting carcinogens

Results are presented as mean for 3 determinations. The experiment was repeated twice with similar results. Spontaneous reversion rates for TA 1535 (N-methyl-N-nitro-N-nitrosoguanidine) and TA 1537 (9-aminoacridine) were 15 ± 1 (SD) and 10 ± 3, respectively.

<table>
<thead>
<tr>
<th>Carcinogen</th>
<th>Concentration (pg/plate)</th>
<th>0 mM</th>
<th>2.8 mM</th>
<th>4.2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNNG*</td>
<td>0.5</td>
<td>2747 ± 186</td>
<td>2577 ± 77</td>
<td>2364 ± 155</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>2724 ± 104</td>
<td>2428 ± 96</td>
<td>1942 ± 144</td>
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<tr>
<td></td>
<td>1.0</td>
<td>2953 ± 302</td>
<td>2497 ± 72</td>
<td>2389 ± 299</td>
</tr>
<tr>
<td>9-Aminoacridine</td>
<td>10</td>
<td>162 ± 39</td>
<td>155 ± 11</td>
<td>117 ± 11</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>470 ± 57</td>
<td>568 ± 79</td>
<td>397 ± 35</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>732 ± 147</td>
<td>988 ± 157</td>
<td>1040 ± 201</td>
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<td></td>
<td>40</td>
<td>1319 ± 166</td>
<td>1296 ± 191</td>
<td>1200 ± 65</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (pg/plate)</th>
<th>0 mM</th>
<th>2.8 mM</th>
<th>4.2 mM</th>
</tr>
</thead>
</table>
| MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.  
| Mean ± SD.  
| catechin concentration of 2.8 mM. Kinetic studies using concentrations of (+)-catechin ranging from 0.06 to 0.18 mM revealed that the inhibition of the NADPH-dependent reduction of cytochrome c was of the competitive type (Chart 4). (+)-Catechin did not interfere with any of the assays at the concentrations used (data not shown).

Administration of (+)-catechin to animals had no effect on mixed-function oxidase activity, NADPH-cytochrome c reductase, or microsomal cytochrome P-450 levels (Table 2). (+)-Catechin interacted with rat hepatic microsomes to generate a reverse Type I spectral change characterized by a maximum at 418 nm and a minimum at 387 nm (Chart 5).

DISCUSSION

Several plant phenols have been shown to modulate the mutagenicity of polycyclic aromatic hydrocarbons and aflatoxin B1, some inhibiting and others enhancing mutagenicity (5, 17). Subsequent studies demonstrated that phenolic plant flavonoids such as ellagic acid could afford protection against chemical carcinogens (1, 2).

In the present study using (+)-catechin, we have demonstrated that the inhibitory effect of flavonoids is not confined to polycyclic aromatic hydrocarbons and mycotoxins but extends also to aromatic amines, a major group of chemical carcinogens, as exemplified by 2-aminofluorene and 4-aminobiphenyl (Chart 1). The mechanism of action may involve either (a) direct interaction between the proximate or ultimate mutagens with (+)-catechin, and/or (b) inhibition of the generation of the ultimate carcinogen by the flavonoid. Addition of (+)-catechin in vitro caused a concentration-dependent decrease in the mutagenicity of N-hydroxy-4-aminobiphenyl, the proximate carcinogen of 4-aminobiphenyl (18) with (+)-catechin. The lack of effect of catechin on the mutagenicity of the direct-acting carcinogens 9-aminoacridine and N-methyl-N'-nitro-N-nitrosoguanidine suggests that its effect on the N-hydroxy derivative of 4-aminobiphenyl is...
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Chart 2. Inhibition of the mutagenicity of 4-hydroxy-3-aminobiphenyl by (+)-catechin. 4-Hydroxy-3-aminobiphenyl (26 nmol) was dissolved in dimethyl sulfoxide. Each point represents the average of 3 plates, and the experiment was repeated twice with similar data. Salmonella typhimurium strain TA 98 was used, and the spontaneous reversion rate [12 ± 1 (SD)] has been subtracted.

specific. Thus, similar to the polycyclic aromatic hydrocarbons (4, 19), direct interaction occurs between the proximate carcinogen of 4-aminobiphenyl and the flavonoid, leading to loss of mutagenicity.

(+)-Catechin may also exhibit its inhibitory effect in the mutagenicity of aromatic amines by impairing the pathways that lead to activation. N-Hydroxylation is recognized as the first step in the bioactivation of aromatic amines, and this pathway may be catalyzed in the liver by the cytochrome P-450-dependent mixed-function oxidases (18, 20) or by the mixed-function amine oxidase system (21). (+)-Catechin at the concentrations used in the mutagenicity studies caused a concentration-dependent inhibition of the N-demethylation of benzphetamine and O-deethylation of ethoxyresorufin, reactions catalyzed by the phenobarbital-induced cytochrome P-450 and the polycyclic aromatic hydrocarbon-induced cytochrome P-448, respectively (12, 22), indicating that the effect of (+)-catechin on the mutagenicity of aromatic amines is at least partly mediated by inhibition of the mixed-function oxidases. This may be brought about by a number of possible mechanisms such as destruction of cytochrome P-450, through competitive inhibition (+)-catechin acting as an alternative substrate, by formation of a ligand complex between (+)-catechin and the heme moiety of the cytochrome preventing substrate oxygenation, or by interfering with the electron transfer from NADPH to the haemoprotein. Incubation of (+)-catechin with hepatic microsomes had no effect on cytochrome P-450 levels, measured as the CO-reduced cytochrome complex (data not shown), indicating the absence of the first mechanism. (+)-Catechin interacted with rat hepatic microsomes to yield a reverse Type I spectral change believed to represent displacement of an endogenous substrate (23). No Type II spectral change, indicative of an interaction with the heme, was obtained at any concentration studied, demonstrating that no ligand formation occurs. Metabolic studies in a number of species, including rat, established that the metabolism of (+)-catechin proceeds through conjugation reactions and methylation catalyzed by the
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1

[nmol of cyt. c reduced/min per g liver] x 10^-3

0.04 0.03 0.02 0.01 0.01 0.02 0.03 0.04 0.05 0.06 0.07

[cytochrome c] mM

Chart 4. Lineweaver-Burke plot of the inhibition of NADPH cytochrome c reductase by (+)-catechin. A, without catechin; Δ, 0.06 mM; O, 0.12 mM; and •, 0.18 mM (+)-catechin.

Table 2

Effect of (+)-catechin administration on the hepatic microsomal mixed function

Animals received single daily i.p. administration of (+)-catechin (200 mg/kg) for 3 days and were killed 24 h after the last administration. Results are presented as mean for 5 animals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>(+)-Catechin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzphetamine N-demethylase</td>
<td>146 ± 16^a</td>
<td>123 ± 21</td>
</tr>
<tr>
<td>Ethoxyresorufin O-deethylase</td>
<td>41 ± 10</td>
<td>45 ± 10</td>
</tr>
<tr>
<td>NADPH-cytochrome reductase</td>
<td>9.4 ± 0.4</td>
<td>9.8 ± 1.7</td>
</tr>
<tr>
<td>Cytochrome P-450</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

* Mean ± SD.

catechol O-methyltransferase system (24, 25). Furthermore, no Type I spectral interaction, indicative of substrate binding, was seen when microsomes were incubated with (+)-catechin. All of these observations argue strongly against (+)-catechin being a substrate of the mixed-function oxidase system. In vitro addition of (+)-catechin, however, caused a marked dose-dependent inhibition of cytochrome c reductase, indicating impairment of the electron flow from the reduced pyridine nucleotide to the cytochrome. Inhibition is competitive, indicating that the flavonoid may act as an electron acceptor. Our findings are compatible to those of Buening et al. (17), who demonstrated that flavonoids containing hydroxy groups are potent inhibitors of the NADPH-dependent reduction of cytochrome c.

Inhibition of mixed-function oxidase activities in vitro by (+)-catechin was observed at concentrations of 10^-4 M, which cannot be achieved in vivo. Indeed, no inhibition of the hepatic microsomal mixed-function oxidase activities was observed following administration of high doses of (+)-catechin (Table 2), in agreement with previous reports (26). It can therefore be concluded that any anticarcinogenic effect of (+)-catechin can only result from direct interaction of the flavonoid with the proximate/ultimate carcinogen. Ellagic acid, administered to mice at doses comparable to those of (+)-catechin used in the present study, inhibited the benzo(a)pyrene-induced pulmonary tumorigenicity (1) and, when applied to the skin, it afforded protection against the 3-methylcholanthrene-induced skin tumorigenesis (2). The latter workers demonstrated that ellagic acid topically applied caused a very marked inhibition of both skin and hepatic aryl hydrocarbon hydroxylase activity and concluded that reduced generation of the reactive metabolites was likely to be the prevailing mechanism of the protective action. Inhibition of the mixed-function oxidases, an enzyme system which not only deactivates xenobiotics but also is involved in the metabolism of many endogenous substrates, will undoubtedly result in complications. (+)-Catechin, which is unlikely to have an in vivo inhibitory effect, may prove a better agent despite the exceptionally high activity of ellagic acid in inhibiting the mutagenicity of polycyclic aromatic hydrocarbons (4).
It is interesting that maximum inhibition of the mutagenicity of 2-aminofluorene was achieved at (+)-catechin concentration of 2.8 mw whereas, for 4-aminobiphenyl, increasing the concentration to 4.2 mw resulted in no further inhibition of mutagenicity. It is likely that the remaining capacity for activation of 2-aminofluorene, not inhibited by the flavonoid, is due to the mixed-function amine oxidase system, which is known to N-hydroxylate this carcinogen (21). No contribution of this enzyme system to the bioactivation of 2-aminofluorene was achieved at a (+)-catechin concentration of 2.8 mw whereas, for 4-aminobiphenyl, increasing the concentration to 4.2 mw resulted in no further inhibition of mutagenicity. It is likely that the remaining capacity for activation of 2-aminofluorene, not inhibited by the flavonoid, is due to the mixed-function amine oxidase system, which is known to N-hydroxylate this carcinogen (21). No contribution of this enzyme system to the bioactivation of 2-aminofluorene was achieved at a (+)-catechin concentration of 2.8 mw whereas, for 4-aminobiphenyl, increasing the concentration to 4.2 mw resulted in no further inhibition of mutagenicity. It is likely that the remaining capacity for activation of 2-aminofluorene, not inhibited by the flavonoid, is due to the mixed-function amine oxidase system, which is known to N-hydroxylate this carcinogen (21). No contribution of this enzyme system to the bioactivation of 2-aminofluorene was achieved at a (+)-catechin concentration of 2.8 mw whereas, for 4-aminobiphenyl, increasing the concentration to 4.2 mw resulted in no further inhibition of mutagenicity. It is likely that the remaining capacity for activation of 2-aminofluorene, not inhibited by the flavonoid, is due to the mixed-function amine oxidase system, which is known to N-hydroxylate this carcinogen (21). No contribution of this enzyme system to the bioactivation of 2-aminofluorene was achieved at a (+)-catechin concentration.
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