The inhibitory effect of propolis and caffeic acid phenethyl ester on cyclooxygenase activity in J774 macrophages

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Summary

The effect of an ethanolic extract of propolis, with and without CAPE, and some of its components on cyclooxygenase (COX-1 and COX-2) activity in J774 macrophages has been investigated. COX-1 and COX-2 activity, measured as prostaglandin E2 (PGE2) production, were concentration-dependently inhibited by propolis (3 \times 10^{-3} – 3 \times 10^{2} \mu g/ml^{-1}) with an IC_{50} of 2.7 \mu g/ml^{-1} and 4.8 \times 10^{-2} \mu g/ml^{-1}, respectively. Among the compounds tested pinocembrin and caffeic, ferulic, cinnamic and chlorogenic acids did not affect the activity of COX isoforms. Conversely, CAPE (2.8 \times 10^{-4} – 28 \mu g/ml^{-1}; 10^{-9} – 10^{-4} M) and galangin (2.7 \times 10^{-4} – 27 \mu g/ml^{-1}; 10^{-9} – 10^{-4} M) were effective, the last being about ten-twenty times less potent. In fact the IC_{50} of CAPE for COX-1 and COX-2 were 4.4 \times 10^{-1} \mu g/ml^{-1} (1.5 \times 10^{-6} M) and 2 \times 10^{-3} \mu g/ml^{-1} (6.3 \times 10^{-9} M), respectively. The IC_{50} of galangin were 3.7 \mu g/ml^{-1}(15 \times 10^{-4} M) and 3 \times 10^{-2} \mu g/ml^{-1} (120 \times 10^{-9} M), for COX-1 and COX-2 respectively. To better investigate the role of CAPE, we tested the action of the ethanolic extract of propolis deprived of CAPE, which resulted about ten times less potent than the extract with CAPE in the inhibition of both COX-1 and COX-2, with an IC_{50} of 30 \mu g/ml^{-1} and 5.3 \times 10^{-4} \mu g/ml^{-1}, respectively. Moreover the comparison of the inhibition curves showed a significant difference (p < 0.001). These results suggest that both CAPE and galangin contribute to the overall activity of propolis, CAPE being more effective.

Key words: cyclooxygenase, CAPE, galangin, J774 macrophages, prostaglandins, propolis

Introduction

Propolis is a resinous material elaborated by honey bees (Apis mellifera) well known for its medical effects, including antiinflammatory, antiviral, immunostimulatory and carcinostatic activities (Dobrovolski et al., 1991). The most used formulation in the folk medicine is the ethanol extract (Mezenes et al., 1999). Standardization of propolis preparations is indeed difficult because of changes in chemical composition and pharmacological activities, resulting from variation in geographical and botanical origin (Ghisalberti, 1997). The wide spectrum of propolis activities was mainly attributed to the large number of flavonoids (Vennat et al., 1995). In addition to flavonoids, propolis contains cinnamic derivatives such as caffeic, ferulic, cinnamic, chlorogenic acids and its esters (Mirzoeva and Calder, 1996). Recently it has been reported that the caffeic acid phenethyl ester (CAPE), a component of propolis, exerts a potent antiinflammatory activity. The antiinflammatory properties of CAPE have been attributed to the suppression of prostaglandin (PG) and leukotrienes synthesis (Mirzoeva and Calder, 1996). It has also been reported that CAPE inhibits in vitro and in vivo histamine release in the rat (Scheller et al., 2000).
PG, produced by cyclooxygenase (E. C. 1.14.99; COX), are important antinflammatory mediators (Vane, 1971). The enzyme is present in constitutive (COX-1) and inducible (COX-2) isoform. The constitutively expressed isoform release PG involved in physiological events in various organs of the body. The inducible isoform, which is expressed by several cell types in response to inflammatory and immunological stimuli, is responsible for the increased PG generation at the inflammation site (Smith and Dewitt, 1995).

Unstimulated J774 macrophages possess a significant COX-1 activity, whereas the immunostimulation by endotoxin, such as lipopolysaccharides, results in a significant induction of COX-2 and a down regulation of COX-1 (Zingarelli et al., 1997).

The present study was designed to investigate the role of CAPE and other components in the overall antinflammatory activity of propolis. To this purpose we have investigated the effect of an ethanolic extract of propolis, with and without CAPE, and some of its components on COX-1 and COX-2 activity, measured as PGE2 production, in J774 macrophages.

Materials and Methods

Drugs tested

• Propolis: The dried ethanol extract of propolis, with and without CAPE, was obtained from Carlo Sessa, Milan, Italy. Propolis extract is a commercial product consisting of propolis deprived of waxes; it is usually obtained from propolis by alcoolic extraction and normally called “propolis”.

• Propolis deprived of CAPE: Ten g of propolis extract were dissolved in 150 ml of methanol and 30 ml of 2N sulphuric acid were added. The vessel was tightly capped and heated for 1 hour in a boiling water bath. After cooling, the solution was neutralized to pH 7.0 with 25% ammonia solution and the methanol was rotary evaporated under vacuum. The hydrolysis products (caffeic acid and phenethyl alcool) were removed from the residual mass by extracting twice with 200 ml water under reflux and discarding the water solutions. The residue was finally dried under vacuum. The yield was about 95%.

Original propolis, water solutions and residual propolis were subjected to thin layer chromatography (TLC) on silica gel using a fluorescent indicator (Kieselgel 60 WF254). The developing solvent was: toluene/ethyl formiate/formic acid 6/3/0.5. Reference compounds were CAPE, caffeic acid and phenethyl alcool, revealed by UV lights (254 nm). Flavonoids and other compounds were detected by a methanol solutions of 1% 2-aminoethyl diphenylborate. CAPE and caffeic acid were also revealed by this reagent (see Fig. 1).

• Other compounds: CAPE, caffeic, ferulic, cinnamic, chlorogenic acids and galangin were obtained from Sigma-Aldrich, Milan, Italy. Pinocembrin was obtained from Trimital, Milan, Italy. Stock solutions of test compounds were prepared in ethanol, an equivalent amount of ethanol was included in control samples.

• Determination of total flavonoids (as galangine) and caffeic acid contents in original and residual propolis: Galangine was determined spectrophotometrically (Ali et al., 1997), while caffeic acid was determined by HPLC (Ali et al., 1997).

Materials

Arachidonic acid was obtained from SPIBIO, Paris, France. [3 H-PGE2] was from NEN Du Pont (Milan, Italy). TLC plates were from Merck (Darmstadt, Germany). Bacterial lipopolysaccharide from Salmonella thyphosa (LPS) and all other reagents and compounds used were obtained from Sigma-Aldrich, Milan, Italy.

Cell culture

The murine monocyte/macrophage J774 cell line was grown in Dulbecco’s modified Eagles medium (DMEM) supplemented with 2mM glutamine, 25 mM Hepes, penicillin (100 µ/ml), streptomycin (100 µg/ml), 10% foetal bovine serum (FBS) and 1.2% Na-pyruvate (Bio Whittaker, Europe). Cells were plated in 24 well culture plates at a density of 2.5×10^5 cells/ml or in 10 cm-diameter culture dishes (1×10^7 cells/10ml/dish) and allowed to adhere at 37 °C in 5% CO2/95% O2 for 2h. Immediately before the experiments, culture medium was replaced by fresh medium without FBS in order to avoid interference with radioimmunoassay (Zingarelli et al., 1997) and cells were stimulated as described.

Assessment of COX-1 activity

Cells were pretreated with test compounds for 15 min and further incubated for 30 min with arachidonic acid (AA) 15×10^-6 M (Zingarelli et al., 1997). At the end of the incubation the supernatants were collected for the measurement of PGE, by radioimmunoassay (Sautelin et al., 1999). Test compounds were the following: propolis, with and without CAPE (3×10^-3–3×10^5 µg/ml), CAPE (2.8×10^-4–28 µg/ml; 10^-9–10^-4 M), galangin (2.7×10^-4–27 µg/ml; 10^-9–10^-4 M), pinocembrin (2.8×10^-4–28 µg/ml; 10^-9–10^-4 M) caffeic acid (1.8×10^-4–18 µg/ml; 10^-9–10^-4 M), ferulic acid.
post hoc test for multiple comparisons. A p value less than 0.05 was considered to be statistically significant.

Results

Analysis of propolis deprived of CAPE
CAPE was undetectable in the residual propolis, whereas caffeic acid and phenethyl alcohol were abundant in water solutions. The amounts of the other detectable components of the original propolis were roughly the same in the residual propolis (Fig. 1).

No evident changes in the content of the original total flavonoids and caffeic acid before and after the procedure, have been observed. Total flavonoid content of propolis with CAPE, expressed as galangin, was 9.04%; CAPE content was 10.44%. Total acidity, expressed as caffeic acid, was 13%. Total flavonoid content of propolis without CAPE, expressed as galangin, was 8.5%.

Inhibition of COX-1 activity by test compounds
Stimulation of J774 macrophages with arachidonic acid (15 \times 10^{-6} M) for 30 min induced a significant increase (p < 0.001) of PGE\(_2\) (5.1 \pm 0.3 ng \times 10^6 cells) levels in comparison to unstimulated control cells (0.32 \pm 0.06 ng \times 10^6 cells).
The inhibitory effect of propolis and caffeic acid phenethyl ester

The ethanolic extract of propolis (3 × 10⁻¹–3 × 10² µgml⁻¹) significantly (p < 0.01 at the lowest concentration; p < 0.001 at all the other concentrations) and concentration-dependently inhibited PGE₂ generation (Fig. 2), with an IC₅₀ of 2.7 µgml⁻¹ (1.3 to 5.8 µgml⁻¹). Same results were obtained with CAPE (28 × 10⁻³–28 µgml⁻¹; 10⁻⁷–10⁻⁴ M) which significantly (p < 0.01 at the lowest concentration; p < 0.001 at all the other concentrations) and concentration-dependently inhibited prostanoid generation (fig. 2). The IC₅₀ was 4.4 × 10⁻¹ µgml⁻¹ (3.3 to 6 × 10⁻¹ µgml⁻¹), corresponding to 1.5 × 10⁻⁶ M. The production of PGE₂ was also significantly inhibited by galangin (27 × 10⁻³–27 µgml⁻¹; 10⁻⁷–10⁻⁴ M), but only from the concentration of 27 × 10⁻² µgml⁻¹ (p < 0.05; p < 0.01 at 27 × 10⁻¹; p < 0.001 at 27 µgml⁻¹), corresponding to 15 × 10⁻⁶ M. The other compounds tested (pinocembrin, caffeic, chlorogenic, ferulic and cinnamic acids) did not inhibit, at all the concentrations tested (10⁻⁷–10⁻⁴ M), prostanoid generation (data not shown).

To investigate the role of CAPE in the overall inhibitory activity of propolis we tested the action of the ethanolic extract of propolis (3 × 10⁻¹–3 × 10² µgml⁻¹) deprived of CAPE. The inhibition was significant only

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**Fig. 2.** Inhibitory effect of an ethanolic extract of propolis with (■) and without (□) CAPE (panel A), CAPE (▼) and galangin (●) (panel B) on PGE₂ production by J774 macrophages incubated with arachidonic acid (15 × 10⁻⁶ M) for 30 min. Cells were pretreated for 15 min with test compounds before the addition of arachidonic acid. The concentrations are expressed as log µgml⁻¹. Each point represents the mean for 4 experiments performed in triplicate.

**Fig. 3.** Inhibitory effect of an ethanolic extract of propolis with (■) and without (□) CAPE (panel A), CAPE (▼) and galangin (●) (panel B) on PGE₂ production by J774 macrophages stimulated with LPS (10 µgml⁻¹) in presence or absence of test compounds. The concentrations are expressed as log µgml⁻¹. Each point represents the mean for 4 experiments performed in triplicate.
from the concentration of 3 µgml⁻¹ (p < 0.05; p < 0.01 at 30, p < 0.001 at 300 µgml⁻¹). This extract was about ten times less potent than the extract containing CAPE (Fig. 2), the IC₅₀ being 30 µgml⁻¹ (10 to 87 µgml⁻¹). Moreover the comparison of the inhibition curves showed a significant difference (p < 0.001) between propolis with and without CAPE.

**Inhibition of COX-2 activity by test compounds**

Stimulation with LPS (10 µgml⁻¹) for 24h induced a significant (p < 0.001) increase of PGE₂ generation (28 ± 3.4 ng × 10⁶ cells) in comparison to unstimulated control cells (1.32 ± 0.1 ng × 10⁶ cells).

In the presence of increasing concentrations of the ethanolic extract of propolis (3 × 10⁻³–30 µgml⁻¹) a concentration-dependent and significant (p < 0.01 at 3 × 10⁻² µgml⁻¹; p < 0.001 at all the other concentrations) inhibition of PGE₂ production was observed (Fig. 3) with an IC₅₀ of 4.8 × 10⁻² µgml⁻¹ (2.5 to 9.2 × 10⁻² µgml⁻¹). CAPE (2.8 × 10⁻²–2.8 µgml⁻¹; 10⁻⁶–10⁻³ M), exerted a similar significant (p < 0.01 at 2.8 × 10⁻² µgml⁻¹; p < 0.001 at all the other concentrations) and concentration-dependent inhibition (Fig. 3), with an IC₅₀ of 2 × 10⁻² (6 × 10⁻⁴ to 6 × 10⁻³ µgml⁻¹) (6.3 × 10⁻⁴ M). Galangin (2.7 × 10⁻⁴–2.7 µgml⁻¹; 10⁻⁰–10⁻⁵ M) was also effective (Fig. 3), but the inhibition was significant only from the concentration of 2.7 × 10⁻² µgml⁻¹ (p < 0.01; p < 0.001 at all the other concentrations). The IC₅₀ was 3 × 10⁻² µgml⁻¹ (2 to 4 × 10⁻² µgml⁻¹) (120 × 10⁶ M). The other compounds tested pinocembrin, caffiec, chlorogenic, ferulic and cinnamic acids did not inhibit, at all the concentrations tested (10⁻⁵–10⁻³ M) prostanoid generation (data not shown).

Next we tested the action of the ethanolic extract of propolis without CAPE. This extract (3 × 10⁻³–30 µgml⁻¹) significantly and concentration-dependently inhibited PGE₂ production but only from the concentration of 3–10⁻¹ µgml⁻¹ (p < 0.05; p < 0.001 at the other concentrations), resulting ten times less potent than the extract contents CAPE (Fig. 3). The IC₅₀ was 5.4 × 10⁻¹ µgml⁻¹ (1.8 × 10⁻¹ to 1.6 µgml⁻¹). Moreover the comparison of the inhibition curves showed a significant difference (p < 0.001) between propolis containing CAPE and propolis without CAPE.

### Discussion

The antinflammatory activity of propolis is well known although the exact mechanism of action is still under debate. The inhibition of PG generation seems to be, at least in part, the mechanism mainly responsible for this property (Mirzoeva and Calder, 1996; Mezenes et al., 1999). Moreover the complicated chemical composi-
In conclusion our results suggest that although both CAPE and galangin contribute to the overall inhibitory activity of propolis, CAPE gives a greater contribution. Thus CAPE could be a useful tool in the control of pathologies characterized by an elevated PG biosynthesis.

References


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