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# Effect of caffeic acid phenethyl ester on gastric acid secretion in vitro

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**Abstract**

Caffeic acid phenethyl ester (CAPE), one of the major components of propolis (honeybee resin), has demonstrated a wide spectrum of activities including suppression of eicosanoids by inhibition of cyclooxygenase-1 and cyclooxygenase-2 enzyme activities. The aim of this study was to investigate the effect of CAPE on basal and secretagogues-stimulated gastric acid secretion in vitro. In the isolated, lumen-perfused, stomach preparation of mouse, CAPE (10–100 μM) did not affect the basal gastric acid secretion nor the secretion stimulated by histamine, pentagastrin, isobutyl methylxanthine and high levels of $K^+$. By contrast, CAPE increased the gastric acid secretion induced by the muscarinic receptor agonist, 5-methylfurmethide (5-MEF). CAPE also inhibited the acetylcholinesterase activity in an in vitro colorimetric assay. Eserine (10 μM), a well known acetylcholinesterase inhibitor, also increased 5-MEF-stimulated acid secretion. Our results show that CAPE increases gastric acid secretion stimulated by an acetylcholine agonist receptor likely through inhibition of acetylcholinesterase activity.


*Keywords:* Caffeic acid phenethyl ester; Acetylcholinesterase inhibitor; Gastric acid secretion; Stomach

## 1. Introduction

Recently a great deal of attention has been focused on caffeic acid phenethyl ester (CAPE), an active component of propolis (honeybee resin). It has been described as anti-inflammatory, anti-viral, anti-bacterial, antineoplastic and antioxidant compound (Fesen et al., 1994; Chiao et al., 1995; Michaluart et al., 1999; Chen et al., 2001; Russo et al., 2002; Parlakpinar et al., 2005) and has been shown to inhibit the ornithine decarboxylase, protein tyrosine kinase and lipoxygenase activities (Sud'ina et al., 1993). It has also been reported that CAPE suppresses lipid peroxidation and inhibits the activation of nuclear transcription factor NF-kB (Natarajan et al., 1996; Okutan et al., 2005). The anti-inflammatory properties of CAPE have been principally attributed to suppression of eicosanoids synthesis by inhibition of arachidonic acid release from cell membranes, suppression of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzyme activities, and inhibition of COX-2 gene expression (Mirzoeva and Calder, 1996; Michaluart et al., 1999; Rossi et al., 2002). The effect of CAPE on COX activity has been demonstrated to be relatively specific for the COX-2 isoform ($IC_{50}$ ratio COX-1/COX-2 : 200–300) (Rossi et al., 2002). Prostaglandins have long been known to be protective to the gastric mucosa and to have inhibitory effects on gastric acid secretion (Barnett et al., 2000). The aim of this study was to investigate the effects of CAPE on basal and secretagogues stimulated gastric acid secretion in vitro.

## 2. Material and methods

### 2.1. Animals

Male ICR mice (25–28 g) were purchased from Harlan Nossan (Correzzana, Italy) and were maintained under controlled conditions of temperature (23±2 °C) and humidity (50±10%) until used. All animals were deprived of food 16 h prior to experimentation but had free access to water. All experiments on animals complied with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European communities Council Directive of 24 November 1986 (86/609/ECC).

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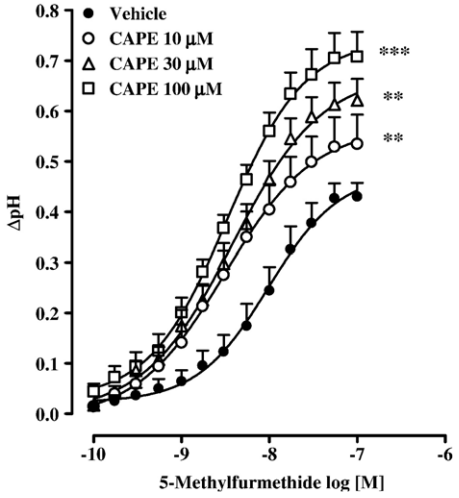


Fig. 1. Acid secretion in isolated mouse stomach stimulated with 5-methylfurmethide (5-MEF) alone and in the presence of caffeic acid phenethyl ester (CAPE, 10–100 μM). Each point represents the mean±S.E.M. of 9 animals for each experimental group. $^{**}P<0.01$; $^{***}P<0.001$ vs vehicle (5-MEF alone).

### 2.2. Isolated, lumen-perfusion stomach preparations

Gastric acid secretion was measured in the isolated, lumen-perfused, stomach preparation of mouse as previously described (Borrelli et al., 2000). Briefly, animals were killed by cervical dislocation, the abdomen opened and the oesophagus ligated close to the stomach. The stomach was removed and two polythene cannulas (2 mm internal diameter) were inserted and tied in, one into the pylorus via the duodenal bulb and the other into a small incision made in the fundus through which the stomach contents was first gently washed out. The stomachs were transferred into a 40 ml organ bath containing buffered serosal solution (mM: NaCl 118, KCl 4.8, $MgSO_4$ 1.2, $KH_2PO_4$ 1.14, $Na_2HPO_4$ 15.9, $CaCl_2$ 0.65, glucose 31.6) maintained at 37 °C and gassed with 95% $O_2$ and 5% $CO_2$. The preparations were continuously perfused from the fundic through the pyloric cannulas (1 ml/min) with warmed, unbuffered, mucosal solution (mM: NaCl 135, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 1.3, glucose 31.6) gassed with 100% $O_2$. This solution was passed over a pH-electrode system set at 12 cm above the preparation to distend the stomach wall.

Five preparations were used simultaneously and showed stable responses after a 60 min period. Any preparations not showing stable response were rejected (<5%). Secretagogues (histamine $10^{-7}$–$10^{-3}$ M, isobutyl methylxanthine $10^{-7}$–$10^{-3}$ M, pentagastrin $10^{-10}$–$10^{-6}$ M and 5-methylfurmetide $10^{-10}$–$10^{-7}$ M; volume not exceeding 1 ml) were added to the serosal solution so as to obtain a single cumulative agonist concentration–effect curve (and usually required 20 to 40 min to reach each plateau). In the experiments conducted in buffer containing a high $K^+$ concentration, stomachs were first set-up as normal and allowed to equilibrate for 60 min in normal serosal solution with continuous perfusion and monitoring as described. The serosal buffer was then replaced with warmed (37 °C) high $K^+$ buffer (mM: KCl 122.8, $MgSO_4$ 1.2, $KH_2PO_4$ 1.14, $NaHPO_4$ 15.9, $CaCl_2$ 0.65, glucose 31.6) and the mucosal perfusate monitored as usual. Changes in pH (ΔpH) following each experimental intervention were recorded. CAPE (10–100 μM), eserine (10 μM) or nifedipine (2 μM) were added 15 min before the start of cumulative agonist concentration–effect curves. In some experiments, the effect of CAPE (10 μM) on 5-methylfurmetide-stimulated gastric acid secretion was evaluated in the presence of nifedipine (2 μM) and eserine (100 μM) plus nifedipine (2μM). All substances were added to the serosal solution in the baths.

### 2.3. Acetylcholinesterase activity

The in vitro inhibition of acetylcholinesterase was determined using a colorimetric method (Henriksen et al., 1997). Briefly, acetylcholinesterase solution (5 μl) was incubated with 2 μl of CAPE (10–100 μM), vehicle or eserine (100 μM), 10 μl of DTNB, 180 μl of $KH_2PO_4$ buffer (pH 8) in a 96-well microplate for 15 min prior to addition of substrate (2 μl). The change in absorbance at 450 nm (Beckman DU-70 spectrophotometer) was recorded and acetylcholinesterase inhibition determined by comparison with controls lacking acetylcholinesterase inhibitors. All samples were analysed in triplicate.

### 2.4. Substances

Drugs were purchased from Sigma (Milan, Italy) unless otherwise indicated. Histamine 2HCl was dissolved in distilled water to give a 1 M stock that was back neutralised to pH 7.4 with NaOH. Isobutyl methylxanthine was dissolved in 90% ethanol to give a 1 M stock solution. Caffeic acid phenethyl ester, eserine and nifedipine were dissolved in dimethylsulphoxide to give a 1 M stock solution and pentagastrin was dissolved in dimethylformamide to give 1 mM stock solution. Subsequent dilutions for pentagastrin were made in distilled water. 5-methylfurmethide iodide (gift from the James Black Foundation, UK) was dissolved in distilled water to give 1 M stock solution. None of the vehicle concentrations and volumes used had an effect on basal acid output.

Table 1
Logistic curve fitting parameters of 5-methylfurmethide ($10^{-10}$–$10^{-6}$ M)-stimulated acid secretion curve alone and in the presence of caffeic acid phenethyl ester (CAPE, 10–100 μM)

| | $n$ | p[$A_{50}$] | $p$ | α |
|---|---|---|---|---|
| 5-Methylfurmethide | 9 | 7.98±0.13 | 1.27±0.21 | 0.52±0.05 |
| 5-Methylfurmethide+ | | | | |
| CAPE 10 μM | 9 | 8.45±0.14[a] | 1.09±0.10 | 0.59±0.07 |
| CAPE 30 μM | 9 | 8.42±0.09 | 0.90±0.05 | 0.67±0.04[a] |
| CAPE 100 μM | 9 | 8.44±0.09 | 0.89±0.13 | 0.80±0.06[b] |

$n$=number of experiments; $p[A_{50}]$=midpoint location; $p$=midpoint slope parameter; α=maximum asymptote. The $p[A_{50}]$ and $p$ results are obtained from the mean data and the ±S.E.M. is therefore a fitting error.

[a] $P<0.05$.

[b] $P<0.01$ vs 5-methylfurmethide.

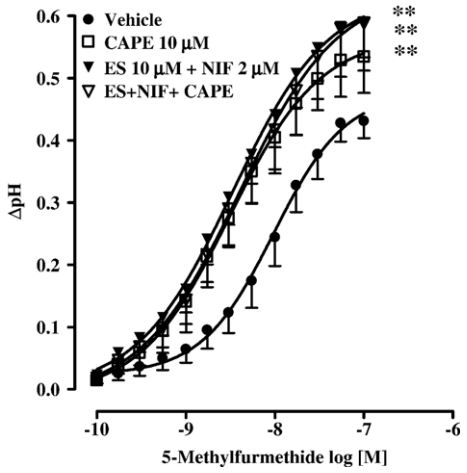


Fig. 2. Acid secretion in isolated mouse stomach stimulated with 5-methylfurmethide (5-MEF) alone and in the presence of caffeic acid phenethyl ester (CAPE, 10 μM), eserine (ES, 10 μM)+nifedipine (NIF, 2 μM) and CAPE (10 μM)+eserine (10 μM)+nifedipine (2 μM). Each point represents the mean±S.E.M. of 9 animals for each experimental group. **$P<0.01$ vs vehicle (5-MEF alone).

Acetylcholinesterase solution: acetilcholinesterase (bovine erythrocytes) was dissolved in 0.1 M $KH_2PO_4$ buffer (pH 8) to a final concentration of 5 U/ml. DTNB: 39.6 mg of 5,5-dithio-bis-2-nitrobenzoic acid and 15 mg of $NaHCO_3$ were dissolved in 10 ml of phosphate buffer (pH 7). Substrate: 0.075 M acetylthiocholine iodine was dissolved in acidified (pH 3) double-distilled water.

### 2.5. Data analysis

Acid secretory responses were expressed as changes in pH. This response metameter was chosen because it has previously been shown on this assay that under basal and stimulated conditions, pH is normally distributed but that the distribution of $[H^+]$ nM/min deviates significantly from normality (Shankley, 1985; Borrelli et al., 2000).

Concentration–effect curve data from individual preparations were fitted by means of an iterative least squares minimisation program to the Hill equation,

$$E = \frac{\alpha[A]^p}{[A_{50}]^p + [A]^p}$$

to provide estimates of the midpoint location ($\log[A_{50}]$), midpoint slope parameter ($p$) and the maximum asymptote ($\alpha$) as described previously (Borrelli et al., 2000).

For analysis the individual computed parameter estimates for each treatment group were expressed as mean±S.E.M.

Computed curve-fitting parameter estimates and absorbance recorded were compared by Analysis of Variance (one-way ANOVA) and the Bonferroni modified $t$-test for multiple comparisons. Values of $P<0.05$ were considered significant.

## 3. Results

### 3.1. Effect of CAPE on basal and secretagogues-stimulated gastric acid secretion

The lumen–perfusion solution, being unbuffered, had a pH of 5.89±0.04 ($n=9$). After 1 h, the stabilised basal outflow from the isolated mouse stomach preparation had a pH of 4.67±0.07 ($n=9$). CAPE (10–100 μM) did not modify the basal pH (ΔpH: 0.01±0.003; 0.005±0.006; 0.01±0.005; for 10, 30 and 100 μM of CAPE, respectively, $n=8$) and had no effect on histamine-($10^{-7}$–$10^{-3}$ M), isobutyl methylxanthine-(IBMX, $10^{-7}$–$10^{-3}$ M), pentagastrin-($10^{-10}$–$10^{-6}$ M) and $K^+$-(122.8 mM) stimulated gastric acid secretion (data not shown). By contrast, CAPE (10–100 μM) significantly ($P<0.01$–$0.001$) increased the gastric acid secretion stimulated by 5-methylfurmethide (5-MEF, Fig. 1 and Table 1). The effect of CAPE (10 μM) on 5-MEF-stimulated gastric acid secretion was not modified by nifedipine (2 μM) (data not shown). Moreover, nifedipine alone (2 μM) neither affected basal pH nor modified the 5-MEF-stimulated acid secretion (data not shown).

### 3.2. Effect of CAPE and eserine on acetylcholinesterase activity

CAPE (10–100 μM) significantly ($P<0.05$) and in a dose-related manner inhibited acetylcholinesterase activity (% of inhibition: 23.44±2.94; 41.33±5.52; 55.41±5.67% for CAPE $10^{-5}$, $3\times10^{-5}$ and $10^{-4}$, respectively). Eserine (100 μM) used as reference drug, inhibited by 70.25±1.39% the enzyme activity.

### 3.3. Effect of eserine on basal and 5-methylfurmethide-stimulated gastric acid secretion

Eserine (10 μM), added in the bath, produced a contraction of mouse stomach preparation which was inhibited after a pre-treatment with nifedipine (2 μM) (data not shown). Eserine (10 μM), in presence of nifedipine (2 μM), significantly ($P<0.01$) increased both basal pH (ΔpH: 0.15±0.005) and 5-MEF-stimulated acid secretion (Fig. 2). Eserine (10 μM) inhibited the effect of CAPE (10 μM) on the gastric acid secretion stimulated by 5-methylfurmethide (Fig. 2).

## 4. Discussion

The isolated, lumen-perfused stomach of the mouse is a standard preparation widely used for studying gastric acid secretory mechanisms. Our experiments show that CAPE selectively increased the acid gastric secretion induced by 5-MEF, a muscarinic receptor agonist. Because CAPE also inhibited the activity of acetylcholinesterase enzyme in an in vitro assay, we hypothesized that such inhibition may be involved in CAPE-induced changes in gastric acid secretion.

We have shown that CAPE did not affect the basal acid secretion and the secretion induced by histamine, pentagastrin,

IBMX and high levels of $K^+$. These experiments exclude an action of CAPE on histamine $H_2$ or gastrin cholecystokinin-2 ($CCK_2$) receptors [located on parietal cells (histamine $H_2$ receptors) or parietal and enterochromaffin-like cells (gastrin $CCK_2$ receptors)], and on cyclic nucleotides-mediated secretory processes (experiments with the phosphodiesterase inhibitor IBMX). Moreover, the lack of effect of CAPE on the acid secretion evoked by high levels of $K^+$, which stimulates gastric acid secretion by directly activating $H^+/K^+$-ATPase pump, excludes a direct action of this natural compound on the $H^+/K^+$-ATPase pump. However, we have shown that CAPE concentration dependently increased acid secretion due to the muscarinic receptor agonist 5-MEF, suggesting that the effect of CAPE is related to the cholinergic pathway leading to acid secretion. Thus, we investigated the CAPE mode of action by evaluating its effect on acetylcholinesterase, the main enzyme involved in acetylcholine catabolism.

Black and Shankley (1987) have previously reported that under basal conditions there is a neural release of acetylcholine which is restricted to the region of the histamine-secreting cells by neural configuration and by cholinesterase activity. Thus, the neurally released acetylcholine acts predominantly to release histamine which, however, is not sufficient to produce a secretory response. In fact, antagonists of histamine or acetylcholine receptors do not affect basal secretion (Welsh et al., 1993). The inhibition of acetylcholinesterase activity allows acetylcholine to diffuse to the location of the oxyntic cells and thus to produce an acid secretory response which can lead to potentiation of stable exogenous acetylcholine (e.g. 5-MEF)-mediated acid secretion (Black and Shankley, 1987). In our experiments CAPE, in a concentration dependent manner, inhibited the activity of acetylcholinesterase, suggesting that this mechanism could be responsible of CAPE-induced hypersecretory effect on the acid secretion stimulated by the muscarinic receptor agonist 5-MEF. Pharmacological experiments seemed to confirm this hypothesis. Indeed, CAPE was without effect on acid secretion induced by 5-MEF in the presence of eserine. It should be noted that 5-MEF is not a substrate for acetylcholinesterase (Welsh et al., 1995). Therefore, the gastric acid hypersecretion induced by CAPE is likely due to an increase of endogenous acetylcholine. Moreover, the ability of acetylcholinesterase inhibitors to increase the maximal response to 5-MEF (as it is the case of CAPE in our experimental conditions) has been previously reported (Black and Shankley, 1987).

In accordance with Welsh et al. (1993), in our experiments eserine produced a contraction of the stomach muscle sufficiently powerful to interfere with lumen–perfusion. Several papers have demonstrated that the major source of $Ca^{2+}$ for contraction of the gastric smooth muscle results from $Ca^{2+}$ entry via L-type $Ca^{2+}$ channels (Paul et al., 1994). Furthermore, it has been reported that the blockade of $Ca^{2+}$ entry with nifedipine resulted in an inhibition of acetylcholine-induced stomach contractions. In our experiments, nifedipine inhibited the eserine-induced contraction. In contrast to eserine, CAPE did not evoked contractions of stomach muscle. This discrepancy could be due to the calcium-dependent antispasmodic effects of CAPE (Cicala et al., 2003). Moreover, other investigators have recently shown that CAPE reduced calcium entry in pituitary GH3 cells (Lin et al., 2004). Therefore, the antispasmodic effects of CAPE could counteract the contractile effect due to acetylcholine sterase inhibition. The effect of CAPE on calcium could also explain why this compound did not affect basal acid secretion, as calcium is well known to be involved in basal acid secretion in the stomach mouse (Soll and Berglindh, 1987; Schubert and Shamburek, 1990; Chew et al., 1992). However, in the present study we have shown that nifedipine, a L-type calcium antagonist, did not modify the effect of CAPE on gastric acid secretion, suggesting that these channels are not involved in CAPE-mediated hypersecretory effects. Consistent with our results, it has been reported that nifedipine inhibited histamine-, but not 5-MEF-induced acid secretion (Erjavec and Stanovnik, 1989; Ostrowski et al., 1993).

In conclusion, our study demonstrated that CAPE selectively increased gastric acid secretion stimulated by an acetylcholine receptor agonist and this effect was associated to inhibition of the enzyme AChE. These results not only broadens the pharmacological effects of CAPE, but could be also relevant for the drug discovery in Alzheimer's disease. This because inhibition of AChE is a target for anti-Alzheimer drugs, and CAPE possesses additional effects relevant to the treatment of Alzheimer disease, such as anti-inflammatory (Montpied et al., 2003; Wei et al., 2004) and antioxidant activities (Ilhan et al., 2004; Parlakpinar et al., 2005).

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