Original Article

CFTR chloride channel as a molecular target of anthraquinone compounds in herbal laxatives

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Aim: To clarify whether CFTR is a molecular target of intestinal fluid secretion caused by the anthraquinone compounds from laxative herbal plants.

Methods: A cell-based fluorescent assay to measure I^- influx through CFTR chloride channel. A short-circuit current assay to measure transcellular Cl^- current across single layer FRT cells and freshly isolated colon mucosa. A closed loop experiment to measure colon fluid secretion *in vivo*.

Results: Anthraquinone compounds rhein, aloe-emodin and 1,8-dihydroxyanthraquinone (DHAN) stimulated I^- influx through CFTR chloride channel in a dose-dependent manner in the presence of physiological concentration of cAMP. In the short-circuit current assay, the three compound enhanced Cl^- currents in epithelia formed by CFTR-expressing FRT cells with EC_{50} values of 73 ± 1.4 , 56 ± 1.7 , and 50 ± 0.5 $\mu\text{mol/L}$, respectively, and Rhein also enhanced Cl^- current in freshly isolated rat colonic mucosa with a similar potency. These effects were completely reversed by the CFTR selective blocker CFTR_{inh}-172. In *in vivo* closed loop experiments, rhein 2 mmol/L stimulated colonic fluid accumulation that was largely blocked by CFTR_{inh}-172. The anthraquinone compounds did not elevate cAMP level in cultured FRT cells and rat colonic mucosa, suggesting a direct effect on CFTR activity.

Conclusion: Natural anthraquinone compounds in vegetable laxative drugs are CFTR potentiators that stimulated colonic chloride and fluid secretion. Thus CFTR chloride channel is a molecular target of vegetable laxative drugs.

Keywords: cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel; anthraquinone compounds; colonic fluid secretion; molecular pharmacology; drug discovery

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Introduction

Vegetable laxative drugs such as Aloe, cascara and senna are widely used all over the world for the treatment of constipation in both prescription and non-prescription forms^[1, 2]. Cathartic ingredients of these laxative drugs are anthranoid compounds (including anthraquinone, anthrone and dianthrone) and possibly their derivatives^[3]. It is generally regarded that anthranoid compounds work exclusively in the colon to stimulate colonic motility and increase electrolyte and fluid transport. These compounds exert their functions by both inhibiting absorption and inducing secretion of fluid in the intestine^[4]. Decrease of net absorption was generally attributed to accelerated intestinal transit^[5, 6]. The molecular mechanism of intestinal net fluid secretion is still unclear.

Intestinal fluid secretion is driven by active Cl^- transport from the basolateral to the apical side of enterocytes. Chloride enters the intestinal epithelial cells via Na^+ , K^+ , $2Cl^-$ cotransporter, and is secreted into the lumen through the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. Both Na^+ and water follow Cl^- paracellularly, resulting in a net iso-osmotic fluid secretion^[7]. Previous studies showed that rhein anthraquinone stimulated Cl^- secretion in colonic mucosa^[8, 9]. However, the precise molecular pathway of such Cl^- secretion has not yet been clarified. Because CFTR is the final common pathway for intestinal Cl^- secretion in response to various agonists^[10], and all stimulant laxatives act by altering fluid and electrolyte transport in the small and/or large intestines, we hypothesized that CFTR Cl^- channel activity played an important role in colonic fluid secretion stimulated by anthraquinone compounds.

In the present study, we determined the potentiating effects of anthraquinone compounds on CFTR chloride channel and

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characterized the role of CFTR in anthraquinone-stimulated colonic fluid secretion.

Materials and methods

Chemicals

Anthraquinone compounds were obtained from the National Institute for the Control of Pharmaceutical and Biological Products in China (purity >99% as determined by analytical HPLC). Compounds were dissolved in DMSO prior to use and stability of stock solution was verified during the study by HPLC analysis. Forskolin (FSK), genistein, F12 Coon's medium and L-glutamine were purchased from Sigma Chemical Co (St Louis, MO, USA); indomethacin, amiloride, amphotericin B were purchased from Sigma-Aldrich company (St Louis, MO, USA); fetal bovine serum (Characterized) was purchased from HyClone; CFTR_{inh}-172 were synthesized as reported before^[11]; other reagents were of analytical grade or higher. cAMP radioimmunoassay kit was purchased from Shanghai Traditional Chinese Medicine University.

Tissue preparation

All *in vivo* studies including isolation of rat colonic mucosa followed institutional guidelines for animal experiments. Rat colonic mucosa was obtained as described previously^[12]. Briefly, Sprague-Dawley (SD) rats (body weight about 200 g) were starved for 24 h prior. Two to three segments of colon were excised after the rat was euthanized with intravenous pentobarbital (100 mg/kg). The segments were immediately stripped of muscularis and bathed in Krebs-Henseleit (KH) solution (contained in mmol/L: NaCl 117, KCl 4.7, MgCl₂ 1.2, KH₂PO₄ 1.2, NaHCO₃ 24.8, CaCl₂ 2.5, glucose 11.1, pH 7.4).

Fluorescence assay of CFTR channel function

Fisher rat thyroid (FRT) epithelial cells coexpressing human wild type CFTR and halide sensitive yellow fluorescent protein YFP-H148Q were generated as described previously^[11, 13]. The FRT cells were seeded in black-walled, clear-bottomed 96-well tissue culture plates (Costar, Corning, NY, USA) and incubated at 37 °C to achieve confluence (about 24 h). After washing 3 times with phosphate-buffered saline (PBS), the FRT cells were incubated with 100 nmol/L FSK and test compounds in a final volume of 40 µL for 10 min. The fluorescence of each well was monitored in a fluorescence plate reader (Fluostar Optima; BMG Laboratory Technologies, Offenburg, Germany) with 2 s before and 12 s after injection of 120 µL of I⁻-containing solution (PBS in which 137 mmol/L Cl⁻ was replaced by equal concentration of I⁻). I⁻ influx rates (d[I⁻]/dt at t=0) were computed from fluorescence time course data by single exponential regression, as described previously^[13].

Electrophysiology

Transepithelial short-circuited current (*I*_{sc}) studies were done on both FRT cells and rat colonic mucosa.

The FRT cells grew on Snapwell inserts, formed monolayer epithelial cells (about 7–9 d) at an air liquid interface as described^[14, 15], and then were placed in a Ussing chamber

system (Vertical Diffusion Chamber, Physiological Instruments, San Diego, CA, USA). Measurements were performed in the presence of a transepithelial Cl⁻ gradient, in which the basolateral side solution contained (in mmol/L): NaCl 130, KCl 2.7, KH₂PO₄ 1.5, CaCl₂ 1, MgCl₂ 0.5, Na-HEPES 10, pH 7.3, and glucose 10; The apical side solution contained the same components but was modified by replacing 65 mmol/L NaCl with sodium gluconate and the concentration of CaCl₂ was increased to 2 mmol/L to compensate for calcium buffering caused by gluconate^[16]. The basolateral membrane of the FRT cells was permeabilized with 250 µg/mL amphotericin B.

For measurements of transepithelial *I*_{sc} on rat colonic mucosa, sheets of tissue were mounted in the Ussing chambers (area 1.03 cm²); the hemichambers were filled with a KH solution. *I*_{sc} values were measured after inhibition of Na⁺ current by the ENaC inhibitor amiloride (10 µmol/L) and prostaglandin synthesis using indomethacin (10 µmol/L), followed by stimulation by forskolin (20 µmol/L) and subsequent inhibitor addition^[12].

Measurements were performed at 37 °C, and solutions were continuously bubbled with air (FRT cells) or with 5% CO₂, 95% air (rat colonic mucosa) during experiments. *I*_{sc} values were recorded with a DVC-1000 voltage clamp (World Precision Instruments, Sarasota, FL, USA) via Ag/AgCl electrodes and 1 mol/L KCl agar bridges.

In vivo intestinal fluid secretion analysis

Fluid accumulation in rat colonic loops was measured as described previously^[11]. Briefly, SD rats were starved for 24 h prior to commencing experiments. Closed colonic loops proximal to cecum were isolated with ligature under intraperitoneal (60 mg/kg) anesthesia. Loops were injected with 100 µL of: PBS alone, PBS containing rhein (2 mmol/L), or PBS containing rhein (2 mmol/L) plus CFTR_{inh}-172 (40 µmol/L). The abdominal wall was then closed, and rats were allowed to recover from anesthesia. After 6 h the rats were euthanized with overdose intraperitoneal injection of pentobarbital (100 mg/kg). Loops were excised, and loop length and weight were measured.

Assay of cAMP activity

cAMP activity was determined by using the cAMP radioimmunoassay kit (Shanghai Traditional Chinese Medicine University).

FRT cells grown in 96-well plates were washed with PBS, then incubated with test compounds for 10 min, and then lysed. cAMP activities were measured in sextuplicate as the manufacture's instruction.

Rat colonic mucosa cAMP contents were measured as described in reference^[17] with some modifications. Briefly, freshly prepared rat colonic mucosa (50 mg) was washed and incubated in KH solution in a 37 °C incubator. The isolated mucosal sheets were exposed to test compounds for 15 min then rapidly frozen in liquid nitrogen and homogenized. The homogenates were centrifuged at 2000×g for 15 min at 4 °C. The supernatant was extracted three times with 3 volumes of

diethyl ether before lyophilization. The supernatants were collected and mixed with equal volume of ethanol, then dried in 37 °C incubator. cAMP levels were assayed by using the radioimmunoassay kit as done on the FRT cells.

Statistical analysis

Data are reported as mean±SEM. Statistical significance of the effects were determined by using the OriginPro 8.0 software.

Results

Anthraquinones potentiate the CFTR chloride channel

First, we analyzed the effect of 5 anthraquinone compounds (Figure 1A) on FRT cells stably expressing CFTR chloride

channel using an iodide-sensitive fluorescent assay. As shown in Figure 1B, three out of the five anthraquinone compounds (rhein, aloë-emodin and DHAN) significantly increased iodide influx into FRT cells in a dose-dependent manner in the presence of 100 nmol/L FSK. The potency and efficacy of the three compounds are lower than the known CFTR potentiator genistein.

To confirm the activity of the three anthraquinone compounds on CFTR, we further analyzed their effect by the more reliable short-circuit current assay in Ussing chamber. Measurements were performed on the FRT cells after basolateral membrane permeabilisation with amphotericin B to measure apical membrane Cl⁻ current. Representative recordings of

Figure 1. Functional analysis of anthraquinones in FRT cells expressing wild-type CFTR. (A) Chemical structures of the tested anthraquinone compounds; (B) Dose-dependent effects of the 5 anthraquinone compounds on CFTR with genistein as a positive control. Mean±SEM. $n=6$.

Figure 2. Short-circuit current analysis of anthraquinone compounds in FRT cells expressing wild-type CFTR. (A–C) representative recordings of short-circuit current potentiated by the indicated concentrations of aloë-emodin, rhein and DHAN in the presence of 100 nmol/L forskolin (FSK) and inhibited by CFTR_{inh}-172 (20 μmol/L). (D) averaged dose-response relationships for potentiation of Cl⁻ currents by the three anthraquinones. Mean±SEM. $n=6$.

I_{sc} are shown in Figure 2A–2C and summarized in Figure 2D. The three positive compounds in the fluorescence assay all potentiated apical membrane I_{sc} in a concentration-dependent manner in the presence of forskolin. In each case, the increased I_{sc} was completely abolished by the specific CFTR blocker CFTR_{inh}-172^[11]. The potency of the three anthraquinone compounds is: Rhein>Aloe-emodin>DHAN. EC₅₀ values for CFTR activation by Rhein, Aloe-emodin and DHAN were (in $\mu\text{mol/L}$): 73 ± 1.4 , 56 ± 1.7 , and 50 ± 0.52 , respectively. Physcion and Emodin did not stimulate significant I_{sc} at 1 mmol/L in the short-circuit current assays (data not shown). Both potency and efficacy of these compounds are significantly lower than genistein.

Stimulation of mucosal short-circuit current and fluid secretion in rat colon by rhein

Further short-circuit current analysis was performed on isolated rat colonic mucosa. Experiments were done after inhibition of Na⁺ current by amiloride and inhibition of prostaglandin generation by indomethacin. Rhein was administered on the serosal and mucosal sides separately. As shown in Figure 3A, rhein stimulated Cl[−] secretion across colonic mucosa in a dose-dependent manner. Although the EC₅₀s are similar in both cases (about 100 $\mu\text{mol/L}$), the serosal application appeared more effective. Addition of CFTR_{inh}-172 (20 $\mu\text{mol/L}$) to the mucosal solution completely abolished the chloride secretion. The effect of rhein on colonic fluid secretion was studied in a closed loop model of rat distal colon. As shown in Figure 3B, left, there was marked fluid accumulation

in rhein-treated loops, whereas control loops filled with saline remained empty. CFTR_{inh}-172 effectively prevented fluid accumulation in the rhein-treated colonic loops. Data from a series of experiments are summarized in Figure 3B, right.

Rhein has no effect on cAMP levels of rat colonic mucosa

To further characterize its mechanism of action, rhein was tested for its ability to elevate cAMP production. As shown in Figure 4, although rhein elicited a small increase of cAMP levels in FRT cells in the presence of 100 nmol/L FSK, it did not affect the cAMP level of rat colonic mucosa. These data suggest that rhein might interact directly with CFTR and potentiate its activity.

Discussion

The purpose of this study was to clarify whether CFTR was a molecular target of intestinal net fluid secretion induced by the anthraquinone compounds. Anthraquinones are a group of small molecules found in herbal laxatives. Previous studies have found that anthraquinone compounds stimulated Cl[−] secretion in colonic mucosa, but molecular targets were not determined^[7, 8]. CFTR is predominantly expressed in colonic crypts where it plays vital roles in regulating the secretion of electrolytes and fluid across the epithelium^[18]. Abnormalities of CFTR function may result in diarrhea^[19–21] or constipation^[22, 23]. Therefore, CFTR modulators might be used to treat diarrhea and constipation^[24].

We demonstrated that natural anthraquinone compounds from laxative herbal plants stimulated CFTR activity both *in*

Figure 3. Stimulation of mucosal short-circuit current and fluid secretion in rat colon by rhein. (A) Representative recordings of rhein-stimulated short-circuit current (I_{sc}) across rat distal colonic mucosa. *Left*, when added from serosal side; *middle*, when added from mucosal side; *right*, data summary. Mean $\pm$ SEM. $n=6$. (B) Stimulation of intestinal fluid secretion in rat intestinal closed loop experiments. *Left*: Photograph of isolated rat descending colon loops at 6 h after luminal injection of KH solution alone, rhein (2 mmol/L) and rhein (2 mmol/L) plus CFTR_{inh}-172 (40 $\mu\text{mol/L}$). *Right*: averaged luminal liquid weight at 6 h. Mean $\pm$ SEM. $n=12$ loops from 6 rats. $^{\circ}P<0.01$.

Figure 4. cAMP stimulating activities of anthraquinones. (A) cAMP content in FRT cells at 10 min after the addition of 400 $\mu\text{mol/L}$ test compounds with and without 100 nmol/L FSK. (B) cAMP content in rat colonic mucosa after the addition of 400 $\mu\text{mol/L}$ rhein. Mean $\pm$ SEM. $n=6$. $^{\circ}P<0.01$.

vitro and *in vivo*. First, *in vitro* functional analysis using a cell-based fluorescent assay^[13] and a short-circuit current assay revealed that anthraquinone compounds potentiated CFTR chloride channel function in a dose-dependent manner in the presence of physiological concentration of cAMP. Second, the increase of chloride secretion across isolated rat colonic mucosa by rhein was completely reversed by CFTR specific blocker CFTR_{inh}-172, further supporting CFTR as the molecular target of anthraquinone compounds. Finally, colonic fluid accumulation stimulated by rhein in *in vivo* closed loop experiment was largely blocked by CFTR_{inh}-172, suggesting that CFTR activation is a major mechanism of anthraquinone-stimulated colonic fluid secretion.

The rhein-induced I_{SC} in rat colonic mucosa is not considered to be mediated by electrogenic Na^+ absorption because it was not inhibited by apical addition of amiloride. Though it is possible that rhein may affect generation of prostaglandins (PG) which are also known to be mediators of other secretagogues of intestinal secretion^[25], secretion of Cl^- stimulated by anthraquinone does not appear to be involved in this pathway, because rhein-induced I_{SC} response was unaffected by the presence of the prostaglandin synthesis inhibitor indomethacin.

CFTR is a cAMP-dependent Cl^- channel. An activator can stimulate CFTR activity by increasing cAMP-dependent phosphorylation of CFTR protein or direct interaction with CFTR. Our data support the idea that anthraquinone compounds interact directly with CFTR, because these compounds exhibited minimal effect on intracellular cAMP level in both FRT cells and rat colonic mucosal epithelial cells. Consistent with our results, Ai *et al* reported that anthracene compounds with similar structure increased P_o of CFTR by prolonging the

mean burst duration and shortening the interburst durations in excised inside-out patches, suggesting potentiation of CFTR activity by directly affecting CFTR gating^[26]. Therefore, it is likely that anthraquinone compounds potentiate CFTR function through direct binding to CFTR protein.

In conclusion, natural anthraquinone compounds in vegetable laxative drugs are CFTR potentiators that stimulated colonic chloride and fluid secretion. Anthraquinone compounds potentiate CFTR function most probably through direct interaction with CFTR protein. Thus CFTR chloride channel is a molecular target of vegetable laxative drugs.

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Author contribution

Hong YANG directed the research and wrote the paper; Li-na XU performed part of the research; Cheng-yan HE performed part of the research; Xin LIU performed part of the research; Rou-yu FANG performed part of the research; Tong-hui MA designed the research and helped writing the paper

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