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Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties

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Caffeic acid phenethyl ester, an active component of propolis extract, inhibits 5-lipoxygenase in the micromolar concentration range. The inhibition is of an uncompetitive type, i.e. the inhibitor binds to the enzyme–substrate complex but not to the free enzyme. Caffeic acid phenethyl ester also exhibits antioxidant properties. At a concentration of 10 μM, it completely blocks production of reactive oxygen species in human neutrophils and the xanthine/xanthine oxidase system.

Caffeic acid phenethyl ester; 5-Lipoxygenase; Enzyme inhibition; Respiratory burst; Xanthine oxidase

1. INTRODUCTION

Caffeic acid phenethyl ester (CAPE) is an active component of propolis extracts [1] and has been demonstrated to be cytostatic [1–3]. Since arachidonic acid metabolites are important mediators in cancer and inflammation we attempted to characterize the anti-lipoxygenase activity of CAPE and compare it with the classical lipoxygenase inhibitors, caffeic acid [4] and nordihydroguaiaretic acid (NDGA) [5]. Keeping in mind that the 5-lipoxygenase pathways of arachidonic acid oxidation by plant and animal lipoxygenases are similar, we used barley 5-lipoxygenase. For the analysis of the inhibitory action of CAPE, we utilized a graphical method of Yoshino's [6], which is applicable for partial and complete competitive, non-competitive, uncompetitive and mixed-type inhibition.

2. MATERIALS AND METHODS

2.1. *Synthesis of CAPE*

Caffeic acid phenethyl ester was synthesized according to Dhaon et al. [7]. The physical constants of the substance obtained [UV (MeOH): 325 nm (ε 118,300), 300 nm (sh., 14,000), 235–245 nm (10,300), melting point: 124–125°C] were identical to those reported by Grunberger [1]. The chemical structure of CAPE was confirmed by ^{1}H NMR spectra (200 MHz, acetone-d_6): 8.52; 8.26 (br.s. 2H, OH), 7.53 (d,J = 16 Hz, 1H, 7-H), 7.35–7.20 (m, 5H,13–17H), 7.17 (d, J = 2, d/z, 1H, 2-H), 7.05 (dd, J = 8.0 Hz, J = 2.0 Hz, 1H, 6-H), 6.84 (d, J = 8.0 Hz, 1H, 5-H), 6.26 (d, J = 16.0 Hz, 1H, 8-H), 4.36 (t, J = 7.0 Hz, 2H, 10-H), 3.00 (t, J = 7,0 Hz, 2H, 11-H).

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Abbreviations: CAPE, caffeic acid phenethyl ester; NDGA, nordihydroguaiaretic acid; PMA, phorbol 12-myristate 13-acetate.

2.2. *Isolation of 5-lipoxygenase*

5-Lipoxygenase, with a specific activity of 38 μmol/min/mg, was isolated from barley grains, variety Risk, by the method described previously [8], with some modifications. After acetone extraction of barley flour, the acetone powder was dried and extracted with 50 mM sodium phosphate buffer containing 50 mM NaCl. The extract was fractionated with ammonium sulfate (30–60%) and subjected to CM–cellulose (Whatman) and DEAE–Sepharose (Pharmacia) chromatography. A column (2.6 × 12 cm) of CM–cellulose was equilibrated with 0.07 M sodium acetate buffer, pH 4.8, and, after application of the sample, eluted with a linear gradient of sodium acetate (0.7–0.77 M). A column (1.6 × 20 cm) of DEAE–Sepharose was equilibrated with 0.02 M sodium phosphate buffer, pH 6.8, and, after application of the sample, eluted with a linear gradient of NaCl (0–0.3 M) in the same buffer. The fractions containing 5-lipoxygenase were dialyzed against water and lyophilized. Dried protein was stored at −20°C. Protein concentration was routinely assayed by measuring the absorbance at 280 nm, and a concentration of 1 mg/ml was attributed to the enzyme solution with an absorbance of 1.6 U, i.e. using the extinction coefficient for potato 5-lipoxygenase [9].

2.3. *Assay of 5-lipoxygenase activity*

The activity of the enzyme was assayed spectrophotometrically according to Holman [10]. The accumulation of the product was estimated from repeated recordings of the characteristic absorption spectra of fatty acid hydroperoxides (λ_{max} = 235 nm) in a Shimadzu UV-240 spectrophotometer at 20°C. The identity of the product was confirmed by reverse-phase HPLC, as described elsewhere [11]. The rate of the 5-lipoxygenase-catalyzed formation of 9-hydroperoxy-10,12,15-octa-decatrienoic acid from linoleic acid was maximal when the enzyme is added to the substrate equilibrated with the buffer. We observed that the residual activity, when related to the activity in the absence of the inhibitor, is independent of the sequence in which the reagents are added. Therefore, the reaction was started by the addition of the substrate to the otherwise complete assay mixture. The standard reaction mixture contained 0.3–1.5 μg/ml barley 5-lipoxygenase, 100 μM ammonium linoleate and 50 mM sodium phosphate, pH 6.8. The inhibitors were added to the enzyme as ethanolic solutions, and the

mixture was preincubated for indicated times before the substrate was added.

2.4. *Assay of reactive oxygen species*

The formation of active oxygen by neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA) was monitored by measuring luminol-enhanced luminescence as described earlier [12]. The neutrophils were isolated from freshly drawn donor blood using a bilayer gradient of Ficoll-Paque (1.077 and 1.125 g/ml specific gravity). Washed neutrophils were resuspended in bicarbonate-free Hanks' solution containing 10 mM HEPES, pH 7.4, and kept at 4°C until use.

Superoxide (O_2^-) production was assayed by measuring luminescence in the luminol/xanthine/xanthine oxidase system, as described [13]. The rate of O_2^- formation in this cell-free system was found to be affected by CAPE, caffeic acid and NDGA. All the inhibitors tested were added to the assay medium containing 50 μM xanthine, 0.1 mM EDTA, 1 mM NaN_3 and 20 mM Na_2CO_3 buffer, pH 10, immediately before initiation of the reaction by the addition of xanthine oxidase.

Chemiluminescence was monitored in a 1251 LKB luminometer, using 1 μM luminol. Measurements were made every 30 s over a 30 min period at 20°C (cell-free system) or 37°C (neutrophils).

2.5. *Chemicals*

Caffeic (3,4-dihydroxycinnamic) acid (Reachim, Latvia) was crystallized from water. Phenethyl alcohol and 4-(dimethylamino)-pyridine used for CAPE synthesis were obtained from Fluka. Linoleic acid, NDGA, PMA, xanthine and xanthine oxidase were Sigma products. Ficoll–Paque was from Pharmacia, and dimethylsulfoxide was from Merck.

3. RESULTS AND DISCUSSION

The purified CAPE prepared in this work was found to suppress 5-lipoxygenase-catalyzed oxygenation of linoleic acid (Fig. 1) and arachidonic acid (data not shown). This compound was also effective as an inhibitor of the analogous reactions catalyzed by 15-lipoxygenase (data not shown).

There is ample evidence in the literature that caffeic acid and its synthetic derivatives can inhibit lipoxygenases [4,14–16]. However, the interaction of CAPE with these enzymes has not yet been studied. A non-competitive mechanism of inhibition was postulated for caffeic acid [4].

The effect of CAPE on 5-lipoxygenase kinetics was characterized using the spectrophotometrical assay of

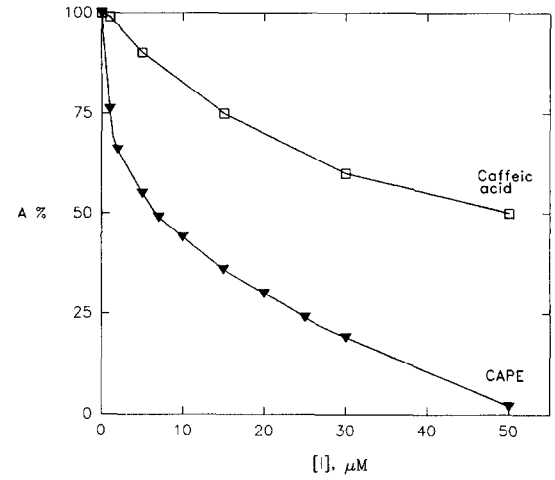


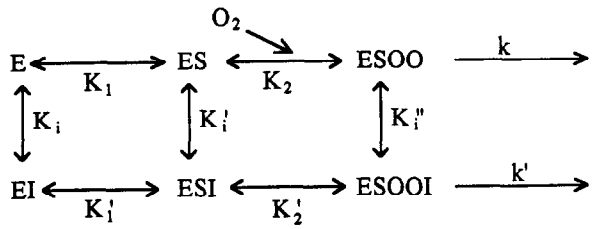
Fig. 1. The dose-dependence of 5-lipoxygenase inhibition by CAPE and caffeic acid. The inhibitors were added to the enzyme 1 min before the substrate (100 μM linoleic acid).

activity. CAPE suppresses markedly 5-lipoxygenase activity after a 1-min incubation. The dose-dependence of the residual activity of 5-lipoxygenase preincubated with caffeic acid and CAPE is shown in Fig. 1. The inhibition is complete as indicated by the linearity of the plot and the fact that enzyme activity decreases to zero when CAPE is in excess.

The fractional-velocity plot, $V/(V_0-V)$, vs. reciprocal inhibitor concentration, consists of straight lines intersecting the abscissa at the origin, with the slope decreasing with increasing substrate concentration (Fig. 2). Here, V and V_0 refer to velocity in the presence and in the absence of the inhibitor, respectively, at a given concentration of the substrate [6]. Yoshino [6] has shown for the Michaelis–Menten mechanism that such a pattern is indicative of a complete uncompetitive inhibition. This inhibition mechanism implies that the inhibitor only combines with the enzyme–substrate complex but not with the free enzyme, and the resulting enzyme–substrate–inhibitor complex is inert. The rate data obtained were analyzed in terms of the model

$$\frac{V}{V_o - V} = \frac{\{1+(K_2/[O_2])(1+K_1/[S])\}(K_i'/[I]+k'/k)}{\{1+K_i'(K_2/[O_2])(1/K_i'+(1/K_1)(K_1/[S]))\}-(k'/k)\{1+(K_2/[O_2])(1+K_1/[S])\}}$$

Eqn. 1.



Scheme 1.

$$\text{tg}\,\alpha = \frac{\{1+([S]/K_1)(1+[O_2]/K_2)\}}{([S]/K_1)([O_2]/K_2+K_i''/K_i')} \cdot K_i''$$

Eqn. 2.

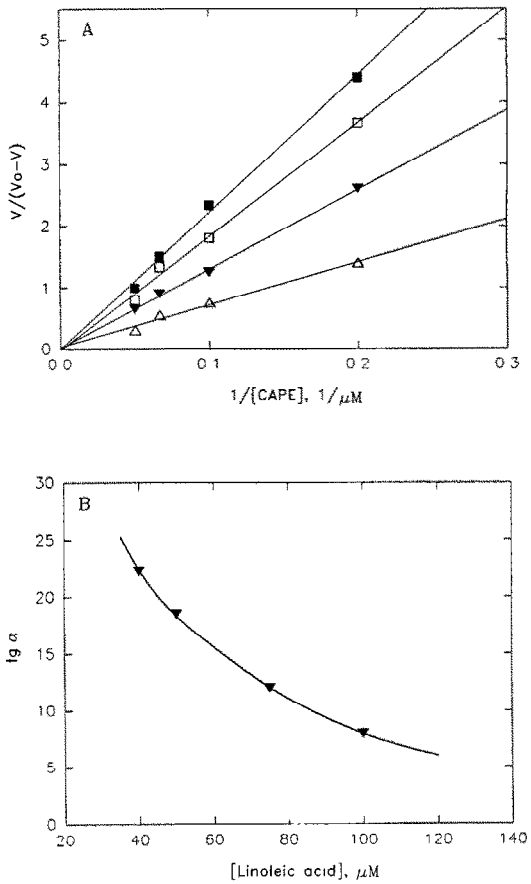


Fig. 2. (A) The fractional-velocity plot for the CAPE inhibition of 5-lipoxygenase-catalized oxidation of linoleic acid. Linoleic acid concentrations (μM): (■) 40; (□) 50; (▼) 75; (△) 100. The inhibitor was added to the enzyme 1 min before the substrate. (B) Re-plot of the slope of the fractional-velocity plot presented in Fig. 2A.

shown in Scheme 1, where E is active lipoxygenase, K_1, K_2, etc. are dissociation constants for the enzyme–ligand complexes, and k and k' are the catalytic constants for the breakdown of the ESOO and the ESOOI complexes, respectively.

Assuming a rapid equilibrium mechanism, one can derive the rate equation shown as Eqn. 1.

That plots of $V/(V_0-V)$ vs. 1/[I] are linear and pass through the origin at constant oxygen pressure (Fig. 2A) suggests that $K_i = \infty$, $K_i' = 0$ and $k' = 0$. The slope of the fractional-velocity plot (tg α) is given in Eqn. 2.

The data shown in Figs. 1 and 2 are consistent with a complete uncompetitive inhibition of 5-lipoxygenase by CAPE. By re-plotting the slopes of the curves in Fig. 2A vs. substrate concentration (Fig. 2B) and assuming $K_i'' = K_i'$, one obtains $K_i'' \leq 8$ μM. The anti-lipoxygenase activity of CAPE is thus greater than that of caffeic acid, though not as high as that of the synthetic caffeic acid derivatives described by Yu et al. [14].

We have also found that CAPE decreases the chemiluminescence of PMA-stimulated neutrophils (Fig. 3) and of a cell-free superoxide-generating xanthine/xanthine oxidase enzyme system (Fig. 4). The effectiveness of CAPE and caffeic acid as lipoxygenase inhibitors correlates with their redox properties (Figs. 1 and 4). The complete uncompetitive type of 5-lipoxygenase inhibition by CAPE is consistent with CAPE being a non-specific antioxidant. One can expect inhibitors with antioxidant properties, like CAPE, to combine with a broad range of reactive enzyme–ligand intermediates with concomitant trapping of radical intermediates. In the reaction mechanism proposed for 15-lipoxygenase [17], enzyme-bound peroxyl and pentadienyl radicals were identified as the intermediates leading to the hydroperoxide products [18]. Based on the reaction mechanism of 15-lipoxygenase, one may speculate that the antioxidant inhibitors block the reduction of the per-

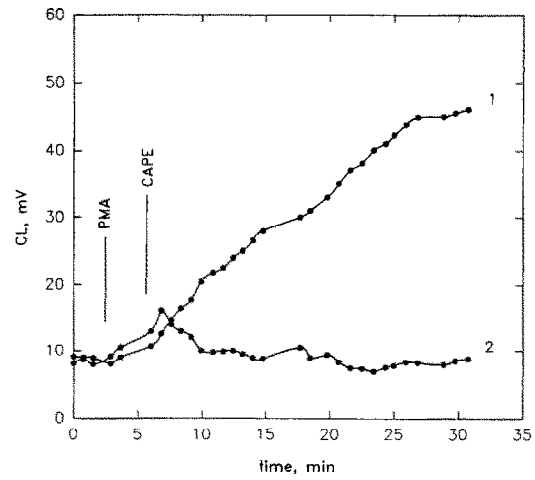


Fig. 3. Effect of CAPE on the luminol-enhanced chemiluminescence produced by human neutrophils (2×10^6 per ml) stimulated with 0.5 ng/ml PMA. At the indicated time 10 μM CAPE (curve 2) or equal volume (6 μl) of ethanol (curve 1) was added.

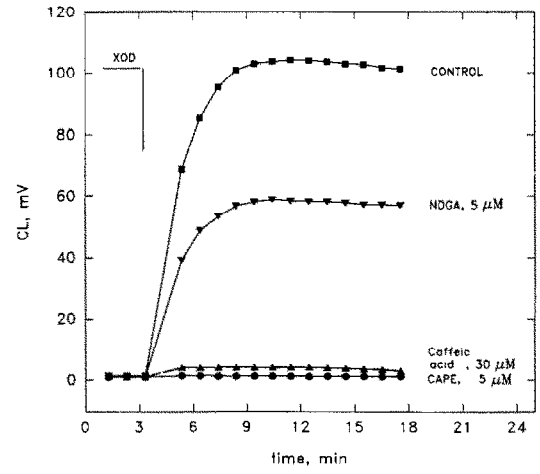


Fig. 4. The effects of CAPE, caffeic acid and NDGA on the luminol-enhanced chemiluminescence produced by the cell-free xanthine/xanthine oxidase system. The indicated concentrations of the inhibitors or 3 μl of ethanol (control) were added 10 s before initiation of the reaction by xanthine oxidase (XOD).

oxyl radical by Fe(II) and, therefore, formation of the activated Fe(III) form of the enzyme and fatty acid peroxide.

The studies described here demonstrate the ability of CAPE to inhibit 5-lipoxygenase by a complete uncompetitive mechanism. One may conclude that CAPE is an antioxidant-based lipoxygenase inhibitor the antioxidant potency of which slightly exceeds that of NDGA. Thus, CAPE may contribute to the anti-inflammatory activity of propolis in vivo by being both a lipoxygenase inhibitor and an antioxidant.

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