Inhibitory effects of caffeic acid phenethyl ester (CAPE) on 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion in mouse skin and the synthesis of DNA, RNA and protein in HeLa cells

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Topical application of caffeic acid phenethyl ester (CAPE), a constituent of the propolis of honeybee hives, to the backs of CD-1 mice previously initiated with 7,12-dimethylbenz[a]anthracene (DMBA) inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion and the formation of 5-hydroxymethyl-2′-deoxyuridine (HMdU) in epidermal DNA. Topical application of 5 nmol TPA twice weekly for 20 weeks to mice previously initiated with 200 nmol of DMBA resulted in 18.8 skin papillomas per mouse. Topical application of 1, 10, 100 or 3000 nmol of CAPE together with 5 nmol of TPA twice a week for 20 weeks inhibited the number of skin papillomas per mouse by 24, 30, 45 or 70%, respectively, and tumor size per mouse was decreased by 42, 66, 53 or 74%, respectively. Topical application of 5 nmol of TPA twice weekly for 20 weeks to mice previously initiated with DMBA produced an average of 12.6 HMdU residues per 10⁴ normal bases in epidermal DNA. Topical application of 1, 10, 100 or 3000 nmol of CAPE with 5 nmol of TPA twice weekly for 20 weeks to DMBA-initiated mice decreased the level of HMdU in epidermal DNA by 40–93%. The in vitro addition of 1.25, 2.5, 5, 10 or 20 μM CAPE to cultured HeLa cells inhibited the synthesis of DNA by 32, 44, 66, 79 or 95%, respectively, the synthesis of RNA was inhibited by 39, 43, 58, 64 or 75%, respectively, and the synthesis of protein was inhibited by 29, 30, 37, 32 or 47%, respectively. The results indicate a potent inhibitory effect of CAPE on TPA-induced tumor promotion and TPA-induced formation of HMdU in DNA of mouse skin as well as an inhibitory effect of CAPE on the synthesis of DNA, RNA and protein in cultured HeLa cells.

Introduction

The propolis of honeybee hives and honey have long been used in folk medicine as anti-inflammatory agents with suspected anticancer activity (1,2). Gribel and Pashinski found that honey possesses antitumor and antimetastatic effects on several rat and mouse tumors and that honey potentiated the antitumor effects of 5-fluorouracil and cyclophosphamide (2). Although the propolis of honeybee hives is a complex mixture of chemicals, caffeic acid esters are prominent constituents that account for ~20% of the total (3). Earlier studies indicated that dietary administration of caffeic acid and some related compounds inhibit benzo[a]pyrene-induced forestomach carcinogenesis in mice (4), and topical application of caffeic acid and some related compounds (ferulic acid, chlorogenic acid, curcumin) inhibit 12-O-tetradecanoylphorbol-13-acetate (TPA*)-induced tumor promotion on mouse skin (5). Recent studies with caffeic acid phenethyl ester (CAPE; Figure 1), one of the constituents of the propolis of honeybee hives, indicated that CAPE is a strong inhibitor of lipoxygenase and xanthine/xanthine oxidase activity in vitro, and it also inhibits TPA-induced formation of superoxide by human neutrophils (6,7). In addition, CAPE selectively inhibits the growth of several transformed cells but not normal cells, and it inhibits adenovirus type 5 EIA-mediated transformation of fibroblasts (1,8,9). Studies by Rao and his colleagues indicated that dietary CAPE and some related compounds inhibited azoxymethane (AOM)-induced aberrant crypts in the colon of rats (11). We recently found that application of CAPE to mouse skin inhibited TPA-induced inflammation, ornithine decarboxylase activity and the formation of oxidized bases in epidermal DNA (12). These results suggested that CAPE may be an inhibitor of TPA-induced tumor promotion. In the present study, we report a strong inhibitory effect of CAPE on TPA-induced tumor promotion and TPA-induced formation of 5-hydroxymethyl-2′-deoxyuridine (HMdU) in DNA of mouse skin as well as an inhibitory effect of CAPE on the synthesis of DNA, RNA and protein in cultured HeLa cells.

Materials and methods

Chemicals and reagents

HMdU and the reagents used for acetylation (4-dimethylpyridine and triethylamine) were purchased from Sigma Chemical Co. (St Louis, MO). [3H]Acetic anhydride (Ac2O) (sp. act. 50 mCi/mmol) was purchased from New England Nuclear (Du Pont Co., Wilmington, DE). The enzymes for DNA purification and hydrolysis, proteinase K, RNase, DNase I, alkaline phosphatase, nuclease P1, ASAP™ and genomic DNA isolation columns were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). HPLC-grade acetic anhydride was purchased from Fisher Scientific (Springfield, NJ).

Treatment of mice

We studied the effect of topical application of CAPE on TPA-induced skin tumor promotion as previously described (5). The dorsal region of 7 week old female CD-1 mice was shaved with an electric clipper. Two days later, groups of 30 mice were treated topically with 200 nmol 7,12-dimethyl-
benzanthracene (DMBA) in 200 μl acetone, and control mice received 200 μl acetone alone. After 1 week, mice were treated topically with 200 μl acetone, 5 nmol TPA, or 5 nmol TPA with CAPE in 200 μl acetone twice weekly for 20 weeks. Tumors of at least 1 mm in diameter were counted and recorded once every 2 weeks, and the results are expressed as the average number of tumors per mouse and the percentage of tumor-bearing mice.

**Isolation of DNA from mouse epidermis**

Mice were killed by cervical dislocation. Isolation of DNA from mouse epidermis was done as previously described (13). The shaven skin was excised and immediately dip-coated in ice-cold Dulbecco's phosphate-buffered saline (PBS). Subcutaneous and connective tissues were scraped off with a razor blade, and the skin was cut into 2 × 1 cm pieces and floated, with the fur side down, on 0.5% trypsin–Hanks' balanced salt solution in a Petri dish. The skin pieces were incubated at 37°C for 0.5 h. The epidermal layers were easily scraped off with a scalpel, minced with scissors, and washed twice with PBS solution. Cells were lysed, and ASAP DNA isolation kits* were used to isolate the DNA, according to the protocol provided by the vendor. Incubation of epidermis from two mice with proteinase K at 55°C for 4 h and RNase A at 37°C for 0.5 h, followed by chromatography on the DNA isolation columns, yielded 200–500 μg of DNA. The isolated DNA was washed twice with 70% ethanol and gently dried under a nitrogen gas stream. The DNA pellets was dissolved in 400 μl 10 mM Tris–HCl (pH 7.0), and a 20-fold dilution sample was used to determine the DNA concentration from the A_{260}/A_{280} ratio, which was normally within the range of 1.7–1.9. DNA was quantitated based on the assumption that 1 A_{260} equals 50 μg double-stranded DNA/ml.

**Enzymatic digestion of DNA**

DNA (100 μg) dissolved in 200 μl of 10 mM Tris–HCl and 100 mM NaCl (pH 7.0) was mixed with 10 μl of nuclelease P1 (5 units) and 10 μl of 10 mM ZnSO_{4} (final concentration 1 mM) were added to the mixtures for 1 h. Finally, the pH was readjusted with 100 μl of 0.4 M Tris–HCl (pH 7.8), and 20 μl of alkaline phosphatase (3 units) were added to the mixtures and incubated for 0.5 h. DNA digest was terminated by adding 5 μl of acetic acid, and the samples were kept at −20°C for 0.5 h. After centrifugation (Beckman J2-21 m, 10 000 r.p.m.), the supernatant was dried under reduced pressure. The DNA hydrolysate was dissolved in 250 μl HPLC-grade water, filtered through a 0.2 μm syringe filter (Acrodisc LC13 PVDF; Gelman) and chromatographed on an HPLC octadecylsilane column under previously described elution conditions (13).

**Acetylation of oxidized nucleosides**

Oxidized nucleosides were acetylated with Ac_{2}O or [3H]Ac_{2}O according to the method of Matsuda et al. (15) with some modifications (14). We found that this method quantitatively acetylates both normal and oxidized nucleosides. HPLC fractions known to contain HMdU were combined and evaporated to dryness. The dried residue was dissolved in 1 ml of HPLC-grade acetonitrile containing 6 mM 4-dimethylpyridine. Triethylamine (10 nmol) and Ac_{2}O (10 umol) acetonitlne (ratio of radioactive and non-radioactive Ac_{2}O or [3H]Ac_{2}O or [3H]HAc_{2}O or [3H]Ac_{2}O (10 μmol) acetoniitlne (ratio of radioactive and non-radioactive Ac_{2}O, 1:10; sp. act. 3300 c.p.m./μmol acetate) were added to the reaction mixtures and incubated at room temperature for 4 h with shaking at 200 cycles/min. One milliliter of methanol was added to decompose the excess Ac_{2}O and terminate the reaction. After drying, samples were dissolved in 250 μl of 20% acetic acid, filtered through a 0.2 μm syringe filter, and injected into an HPLC octadecylsilane column together with acetylated non-radioactive HMDU as a marker (14).

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**Results**

**Inhibitory effect of CAPE on TPA-induced tumor promotion in mice previously initiated with DMBA**

Female CD-1 mice initiated with 200 nmol of DMBA and promoted with 5 nmol of TPA twice a week for 20 weeks developed an average of 18.8 tumors per mouse. Topical application of 1, 10, 100 or 3000 nmol of CAPE together with 5 nmol of TPA to the backs of mice twice a week for 20 weeks decreased the number of skin tumors per mouse by 24, 30, 45 or 70% respectively (Figure 2, Table I), and tumor volume per mouse was inhibited by 42, 66, 53 or 74% respectively (Table I). It is of interest that the effects of low doses of CAPE on tumor size per mouse were greater than the effects of low doses of CAPE on the number of tumors per mouse. The four dose levels of CAPE used in this study had no effect on body weight (data not presented).

**Inhibitory effect of CAPE on TPA-induced formation of HMDU in epidermal DNA from mice previously initiated with DMBA**

HMDU is one of many oxidized bases in DNA formed through the action of reactive oxygen species. Most of these oxidized bases could serve as markers of oxidative stress. Frequently, it is 8-hydroxyl-2′-deoxyguanosine (8-OHdG) that is used as a dosimeter due to the ease of its determination by HPLC with an electrochemical detector. However, rings of 8-OHdG as well as of thymidine glycol—one of the most abundant oxidized DNA base products—can be easily opened. Thus, these products can exist in DNA in several forms, while electrochemical detection measures only intact 8-OHdG. In contrast, HMDU is chemically very stable, and therefore we believe it is a more reliable measure of oxidative DNA damage. Our laboratory (K.F.) previously measured all three (HMdU, 8-OHdG and 8-OHdG) oxidized DNA base derivatives using an H postlabeling technique which can sensitively measure all of them at the same time. We concluded that the determination of HMDU alone provides the information being sought. The utilization of this assay also requires less
Inhibitory effect of CAPE on the number and size of TPA-induced tumors in mice previously initiated with DMBA

<table>
<thead>
<tr>
<th>Duration of treatment (weeks)</th>
<th>Treatment</th>
<th>% mice with tumors</th>
<th>% inhibition</th>
<th>Tumors/mouse</th>
<th>% inhibition</th>
<th>Tumor volume (mm³)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>TPA</td>
<td>63</td>
<td>16</td>
<td>4.68±1.22</td>
<td>22</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>TPA + CAPE (1 nmol)</td>
<td>53</td>
<td>16</td>
<td>3.67±1.10</td>
<td>93</td>
<td>–</td>
<td>–</td>
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<td>TPA + CAPE (10 nmol)</td>
<td>47</td>
<td>25</td>
<td>3.79±1.20</td>
<td>19</td>
<td>–</td>
<td>–</td>
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<td></td>
<td>TPA + CAPE (100 nmol)</td>
<td>43</td>
<td>32</td>
<td>2.47±0.76</td>
<td>47</td>
<td>–</td>
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<td>TPA + CAPE (3000 nmol)</td>
<td>30</td>
<td>52</td>
<td>0.47±0.16</td>
<td>90</td>
<td>–</td>
<td>–</td>
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<tr>
<td>20</td>
<td>TPA</td>
<td>97</td>
<td>4</td>
<td>18.8±1.9</td>
<td>24</td>
<td>121±29</td>
<td>42</td>
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<tr>
<td></td>
<td>TPA + CAPE (1 nmol)</td>
<td>93</td>
<td>4</td>
<td>14.3±2.0</td>
<td>45</td>
<td>98±29</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>TPA + CAPE (10 nmol)</td>
<td>77</td>
<td>21</td>
<td>13.1±2.2</td>
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<td>70±17</td>
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<td>14</td>
<td>10.3±1.6</td>
<td>45</td>
<td>98±29</td>
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<td></td>
<td>TPA + CAPE (3000 nmol)</td>
<td>87</td>
<td>10</td>
<td>5.7±1.0</td>
<td>70</td>
<td>55±17</td>
<td>74</td>
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</table>

Female CD-1 mice (7–8 weeks old; 30 per group) were treated topically with 200 nmol of DMBA in 200 μl acetone. One week later, the mice were treated with 5 nmol TPA alone or with 5 nmol TPA and CAPE in 200 μl acetone twice weekly for 20 weeks. The number of tumors and the size of each tumor were determined except that tumor size was not measured at 8 weeks. Data represent the mean±SE.

*Significantly different from TPA group (P < 0.05) as determined by the Student's t-test.

**Significantly different from TPA group (P < 0.01) as determined by the Student's t-test.

Table II. Effects of CAPE on the formation of HMdU in the DNA of DMBA/TPA-treated CD-1 mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>HMdU/10⁴ bases</th>
<th>% decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone/TPA</td>
<td>4</td>
<td>2.29±1.97</td>
<td>–</td>
</tr>
<tr>
<td>DMBA/TPA</td>
<td>9</td>
<td>12.5±4.88</td>
<td>–</td>
</tr>
<tr>
<td>DMBA/TPA + CAPE (1 nmol)</td>
<td>4</td>
<td>7.51±2.49</td>
<td>40</td>
</tr>
<tr>
<td>DMBA/TPA + CAPE (10 nmol)</td>
<td>4</td>
<td>0.92±0.62</td>
<td>93</td>
</tr>
<tr>
<td>DMBA/TPA + CAPE (100 nmol)</td>
<td>4</td>
<td>1.33±1.00</td>
<td>89</td>
</tr>
<tr>
<td>DMBA/TPA + CAPE (3000 nmol)</td>
<td>4</td>
<td>4.15±0.96</td>
<td>67</td>
</tr>
<tr>
<td>DMBA/acetone</td>
<td>5</td>
<td>5.53±1.38</td>
<td>–</td>
</tr>
</tbody>
</table>

CD-1 female mice were initiated with 200 nmol DMBA followed 1 week later by topical application of 5 nmol TPA alone or together with CAPE twice a week for 20 weeks. Other animals were treated with acetone followed by TPA twice a week or with DMBA followed by acetone twice a week for 20 weeks. HMdU lesions in DNA were measured. Each value represents the mean ± SE of n independent determinations using DNA obtained from different mice.

*Significantly different from the acetone/TPA group (P < 0.05) as determined by the Student's t-test.

**Significantly different from the DMBA/TPA group (P < 0.05) as determined by the Student's t-test.

***Significantly different from the DMBA/TPA group (P < 0.10) as determined by the Student's t-test.

DNA for analysis than for 8-OHdG detection by ³H-postlabeling.

The epidermal DNA of mice treated topically with 5 nmol TPA twice a week for 20 weeks had 2.29 HMdU residues per 10⁴ normal bases, and this was increased 5.5-fold when the animals were initiated with DMBA prior to TPA treatment for 20 weeks (Table II). Treatment of DMBA-initiated mice with 1, 10, 100 or 3000 nmol of CAPE together with TPA twice a week for 20 weeks decreased the level of HMdU in DNA by 40–93%, but a dose-dependent effect was observed only at lower doses (Table II). The three highest doses of CAPE, administered together with TPA to DMBA-initiated mice, decreased the amounts of HMdU in DNA to levels that were below those observed in animals initiated with DMBA but not promoted with TPA (Table II). The results indicate a strong inhibitory effect of CAPE on TPA-induced formation of HMdU in epidermal DNA.

Inhibitory effect of CAPE on the synthesis of DNA, RNA and protein in HeLa cells

CAPE was added to a 1.5 ml suspension of HeLa cells (3 × 10⁶ cells/ml) suspended in Eagle's medium. After 1 min, [³H]thymidine (2 nmol, 50 μCi/μmol), [³H]uridine (2 nmol; 55 μCi/μmol) or [³H]leucine (1 nmol; 150 μCi/μmol) was added, and the cell suspensions were incubated at 37°C for 60 min. The average amounts of radioactivity in protein, DNA and RNA samples from eight control samples were 23 715, 17 254 and 20 550 c.p.m./3 × 10⁶ cells respectively. This amount of radioactivity was at least 10-fold higher than was present in control samples that were not incubated.

Fig. 3. Inhibitory effect of CAPE on the synthesis of DNA, RNA and protein in HeLa cells.

Inhibitory effect of CAPE on the synthesis of DNA, RNA and protein in HeLa cells

CAPE at 1.25, 2.5, 5, 10 or 20 μM inhibited the incorporation of [³H]thymidine into DNA in cultured HeLa cells by 32, 44, 66, 79 or 95%, respectively (Figure 3). The incorporation of [³H]uridine into RNA was inhibited by 39, 43, 58, 64 or 75%, respectively, while the incorporation of [³H]leucine into protein was inhibited by 29, 30, 37, 42 or 47%, respectively (Figure 3). The results indicate that CAPE is a potent inhibitor of DNA synthesis but is somewhat less effective at inhibiting RNA synthesis and is least effective at inhibiting protein synthesis (Figure 3).

Discussion

The results of the present study demonstrate an inhibitory effect of topical application of very low doses of CAPE on
TPA-induced increases in oxidized bases in epidermal DNA and on tumor promotion. Doses as low as 1–10 nmol of CAPE together with 5 nmol of TPA twice a week for 20 weeks decreased the number of TPA-induced tumors per mouse by 24–30%, and tumor volume per mouse was decreased by 42–66% (Table I). The number of HMDU residues per 10^6 normal bases was decreased by 40–93% (Table II). Increasing the dose of CAPE to 100 or 3000 nmol per application (together with 5 nmol of TPA) decreased the number of tumors per mouse by 45 and 70% respectively, the tumor volume per mouse was decreased by 53–74%, and the number of HMDU bases per 10^7 normal bases in epidermal DNA was decreased by 67–89% (Tables I and II). CAPE exerts several biochemical and cellular effects that may be related to its potent inhibitory effect on tumor promotion. CAPE has antioxidant and anti-inflammatory activity (6,7,12), and it inhibits lipoxygenase and xanthine/xanthine oxidase activities (6), inhibits TPA-induced epidermal ornithine decarboxylase activity, neutrophil infiltration, hydrogen peroxide formation and the formation of oxidized DNA bases in the epidermis (12). It is of particular interest that CAPE enhances apoptosis in cultured adenosvirustransformed rat embryo fibroblasts (17), selectively inhibits the growth of several transformed cells but not normal cells (1,8,9), inhibits adenosvirustype 5 EIA-mediated transformation in rat embryo fibroblasts (8) and causes differentiation of human promyelocytic leukemia (HL-60) cells (7,18). The results of our studies with cultured HeLa cells indicate a potent inhibitory effect of CAPE on DNA and RNA synthesis (IC_{50} = 3 \mu M), but considerably higher concentrations are required for inhibition of protein synthesis (IC_{50} = 20 \mu M). In an earlier study, CAPE was shown to strongly inhibit TPA-mediated formation of H_2O_2, 8-OHdG and HMDU, sometimes even below the background levels, in HeLa cells (19). The concentration of CAPE required to inhibit H_2O_2 formation by 50% in this earlier study was ~5 \mu M, which is similar to that observed for inhibition of DNA and RNA synthesis in the present study (Figure 3). The potent inhibitory effect of CAPE on DNA and RNA synthesis may play a role in the inhibitory effect of CAPE on the formation and growth of skin tumors observed in the present study. Studies by Hennings and his colleagues indicated that CAPE inhibits focal growth of papilloma cells in a keratinocyte coculture assay for tumor promotion (20). Earlier work indicating an inhibitory effect of CAPE on DNA synthesis in cultured melanoma and breast cancer cells (1) coupled with our studies on the potent inhibitory effect of CAPE on DNA and RNA synthesis in HeLa cells (Figure 3) suggest a need for further research on the mechanism of the inhibitory effects of CAPE on DNA and RNA synthesis.

Recent studies have indicated an inhibitory effect of CAPE and certain other caffeic acid esters on AOM-induced increases in colonic lipoxygenase, ornithine decarboxylase and tyrosine protein kinase activities and on the formation of aberrant crypt foci in the colon of rats (11). Studies with curcumin (diferuloylmethane), which is structurally related to CAPE, indicate a potent inhibitory effect of this compound on TPA-induced formation of HMDU in the epidermal DNA of mice and on tumor promotion, but the inhibitory effects of curcumin on tumor promotion at the lowest dose levels were less than those of CAPE (M.-T.Huang et al., unpublished observations). Additional studies indicated a strong inhibitory effect of dietary curcumin on N-ethyl-N' -nitro-N-nitrosoguanidine-induced duodenal cancer, benzo[a]pyrene-induced forestomach cancer and AOM-induced colon cancer in mice (21) or rats (22). The results of our studies in mice and those by Rao and his colleagues in rats (11,22) suggest that further research is needed to evaluate the possible effects of CAPE on gastrointestinal carcinogenesis.

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