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Characteristics of nobiletin-induced effects on jejunal contractility

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ABSTRACT

Nobiletin, a citrus polymethoxylated flavone, exhibits multiple biological properties including anti-inflammatory, anti-carcinogenic, and anti-insulin resistance effects. The present study found that nobiletin exerted significant stimulatory effects on the contractility of isolated rat jejunal segments in all 6 different low contractile states, and meanwhile significant inhibitory effects in all 6 different high contractile states, showing characteristics of bidirectional regulation (BR). Nobiletin-exerted BR on jejunal contractility was abolished in the presence of c-kit receptor tyrosine kinase inhibitor imatinib or Ca^{2+} channel blocker verapamil. In the presence of neuroxin tetrodotoxin, nobiletin only exerted stimulatory effects on jejunal contractility in both low and high contractile states. Hemicholinium-3 and atropine partially blocked nobiletin-exerted stimulatory effects on jejunal contractility in low-Ca^{2+}-induced low contractile state. Phentolamine or propranolol or L-NG-nitro-arginine significantly blocked nobiletin-exerted inhibitory effects on jejunal contractility in high-Ca^{2+}-induced high contractile state respectively. The effects of nobiletin on myosin light chain kinase (MLCK) mRNA expression, MLCK protein content, and myosin light chain phosphorylation extent were also bidirectional. In summary, nobiletin-exerted BR depends on the contractile states of rat jejunal segments. Nobiletin-exerted BR requires the enteric nervous system, interstitial cell of Cajal, Ca^{2+}, and myosin phosphorylation-related mechanisms.

1. Introduction

The dried immature fruit of citrus is widely used as a traditional medicine with multiple pharmacological effects; the use of citrus for digestive diseases, such as abdominal pain, constipation, and dysenteric diarrhea, is widely reported [1,2]. Flavonoids are polyphenolic compounds found in food of citrus. O-methylated flavonoids are flavonoids with methylations on hydroxyl groups [3]. Nobiletin (Fig. 1), one of the widely distributed citrus O-methylated flavonoids, has attracted much attention recently for its beneficial effects on human health [4]. From 10 kg of citrus peel, 84 mg of nobiletin can be extracted [5]. The absolute bioavailability of nobiletin after oral administration in rat is about 56% [6].

Nobiletin shows anti-inflammation, anti-cancer, and anti-insulin resistance activities [7–9]. Nobiletin exhibits concentration-dependent inhibition on the contractions induced by acetylcholine, transmural electrical stimulation, and histamine in isolated guinea pig ileum [10]. However, whether nobiletin could be used to relieve intestinal motility-related disorders remains unknown. Intestinal motility-related disorders include decreased or increased motility. For instance, evidence indicates that compared with controls (85.3 ± 37.3 min), the transit time (a measurement of bowel movement) obtained in constipation-predominant (CP) (108.4 ± 34.3 min) and diarrhea-predominant (DP) (67.4 ± 19.6 min) patients with IBS was decreased or increased, respectively ($p < 0.05$) [11]. Our pre-experiment showed that

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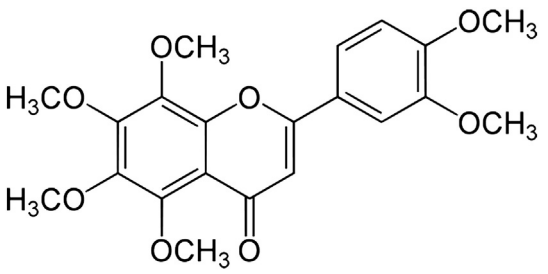

Fig. 1. The chemical structure of nobiletin.

nobiletin, at a fixed dose, exerted both stimulatory and inhibitory effects on isolated rat jejunal contractility in low and high contractile states of jejunal segments respectively. Based on the observations, the present study was designed to characterize nobiletin-induced bidirectional regulation (BR) on the isolated rat intestinal motility, to reveal the related mechanisms, and to evaluate the potential clinical implication of nobiletin in modulating alternating hyper- and hypo-intestinal motility.

Small intestine motility is modulated by enteric nervous system (ENS), which is able to fulfill pivotal functions even when isolated from the body [12], suggesting that isolated intestinal segment can be used to evaluate drug effects. To characterize the effects of nobiletin, different low and high contractile states of rat intestinal segments were established by changing ionic concentration or by using inhibitory and stimulatory neurotransmitters respectively or by using exogenous inhibitors and stimulators respectively or by using jejunal segments isolated from CP and DP rats respectively. Considering that the jejunum is the main portion of the intestine involved in the digestion and absorption of nutrients [13], we chose to characterize nobiletin-induced BR on jejunal contractility as well as its underlying mechanisms.

2. Materials and methods

2.1. Materials

Eighty Sprague-Dawley rats, half male and half female, 200–250 g, were provided by Laboratory Animal Center, Dalian Medical University (Certificate of Conformity: No. SCXK (Liao) 2008–0002). All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Animals were housed 5 per cage in a temperature-controlled room with a 12-h light–dark cycle. Food and water were available for *ad libitum* consumption.

2.2. Experimental models of constipation and diarrhea

CP rats were established by daily gavage with cool water (0–4 °C) for 14 days while the control rats were gavaged with water at room temperature [14]. Intracolonic instillation of 4.0% (V/V) acetic acid and restraint stress were used to establish DP rat model [15]. Control rats underwent intracolonic instillation with saline. CP and DP rats were established by assessing the decreased and increased contractility of isolated jejunal segments which were obtained from CP and DP rats respectively. After treatment with nobiletin (20.0 mg/kg) for 7 days; jejunal segments isolated from control rats, CP, nobiletin-treated CP, DP, and nobiletin-treated DP rats were used in the determination of jejunal contractility, mRNA expression of myosin light chain kinase (MLCK), protein content of MLCK, phosphorylation extent of the 20 kDa myosin light chain (p-MLC_{20}). The granules and moisture content of the feces from each group were calculated daily, and the body mass was recorded once every 3 days.

2.3. Tissue preparation and jejunal contractility determination

Jejunal segments were isolated from the intact jejunum of normal, CP, and DP rats respectively as described previously [16]. Jejunum was cut into approximately 2.0 cm in length. One end of jejunal segment was fixed to the wall of the tissue bath chamber (20.0 mL volume), and the other end was connected to a force-displacement transducer in longitudinal direction. Contractile amplitude of isolated jejunal segments was recorded and identical time-interval of each assay with same start and stop time was chosen to compare the amplitude of contractions before and after drug treatment in all assays [17]. The mean amplitude was calculated from the results of 6 separated assays.

2.4. Isolated jejunal segments in different contractile states

The contractility of jejunal segments was measured in Krebs buffer and selected as the normal contractile state. Since intestinal spontaneous contractions are paralleled to the intracellular Ca^{2+} concentration [18], jejunal contractility measured in a modified low Ca^{2+} (1.3 mM) and high Ca^{2+} (5.0 mM) Krebs buffer was selected as the representative low contractile state (RLCS) and representative high contractile state (RHCS) respectively. Jejunal segments isolated from CP and DP rats respectively were chosen as a pair of low-high contractile states. Jejunal segments pre-incubated in modified low K^+ (2.5 mM)–high K^+ (10.0 mM) Krebs buffer, low Na^+ (100.0 mM)–high Na^+ (150.0 mM) Krebs buffer, adrenaline (5.0 μM)–acetylcholine (ACh) (5.0 μM) Krebs buffer, and nitric oxide donor sodium nitroprusside (SNP) (5.0 μM)–erythromycin (10.0 μM) Krebs buffer respectively were established as the other 4 pairs of low-high contractile states.

2.5. Western blot analysis

As described previously [19], the extent of p-MLC_{20} and protein content of MLCK in jejunal segments were examined by using Western blot analysis. Jejunal segments, isolated from normal control, CP, nobiletin-treated CP, DP, and nobiletin-treated DP rats, were frozen and stored in liquid nitrogen for Western blot or RT-PCR analysis. Ground product was incubated for 30 min in ice-cold homogenization buffer. The blots on nitrocellulose filter membrane were probed with phosphor-myosin light chain 2 (Ser 19) antibody (1:1000) (No. 3671, Cell Signaling Technology, Inc. (CST), Beverly, MA, USA) and myosin light chain 2 (total myosin light chain) antibody (1:1000) (No. 3672, CST, Beverly, MA, USA) respectively, at 4 °C with gentle shaking, over night. For MLCK analysis, the blots on nitrocellulose filter membrane were probed with MLCK antibody (1:1000) (No. ab76092, Abcam (Hong Kong) Ltd. UK). Anti-rabbit IgG secondary antibodies were assayed at