Effect of Green Tea Extract on the Induction of Ornithine Decarboxylase and the Activation of Extracellular Signal-Regulated Kinase in Bladder Carcinoma ECV304 Cells

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Abstract: According to several studies, green tea and individual catechins can inhibit the induction of ornithine decarboxylase (ODC), the key enzyme in the biosynthesis of polyamines. It has been suggested that the inhibition of ODC induction may offer an explanation to the anticancer and chemopreventive activities of green tea. In the present study, however, treatment of bladder carcinoma ECV304 cells with green tea extract (GTE) was not able to reduce the induction of ODC by fetal calf serum. Actually, in the absence of serum, GTE provoked a dose-dependent and remarkable induction of ODC activity. The induction of ODC, which could be elicited also by (-)-epigallocatechin 3-gallate, a major green tea component, required an early activation of extracellular signal-regulated kinase 1 and 2 (ERK), and both events appeared to be dependent on an alteration of the status of cellular thiol groups. Pretreatment with specific ERK or ODC inhibitors was able to prevent a late caspase activation but hardly affected the loss of cell viability provoked by GTE. In conclusion, to our knowledge, this is the first study showing that GTE can promote ODC induction in a tumor cell line.

Introduction

Green tea, a beverage widely consumed, especially in Eastern countries, is being extensively investigated as a potential dietary tool against cancer. Laboratory and animal studies have shown a protective effect of green tea on cancer of different sites, although epidemiological evidence is still limited and, in the case of some cancers such as bladder cancer, even controversial with opposite results (1–4). The anticancer and chemopreventive properties of green tea have been attributed mainly to its content in polyphenols, which comprise about 30% of the weight of the dry leaves. Most of the polyphenols are flavanols and include (-)-epigallocatechin 3-gallate (EGCG), the most abundant, (-)-epigallocatechin (EGC), and other catechins.

Whole green tea extract (GTE) and single components, such as EGCG and EGC, have been shown to exert a wide range of cellular, biochemical, and pharmacological activities, including anti- and pro-oxidant activities, inhibition of cell proliferation, oncogene expression and carcinogenesis, induction of tumor cell death and apoptosis, and alteration of signaling pathways, such as those involving mitogen-activated protein kinases (MAPKs) (4–8). In particular, activation of MAPKs, such as p44/42 MAPK (also called extracellular signal-regulated kinase 1 and 2, ERK), has been related to the pro-oxidant and apoptotic effects of green tea components (7–9).

According to several studies (5,6,10,11), green tea and catechins can also inhibit the induction of ornithine decarboxylase (ODC), which catalyzes the rate-limiting step in the biosynthesis of polyamines. Although it has been long known that polyamine biosynthesis is essential for cell proliferation (12–14), growing evidence suggests a role for polyamines even in the control of cell death and apoptosis (14,15 and references therein). ODC may be considered to be a proto-oncogene, and its expression is strictly related to cell growth and carcinogenesis (6,12–14). Furthermore, ODC inhibitors, such as difluoromethylornithine (DFMO), have been proposed and used as agents for cancer chemoprevention and therapy. Thus, prevention of ODC induction and polyamine accumulation has been suggested as an important mechanism by which green tea could exert its cancer chemopreventive and tumor growth–inhibiting actions (6).

In the present study we have investigated the effect of GTE and EGCG on ODC induction in bladder carcinoma cell line ECV304 (16). We found that GTE could not reduce the ODC induction elicited in these cells by serum. Actually, it proved able to cause a large increase in basal ODC activity, which appeared to be dependent on an early activation of ERK and was followed by a late activation of caspases, the proteases that execute the apoptotic death program (17).

Materials and Methods

Materials

Anti-ERK and anti-phospho-specific ERK (Thr202/Tyr204) antibodies were purchased from New England Biolabs and
anti-poly (ADP-ribose) polymerase (PARP) antibodies were from BIOMOL. DFMO and Ac-Asp-Glu-Val-Asp-chloromethylketone (AcDEVD-CMK) were obtained from Calbiochem and GTE (a decaffeinated preparation) was from Indena (Milan, Italy). EGCG and the inhibitors of signal transduction pathways were purchased from Alexis Corp., except U0126 (from Promega). N-Acetylcyesteine (NAC) and TEMPO were from Sigma.

Cell Culture and Treatments

Human ECV304 cells were routinely grown in M199 medium containing 10% fetal calf serum and antibiotics (100 U/ml of penicillin and 100 μg/ml streptomycin) and tested for mycoplasma contamination. For experiments, confluent cells were kept in a serum-free medium for 2 days before treatment with GTE, EGCG, or 10% serum. Inhibitors were added to the cells 30 min before these treatments. Some inhibitors were added in dimethylsulfoxide (final concentration: 0.1%). Control cells received equal amounts of the vehicle. To obtain polyamine depletion, DFMO was added during the last subculturing of the cells before experiments. Cell viability was determined by Trypan blue exclusion. ECV304 cell line has long been considered to be derived from HUVVEC (endothelial) cells but has been recently reclassified as a bladder carcinoma cell line (16). The effect of GTE on ODC activity was investigated in cells other than ECV304, such as rat neonatal cardiomyocytes, human immortalized chondrocytes, and rat H9C2 cardiac myoblasts.

Determination of ODC Activity and Polyamine Content

At the time indicated after treatment, cells extracts were prepared and assayed for ODC activity as previously described (18). Duplicate determinations were carried out on each sample. Specific ODC activity is expressed as units/mg protein, where 1 unit corresponds to 1 nmol of CO₂/h of incubation. Polyamines were separated and quantified by HPLC after derivatization with dansyl chloride (19) and expressed as nmol/mg protein.

Determination of Caspase Activity

At the time indicated after treatment, cells were detached and harvested to be assayed for caspase activity. The activity of caspase enzymes was measured by the cleavage of the fluorogenic peptide substrate Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AcDEVD-AMC) during a 15-min incubation at 37°C (15). Because the sequence DEVD represents a substrate for caspase-3 and other members of the caspase family, this activity is referred to as caspase activity. Duplicate determinations were carried out on each sample. Caspase activity is expressed as units/mg protein, where 1 unit is defined as the amount of enzyme activity cleaving 1.0 nmol of substrate per minute.

TUNEL Assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed using a colorimetric reagent kit (Titer/TACS, Trevigen, Inc.), which allows quantitative labeling of the 3'-OH ends of fragmented DNA in cultured cells. Briefly, ECV304 cells were fixed, washed in PBS, and labeled according to manufacturer's instructions. After washing the samples with PBS, colorimetric reaction was allowed to occur and absorbance was read at 450 nm on a microplate reader (Victor2, Perkin Elmer).

Western-Blot Analysis

Western-blot analysis of total and phosphorylated ERK was performed by using specific antibodies as previously described (18). About 10⁵ cells were resuspended in 0.1 ml of lysis buffer (20 mM Tris/Cl, pH 8, 100 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 1 mM benzamidine, 1% Nonidet P40, 1 mM PMSF, 10 mM p-nitrophenylphosphate, 1 mM dithiothreitol, 10 mM β-glycerophosphate, and 1 μg/ml aprotinin, leupeptin, and pepstatin), sonicated, and centrifuged. The supernatant was boiled in loading buffer, and an aliquot corresponding to 80 μg of protein was analyzed by SDS-PAGE (12% gel). Separated proteins were transferred to a nitrocellulose membrane for 1 h. The membrane was saturated with 5% powdered milk, 0.05% Tween 20 in 10 mM Tris, pH 8, and 150 mM NaCl for 1 h and then incubated with anti-ERK or anti-phosphospecific ERK antibody at 4°C overnight. Bands were revealed by the Amersham ECL detection system. PARP and its apoptosis-related cleavage fragment were detected by Western blot (19) in nuclear extracts isolated using the Nuclear Extract Kit obtained from Active Motif.

Statistical Analysis

The data presented in Table 1 and Figs. 2, 4C and D, and 5 were analyzed for statistical significance (P < 0.05) by unpaired t-test.

Results and Discussion

ODC Induction and ERK Activation by GTE

It has been suggested that the inhibition of ODC induction by green tea may offer an explanation to the chemopreventive and anticancer activities of green tea (6). However, in preliminary experiments we found that treatment of carcinoma bladder ECV304 cells with GTE could not reduce the induction of ODC by 10% fetal calf serum (Fig. 1A), a strong ODC inducer in these cells (18) as well as in other cell types. Actually, in the absence of serum, addition of GTE resulted in a dose-dependent induction of ODC activity. Although as low as 10 μg/ml GTE was able to increase ODC activity, a
Table 1. Effect of Various Inhibitors on ODC Induction by GTE and EGCG

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Specificity</th>
<th>Concentration</th>
<th>Treatment</th>
<th>ODC Activity (% of GTE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>11.9 ± 1.3* (12)</td>
</tr>
<tr>
<td>PD98059</td>
<td>MEK 1/2 inhibitor</td>
<td>50 µM</td>
<td>GTE</td>
<td>100 ± 3.4* (12)</td>
</tr>
<tr>
<td>U0126</td>
<td>MEK 1/2 inhibitor</td>
<td>1 µM</td>
<td>GTE</td>
<td>65.2 ± 8.0* (4)</td>
</tr>
<tr>
<td>SB203580</td>
<td>p38 MAPK inhibitor</td>
<td>10 µM</td>
<td>GTE</td>
<td>53.8 ± 1.5* (3)</td>
</tr>
<tr>
<td>NAC</td>
<td>Thiol reductant</td>
<td>10 µM</td>
<td>GTE</td>
<td>7.3 ± 0.7* (6)</td>
</tr>
<tr>
<td>TEMPO</td>
<td>Antioxidant</td>
<td>10 µM</td>
<td>GTE</td>
<td>120.2 ± 4.4* (6)</td>
</tr>
<tr>
<td>Go6976</td>
<td>PKC inhibitor</td>
<td>2 µM</td>
<td>GTE</td>
<td>26.3 ± 5.8* (3)</td>
</tr>
<tr>
<td>Go6976</td>
<td>PKC α, β, γ inhibitor</td>
<td>10 µM</td>
<td>GTE</td>
<td>6.2 ± 0.43* (3)</td>
</tr>
<tr>
<td>Rottlerin</td>
<td>PKC δ inhibitor</td>
<td>10 µM</td>
<td>GTE</td>
<td>4.7 ± 0.6* (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EGCG</td>
<td>32.5 ± 1.5* (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EGCG</td>
<td>124.6 ± 28.2 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EGCG</td>
<td>11.1 ± 6.2* (4)</td>
</tr>
</tbody>
</table>

Experiment B

| U0126        | MEK 1/2 inhibitor            | 10 µM         | EGCG      | 22.8 ± 2.0 (3)          |
| SB203580     | p38 MAPK inhibitor           | 10 µM         | EGCG      | 2.6 ± 0.20* (3)         |
|              |                              |               | EGCG      | 39.6 ± 1.0* (3)         |

*P < 0.05 vs. GTE-treated cells (experiment A) or EGCG-treated cells (experiment B).

very large induction was observed at 50–100 µg/ml. It should be noted that this induction was remarkable, being about two- to threefold higher than that elicited by serum. The ODC induction by 50 µg/ml GTE showed a broad peak at 6–12 h and then decreased to reach values similar to that of control after 24 h (Fig. 1B). The levels of the three polyamines in ECV304 cells treated with GTE are shown in Fig. 2. The increase in ODC activity was accompanied by an increase in the content of putrescine, the product of the enzyme. Pretreatment of the cells with DFMO, a specific ODC inhibitor, completely prevented the increase in putrescine level. DFMO also reduced the content of spermidine, whereas that of spermine was not affected significantly, as generally reported for this drug (12–15).

In previous studies we have shown that ODC induction elicited in ECV304 cells and in other cells by various stimuli was dependent on ERK activation (18,20,21). Moreover, it has been shown that GTE and single catechins such as EGCG or EGC are able to reduce the level of cellular thiol groups and provoke a pro-oxidant-dependent activation of MAPKs, including ERK, in tumor cells (7–9). Thus, to identify possible events involved in the ODC induction by GTE, several inhibitors of signaling pathways were tested, and their effects on ODC are shown in Table 1 (experiment A). ODC induction was reduced or prevented by two different ERK pathway inhibitors, that is, PD98059 and U0126. U0126 was more effective in accordance with the notion that it is a more potent MEK1/2 inhibitor than PD98059 (22). On the contrary,

Figure 1. Effect of GTE on ODC activity in ECV304 cells. (A) Confluent, serum-starved cells were treated with the indicated concentrations of GTE in the presence or absence of 10% fetal calf serum. After 6 h cells were harvested and cell extracts were assayed for ODC activity. (B) Confluent, serum-starved cells were treated with 50 µg/ml GTE. At the time indicated, cells were harvested and assayed for ODC activity. Results represent means ± SEM (N = 3) obtained from one experiment representative of two.
SB203580, a p38 MAPK inhibitor, enhanced ODC induction, as previously found for other ODC-inducing stimuli in ECV304 cells (18). Other effective agents were antioxidants such as TEMPO and N-acetyl cysteine (NAC), which is able to replenish and protect thiol groups (9), and Go6850, a general PKC inhibitor. In this regard, it has been described how ODC expression is induced in papilloma PE cells exposed to oxidative stress via PKCδ (23). Accordingly, we found that Go6976, an inhibitor specific for the conventional PKC isoforms, was not effective, whereas the selective PKCδ inhibitor rottlerin (24) was active.

Table 1 (experiment B) shows that EGCG, a major component of GTE extract, was also able to induce ODC activity in ECV304 cells, although less effectively with respect to the complete GTE extract, suggesting an effect of other GTE components in addition to EGCG. Likewise, the ODC increase by EGCG was blocked by U0126 and enhanced by SB203580.

In the light of the efficacy of ERK pathway inhibitors in reducing ODC induction by GTE and EGCG, we have assessed the ability of GTE to activate ERK by measuring the amount of phosphorylated, active ERK. Figure 3A shows that GTE caused a marked activation of ERK, which was transient and preceded the ODC induction. Furthermore, ERK phosphorylation was inhibited by U0126, NAC, and, although less effectively, Go6850 (Fig. 3B). It should be noted that the amount of total ERK was not affected by these treatments. Together these results indicate that the marked induction of ODC by GTE requires an early activation of

**Effects of Inhibitors on GTE-Induced Caspase Activation and Loss of Cell Viability**

ERK activation has been generally correlated to cell survival; however, growing evidence suggests that in some conditions ERK can act in signaling pathways leading to apoptosis (28 and references therein). In particular, the prooxidant–dependent activation of ERK by GTE or pure catechins has been related to the ability of green tea components to promote apoptosis (8,9). Changes in mitochondrial integrity involving cytochrome c release and subsequent activation of caspase proteases have been documented in tumor cells treated with GTE (29). On the other hand, it has been described how excessive intracellular polyamine levels may also trigger cell death and even apoptosis via cytochrome c release and caspase activation (14,19,30). Moreover, polyamine depletion by DFMO can prevent caspase activation and protect cells stimulated by some apoptotic stimuli (15,31–34). Therefore, the ability of GTE to induce caspase activation, as a critical event in apoptosis, has been investi-
igated, and the relevance of polyamines and ERK pathways in this respect has been assessed by using specific inhibitors (Fig. 4). GTE provoked a progressive increase in caspase activity in ECV304 cells up to about fivefold after 3 days of treatment (Fig. 4A). We also detected the 85-kDa cleavage fragment of PARP, a caspase substrate, by Western blot (Fig. 4B). The production of this proteolytic fragment is considered a hallmark of apoptosis (17). The occurrence of apoptosis after GTE treatment was indicated even by TUNEL assay (Fig. 4D). Interestingly, EGCG has recently been shown to induce apoptosis in ECV304 cells (35). Figure 4C depicts how DFMO pretreatment prevented the increase in caspase activity. Furthermore, even NAC and U0126, able to block both ODC induction and ERK activation, inhibited caspase activation as well. These findings indicate that ERK and polyamines are required for the GTE-induced caspase activation. Similar results were obtained with the TUNEL assay (Fig. 4D). However, GTE provoked a partial loss in the number of viable cells (Fig. 5) after 1 day of treatment, when caspase activity was hardly affected. Moreover, pretreatment with DFMO, U0126, or AcDEVD-CMK, a cell-permeable irreversible caspase inhibitor, was not effective in restoring cell viability. On the contrary, NAC protected ECV304 cells efficiently. Therefore, GTE is able to provoke in these tumor cells toxic effects that may involve alteration of thiol group status but appear to be largely independent of polyamine, ERK, and caspase pathways.

**Conclusion**

The present results indicate that ODC inhibition and polyamine depletion may not represent a constant motif in the action of green tea or individual catechins because GTE

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**Figure 4.** Induction of apoptosis in ECV304 cells after GTE and effect of pharmacological agents. Confluent, serum-starved cells were treated with 50 μg/ml GTE for (A) the time indicated or for (B–D) 3 days in the absence or presence of (B–D) 10 μM U0126, 2 mM NAC, or 5 mM DFMO. Cells were then harvested and analyzed for (A, C) caspase activity, (B) PARP detection by Western blotting, or (D) DNA breaks by TUNEL assay. (B) The Western blot picture is representative of two experiments. Graph results represent means ± SEM (N = 3) obtained from one experiment representative of (A, D) two or (C) three. *P < 0.05 vs. (C, D) GTE-treated cells.
can actually promote a remarkable ODC induction under some circumstances, as we report here for ECV304 carcinoma bladder cells in serum-starved conditions. It should be noted that these cells show relatively high levels of ODC activity (18), as expected for a tumor cell line. In non-tumor-derived cell types tested, GTE failed to affect the very low basal activity of ODC (not shown), suggesting that the phenomenon may be cell specific. The marked ODC induction in ECV304 cells is probably provoked by ERK activation through thiol status-dependent events. Both ERK and polyamine pathways are required for a GTE-induced caspase activation but seem largely dispensable for the caspase-independent component of cell death provoked by GTE in these cells. These results suggest that the apoptotic (caspase-dependent) action of green tea reported by various authors may require active ERK and adequate levels of polyamines but also raise the possibility that green tea might actually favor tumor growth and promotion in particular contexts where its ERK- and ODC-stimulating effects were dissociated from its cell toxic action.

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