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Antispasmodic and myorelaxant effects of the flavoring agent methyl cinnamate in gut: Potential inhibition of tyrosine kinase

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ABSTRACT

Methyl cinnamate (MC) is a safe flavoring agent useful to food industry. Although chemically analog to tyrosine kinase inhibitors, there is little information regarding its biological actions. Here, we aimed at assessing the MC effects on gastrointestinal contractility and the putative involvement of tyrosine kinase in the mediation of these effects. Isometric contractions were recorded in rat isolated strips from stomach, duodenum and colon segments. In gastric strips, MC (3–3000 μM) showed antispasmodic effects against carbachol-induced contractions, which remained unchanged by either L-NAME or tetraethylammonium pretreatment and occurred with potency similar to that obtained against contractions evoked by potassium or U-46619. In colon strips, MC was four times more potent than in gastric ones. MC and the positive control genistein inhibited phasic contractions induced by acetylcholine in Ca^{2+}-free medium, an effect fully prevented by sodium orthovanadate. Both MC and genistein decreased the spontaneous contractions of duodenal strips and shortened the time necessary for gastric fundic tissues to reach 50% of maximal relaxation. In freshly isolated colon myocytes, MC decreased the basal levels of cytoplasmic Ca^{2+}, but not the potassium-elicited cytoplasmic Ca^{2+} elevation. Colon strips obtained from rats subjected to intracolonic acetic acid instillation showed reduced contractility to potassium, which was partially recovered in MC-treated rats. Inhibitory effect of nifedipine against cholinergic contractions, blunted in acetic acid-induced colitis, was also recovered in MC-treated rats. In conclusion, MC inhibited the gastrointestinal contractility with a probable involvement of tyrosine kinase pathways. *In vivo*, it was effective to prevent the deleterious effects of colitis resulting from acetic acid injury.

1. Introduction

Methyl cinnamate (MC; Fig. 1; CAS number: 103-26-4) is a compound widely used in foodstuffs as a flavoring agent or to control food browning (Huang et al., 2009). As a food additive, MC is considered safe for human consumption (*e.g.* US Food and Drug Administration Code: 21CFR172.515), with an estimated median 50% lethal dose as high as 2.61 g/kg in rats (Bhatia et al., 2007). However, its biological activities are poorly known yet.

The dihydroxylated analog of MC, methyl 2,5-dihydroxycinnamate (Fig. 1), was reported as the more stable analog possessing the tyrosine kinase inhibitory properties of erbstatin (Fig. 1) (Umezawa et al., 1986). It is worth to note that tyrosine kinase signaling cascades can modulate the functioning of the gastrointestinal smooth muscle by altering the balance between phosphorylation and dephosphorilation of tyrosine by kinases and phosphatases, respectively. These pathways were already reported to interfere with contractile responses mediated by cholinergic stimulation, a cellular event potentially influenced by inflammatory stimuli (Yang et al., 1992; Laniyonu et al., 1994; Singer et al., 2002; Shi and Sarna, 2004).

The present study was designed based on the following presuppositions: (i) MC has a strong structural analogy with the tyrosine kinase inhibitor methyl 2,5-dihydroxycinnamate (Fig. 1) and (ii) tyrosine kinase activity interferes with the contractility of gastrointestinal smooth muscle. Based on these two facts, our main focus was to verify by a pharmacological approach the efficacy of MC *in vitro* in rat isolated gastrointestinal tissues and the potential involvement of tyrosine kinase pathways in its effects. In addition, because cinnamic acid derivatives and analogs are compounds possessing anti-inflammatory properties (Zhang and Ji, 1992; Godoy et al., 2000), we evaluated whether *in vivo* MC can prevent the deleterious effects in gut contractility caused by a colitis-inducing inflammatory stimulus.

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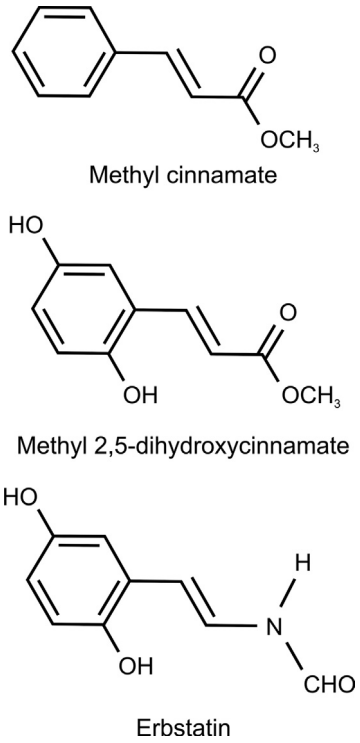

Fig. 1. Chemical structure of methyl cinnamate and its analogs.

2. Materials and methods

2.1. Animals

Male Wistar rats (200–250 g) obtained from our institutional vivarium were housed under standard conditions with free access to food and water. All animals were handled in accordance with the Ethical Principles for Care and Use of Laboratory Animals, published by the Brazilian National Council for Animal Experimentation (RN12/2013) after approval by the local animal ethics committee (process #11/2012).

2.2. Solutions and drugs

In vitro experiments were conducted in modified Tyrode's solution of the following composition: NaCl 136.0 mmol/l; KCl 5.0 mmol/l; $MgCl_2$ 0.98 mmol/l; NaH_2PO_4 0.36 mmol/l; $NaHCO_3$ 11.9 mmol/l; $CaCl_2$ 2.0 mmol/l; glucose 5.5 mmol/l. Acetylcholine (PubChem CID: 6060), carbachol (PubChem CID: 5831), tetraethylammonium (TEA, PubChem CID: 5413), Nω-Nitro-L-arginine methyl ester (L-NAME, PubChem CID: 550788), sodium orthovanadate (OV, PubChem CID: 61671), genistein (PubChem CID: 5280961), tetrodotoxin (TTX, PubChem CID 6324668), papain, collagenase, prednisolone (PubChem CID: 57369545), nifepidine (PubChem CID: 4485), U-46619 (PubChem CID: 5311493), DL-dithiothreitol (PubChem CID: 446094), Fluo-4 AM, pentobarbital sodium (PubChem CID: 14075609) and MC (PubChem CID: 7644) were purchased from Sigma (St Louis, MO, USA). MC was dissolved directly in Tyrode containing Tween 80 (maximum 0.1% v/v) and sonicated just before use. For *in vivo* experiments, MC was dissolved in 0.9% NaCl containing 1% Tween 80 as vehicle.

2.3. Contractility of gut smooth muscle preparations

After animal euthanasia by stunning and cervical dislocation, longitudinal strips of gastric fundus, duodenum or colon were suspended in a 5 ml organ bath containing Tyrode continuously aerated at 37 °C (pH 7.4) and basal tension of 1 g. Strips were attached to a fixed pin in the bath and to a force transducer connected to a digital recording device (PowerLab 8/30, ADInstruments) to record tension under isometric conditions.

2.4. Measurement of the intracellular levels of Ca^{2+}

Colon strips were kept in Tyrode under a stereomicroscope (EZ4, Leica Microsystems), the colon lumen was exposed and the adherent mucosal layer was removed by gentle scraping. The remaining tissue was carefully cut in small pieces that were subjected to two-steps enzymatic digestion at 34 °C, firstly during 30 min with papain (6 mg/ml) in presence of DL-dithiothreitol (0.5 mg/ml), followed by other 30 min period with collagenase 1 mg/ml in presence of soybean trypsin inhibitor (0.3 mg/ml). Cells were separated by repeated mechanical dissociation and incubated with 3 μM Fluo-4 AM at room temperature for 20 min. After Fluo-4 AM excess removal, cells were analyzed on an inverted confocal microscope (Olympus, IX81) at excitation/emission wavelengths of 488 nm/500–550 nm. The fluorescence background was subtracted and the results were expressed as ratio of this initial fluorescence (F/F_0). Data were acquired with a rate of 1 frame/5 s.

2.5. Experimental colitis induced by acetic acid

Colitis was induced by the intracolonic instillation of acetic acid (5% v/v). After an overnight fast, the rats were anesthetized with pentobarbital (30 mg/kg, i.p.) and an 8-cm polyethylene cannula was inserted into the colonic lumen *via* the anus. Initially, each rat received 1 ml saline (NaCl 0.9%) flush to remove fecal matter. Next, 1 ml of acetic acid (5% v/v in NaCl 0.9%) was instilled during 30 s into the distal colon, followed by a second colonic wash with saline (1.5 ml). Sham animals received 1 ml saline with vehicle (for 30 s). After 5 h the rats were gavaged once daily and for 3 days with saline (1 ml), MC (50 mg/kg/day) or prednisolone (1 mg/kg/day). At the end, they were euthanized by stunning and cervical dislocation. Strips of the distal colon were removed and disposed as a ring in the bath chamber for evaluation of the contractile behavior as described above.

2.6. Statistical analysis

Results are expressed as mean ± S.E.M. Peak deflections were used to measure the magnitude of the concentration–response curves, which were expressed as a percentage of a given contractile agent (in the absence of MC). The IC_{50} value was defined as the MC concentration (μM) required for producing a half-maximum reduction of a given contractile stimulus. It was calculated by interpolation from semi-logarithmic plots, and was expressed as geometric mean [95% confidence interval]. The significance ($P < 0.05$) of results was assessed by paired Student's *t*-test, Mann–Whitney *U*-test, and one- or two-way analysis of variance (ANOVA), followed by Holm–Sidak or Tukey multiple comparison tests when appropriate.

3. Results

3.1. Antispasmodic effects of MC on gut isolated smooth muscle preparations

In isolated strips of gastric fundus (Fig. 2A and B), MC (3–3000 μM, $n=6$) completely inhibited the contractions elicited by the muscarinic agonist carbachol (1 μM) with an IC_{50} value (432.4 [346.6–539.6] μM) that remained unchanged (Table 1; $P > 0.05$, Mann–Whitney) by pretreatment with either L-NAME (300 μM; a nitric oxide synthase inhibitor; $n=6$) or TEA (3 mM; a potassium channel blocker; $n=6$). In fundic strips, MC (3–3000 μM, $n=6$) also displayed concentration-