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Original Article

Naringenin inhibits pacemaking activity in interstitial cells of Cajal from murine small intestine

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ABSTRACT

Background: Naringenin (NRG) is a common dietary polyphenolic constituent of fruits. NRG has diverse pharmacological activities, and is used in traditional medicine to treat various diseases including gastrointestinal (GI) disorders. Interstitial cells of Cajal (ICCs) are pacemaker cells of the GI tract. In this study, the authors investigated the effects of NRG on ICCs and on GI motility *in vitro* and *in vivo*.

Methods: ICCs were dissociated from mouse small intestines by enzymatic digestion. The whole-cell patch clamp configuration was used to record pacemaker potentials in cultured ICC clusters. The effects of NRG on GI motility were investigated by calculating percent intestinal transit rates (ITR) using Evans blue in normal mice.

Results: NRG inhibited ICC pacemaker potentials in a dose-dependent manner. In the presence of tetraethylammonium chloride or iberiotoxin, NRG had no effect on pacemaker potentials, but it continued to block pacemaker potentials in the presence of glibenclamide. Preincubation with SQ-22536 had no effect on pacemaker potentials or on their inhibition by NRG. However, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one blocked pacemaker potential inhibition by NRG. In addition, L-NG-nitroarginine methyl ester blocked pacemaker potential inhibition by NRG. Furthermore, NRG significantly suppressed murine ITR enhancement by neostigmine *in vivo*.

Conclusion: This study shows NRG dose-dependently inhibits ICC pacemaker potentials via a cyclic guanosine monophosphate/nitric oxide-dependent pathway and Ca^{2+}-activated K^+ channels *in vitro*. In addition, NRG suppressed neostigmine enhancement of ITR *in vivo*.

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1. Introduction

Natural products derived from foods and traditional herbs have been traditionally used to treat infections, inflammatory and gastrointestinal (GI) diseases, and other disease types.[1–3] Naringenin [NRG; 5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one] is a type of natural flavonoid, and is found in grapefruit, bitter orange, and other fruits.[2] NRG has wide range of biological and pharmacological activities, which include anti-inflammatory,[4] antimutagenic,[5] antiatherogenic,[6] antioxidant,[7] and anticancer[8] effects, and has a relaxant effect on vascular smooth muscle[9] and a regulatory effect on GI function.[10,11]

Interstitial cells of Cajal (ICCs) are the pacemaker cells of GI muscularis propria, and are involved in the generation of primary electrical pacemaker activity, which controls GI motility.[12–14] These cells also form a bridge between enteric motor nerve terminals and smooth muscle cells.[14] Because of the central role played by ICCs in GI motility, loss of these cells is extremely detrimental to GI functions.[15]

As the effects of NRG on ICCs and GI motility have not been previously investigated, we undertook to investigate its effects on the pacemaker potentials of clusters of murine small intestine ICCs *in vitro* and on GI motor functions *in vivo* by measuring intestinal transit rates (ITRs) in Evans blue in mice.

2. Methods

2.1. Ethics

Animal care and experiments were conducted in accordance with the guidelines issued by the ethics committee of Pusan National University (Busan, Korea; Approval no. PNU-2016-1370) and those issued by the National Institute of Health 'Guide for the Care and Use of Laboratory Animals' (2013).

2.2. Preparation of cells and cell cultures

BALB/c mice (aged 2–6 days; weight, 2.1–2.3 g; Samtako Bio Korea Co., Ltd., Osan, Korea) were anesthetized with 0.1% ether (Sigma–Aldrich, St Louis, MO, USA; Merck Millipore, Darmstadt, Germany) and sacrificed by cervical dislocation. Mice were maintained under controlled conditions (21 ± 2 °C; relative humidity 50 ± 5%; 12-h light/dark cycle) and allowed free access to food and water. Small intestines were removed and opened along the mesenteric border, and luminal contents were removed by washing with Krebs–Ringer bicarbonate solution. Small intestine mucosae were removed by sharp dissection and small strips of intestine muscle were equilibrated in Ca^{2+}-free physiological salt solution (125 mM NaCl, 5.36 mM KCl, 0.34 mM NaOH, 0.44 mM Na_2HCO_3, 10 mM glucose, 2.9 mM sucrose, and 11 mM HEPES buffer) for 20 min and then dispersed using an enzyme solution containing 1.5 mg/mL collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA), 2.5 mg/mL bovine serum albumin (Sigma–Aldrich; Merck Millipore), 2.5 mg/mL trypsin inhibitor (Sigma–Aldrich; Merck Millipore), and 0.5 mg/mL adenosine triphosphate (ATP; Sigma–Aldrich; Merck Millipore). Cells were plated on glass coverslips coated with 0.01% poly-L-lysine solution (Sigma–Aldrich; Merck Millipore) and cultured in a 95% O_2/5% CO_2 atmosphere in smooth muscle basal medium (Clonetics Corp.; Lonza, Walkersville, MA, USA) supplemented with stem cell factor (5 ng/mL; Sigma–Aldrich; Merck Millipore) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37 °C.

2.3. Patch-clamp experiments

The cells were transferred onto a solution chamber on the stage of an invert microscope (IX70; Olympus, Tokyo, Japan). The whole-cell patch-clamp technique was used to record the membrane potentials (current clamp) of cultured ICC as described previously,[6,7] and an Axopatch 200 B amplifier (Axon Instruments, Foster City, CA, USA) was used to amplify membrane potentials. Bath solution (Normal Tyrode) contained 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES with a pH that was adjusted to 7.4 using NaOH. The internal solution contained 145 mM Cs-glutamate, 8 mM NaCl, 10 mM BAPTA, and 10 mM HEPES-CsOH, with pH adjusted to 7.2 with CsOH. All experiments were performed at 30 °C.

2.4. In vivo intestine motility measurements

Mice were randomly allocated into four groups: the normal group (saline controls); neostigmine (0.2 mg/kg) group; neostigmine and low-dose naringenin (10 mg/kg) group; and the neostigmine and high-dose naringenin (20 mg/kg) group. Animals were fasted for 12 h prior to the intraperitoneal administration of drugs or saline solution. Immediately after treatment, 0.5 mL of Evans blue was administered by gavage. Mice were sacrificed 30 min after Evans blue administration and entire intestines (from pylorus to the anus (anal ring?)) were excised. Total intestinal lengths and distances traveled by Evans blue were then measured. Intestine transit rate (ITR) was defined as the distance traveled by Evans blue expressed as a percentage of total intestinal length, as described elsewhere.[10,16,17]

2.5. Drugs

Drugs were purchased from Sigma–Aldrich. To produce stock solutions, all drugs were dissolved in distilled water or dimethylsulfoxide (DMSO) and stored at −20 °C. The final concentration of DMSO in bath solution was always <0.1%, and at this level DMSO did not affect the results.

2.6. Statistics

Results are expressed as mean ± standard error. The analysis was performed using the Student *t* test or by analysis of variance (ANOVA) followed by Tukey's multiple comparison test, as appropriate, using GraphPad Prism version 6. Statistical significance was accepted for p values <0.05. The n values reported in the text refer to the number of cells used in patch-clamp experiments