Basic Study

Carbon monoxide contributes to the constipating effects of granisetron in rat colon

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Abstract

AIM

To investigate the mechanisms underlying the potential contribution of the heme oxygenase/carbon monoxide (HO/CO) pathway in the constipating effects of granisetron.

METHODS

For in vivo studies, gastrointestinal motility was evaluated in male rats acutely treated with granisetron [25, 50, 75 µg/kg/subcutaneous (sc)], zinc protoporphyrin IX [ZnPPIX, 50 µg/kg/intraperitoneal (ip)] and hemin (50 µmol/L/kg/ip), alone or in combination. For in vitro studies, the contractile neurogenic response to electrical field stimulation (EFS, 3, 5, 10 Hz, 14 V, 1 ms, pulse trains lasting 10 s), as well as the contractile myogenic response to acetylcholine (ACh, 0.1-100 µmol/L) were evaluated on colon specimens incubated with granisetron (3 µmol/L, 15 min), ZnPPIX (10 µmol/L, 60 min) or CO-releasing molecule-3 (CORM-3, 100, 200, 400 µmol/L) alone or in combination. These experiments were performed under co-treatment with...
or without atropine (3 µmol/L, a muscarinic receptor antagonist) or N- nitro-L-Arginine (L-NNA, 100 µmol/L, a nitric oxide synthase inhibitor).

RESULTS
Administration of granisetron (50, 75 µg/kg) in vivo significantly increased the time to first defecation (P = 0.045 vs vehicle-treated rats), clearly suggesting a constipating effect of this drug. Although administration of ZnPPIX or hemin alone had no effect on this gastrointestinal motility parameter, ZnPPIX co-administered with granisetron abolished the granisetron-induced constipation. On the other hand, co-administration of hemin and granisetron did not modify the increased constipation observed under granisetron alone. When administered in vitro, granisetron alone (3 µmol/L) did not significantly modify the colon’s contractile response to either EFS or ACh. Incubation with ZnPPIX alone (10 µmol/L) significantly reduced the colon’s contractile response to EFS (P = 0.016) but had no effect on contractile response to ACh. Co-administration of ZnPPIX and atropine (3 µmol/L) abolished the ZnPPIX-mediated decrease in contractile response to EFS. Conversely, incubation with CORM-3 (400 µmol/L) alone increased both the contractile response to EFS at 10 Hz (10 Hz: 71.02 ± 19.16 vs 116.25 ± 53.70, P = 0.01) and the contractile response to ACh (100 µmol/L) (P = 0.012). Co-administration of atropine abolished the CORM-3-mediated effects on the EFS-mediated response. When granisetron was co-incubated in vitro with ZnPPIX, the ZnPPIX-mediated decrease in colon contractile response to EFS was lost. On the other hand, co-incubation of granisetron and CORM-3 (400 µmol/L) further increased the colon’s contractile response to EFS (at 5 Hz: P = 0.007; at 10 Hz: P = 0.001) and to ACh (ACh 10 µmol/L: P = 0.001; ACh 100 µmol/L: P = 0.001) elicited by CORM-3 alone. L-NNA co-administered with granisetron and CORM-3 abolished the potentiating effect of CORM-3 on granisetron on both the EFS-induced and ACh-induced contractile response.

CONCLUSION
Taken together, findings from in vivo and in vitro studies suggest that the HO/CO pathway is involved in the constituting effects of granisetron.

Key words: Granisetron; Carbon monoxide; Heme oxygenase; Colon; Contraction; Neurogenic response; Myogenic response

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Core tip: We studied whether in vivo and in vitro effects of granisetron might be influenced, at least in part, by the heme oxygenase/carbon monoxide (HO/CO) pathway. Our findings demonstrate for the first time that the HO/CO pathway takes part in the contractile colon activity in rats. Interestingly, the constipating effects of granisetron are positively correlated with levels of carbon monoxide, thus suggesting that treatments able to modulate carbon monoxide levels may potentially reduce the constipation mediated by granisetron.

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INTRODUCTION
In recent decades, the role played by carbon monoxide (CO) in several biochemical processes has been increasingly recognized[1-3]. Once considered only for its lethal effects, the therapeutic use of CO has been proposed after the discovery of its potential “positive” functions (http://clinicaltrials.gov/ct2/search, “carbon monoxide”).

CO is a gas that is produced, together with iron and biliverdin, from the catalysis of heme by the microsomal heme oxygenase (HO) enzyme. Of the two HO isoforms, HO-2 is the constitutive one, whereas HO-1 is a highly inducible isoform whose activity is intended to provide protection against oxidative stress, injury and inflammation[1-5].

The first physiological role suggested for CO was in non-adrenergic non-cholinergic (NANC) neurotransmission at the gastrointestinal level[6]. The hypothesis of CO as a neurotransmitter is strongly supported by the wide expression of HO-2 throughout the gastrointestinal tract in the enteric nerves, as well as in the non-neuronal cells of the mucosal epithelium, smooth muscle cells, endothelium of blood vessels and interstitial cells of Cajal[3-5]. Moreover, HO-1 is upregulated in several gastrointestinal pathologies such as colitis, inflammatory bowel disease and gastric ulcers (see[5] for references). Because endogenously produced CO diffuses to blood where it binds to hemoglobin, increased HO-1 expression may result in augmented blood levels of carboxyhemoglobin (normal levels 0.8%). However, high levels of carboxyhemoglobin are more typically the consequence of smoking habits or environmental pollution[6]. Either from endogenous or exogenous sources, altered CO levels may affect physiological processes or modulate pathological conditions via several distinct mechanisms[6]. Ion channels have been shown to be, among others, the target of CO; thus, it is possible that CO may modulate the effects of other signals by acting directly on the same target or indirectly on the shared pool of second messengers[6-8]. A similar modulating activity of CO might also be plausible toward specific drugs; indeed, in a previous report, we observed the involvement of the
HO/CO pathway in granisetron-mediated effects on rat duodenal motility[9].

Granisetron is a highly selective competitive antagonist of the 5-HT₃ receptor, the only serotonin-gated ion channel that, if activated, allows an influx of cations[10]. Granisetron is currently used for the chemotherapy-induced nausea and vomiting[11], and constipation is reported among its side effects[12]. On the other hand, constipation is the desired effect for 5-HT₃ receptor antagonists such as alosetron and cilansetron in the treatment of irritable bowel syndrome with diarrhea[13] in which the delayed transit in the large bowel may reduce pain and discomfort in those patients[14]. Unfortunately, despite their clinical efficacy, the potential use of these drugs has been restricted due to reports of severe ischemic colitis (see[15] for review). Nevertheless, these observations support the ability of 5-HT₃ receptor antagonists to induce constipation.

To explore potential mechanisms linking the activity of the HO/CO pathway to granisetron-induced constipation, we investigated whether the constipating effects of granisetron administered in vivo may be modulated by agents that induce (such as hemin) or inhibit (such as zinc protoporphyrin, ZnPPIX) the endogenous HO activity. A 3 μmol/L concentration of granisetron was chosen for the present investigation based on dose-response curves previously obtained[9]. Moreover, because constipation has been ascribed to abnormalities of various contractile activities of the colon[16-20], parallel in vitro studies on isolated colon preparations were performed to evaluate (1) the neurogenic contractile responses to electrical field stimulation indicative of cholinergic and non-cholinergic transmitter release from enteric neurons[20,21] in the absence and in the presence of the muscarinic antagonist atropine as well as the nitric oxide synthase inhibitor L-NNA; and (2) the myogenic contractile response to ACh, one of the major contractile neurotransmitters at the gastrointestinal level in the absence and in the presence of L-NNA.

**MATERIALS AND METHODS**

**Experimental animal model**

All experimental procedures were performed in accordance with the Guidelines and Authorization for the Use of Laboratory Animals (Italian Government, Ministry of Health) and according to the European Community Council Directive of 24 November 1986 - 86/609/EEC.

Ten-week-old male Sprague-Dawley rats weighing 220-250 g at arrival (Envigo, San Pietro al Natisone, Udine, Italy) were used. The animal protocol was designed to minimize pain or discomfort to the animals.

Rats were housed in an animal facility with monitored temperature and light (12-h cycle and 21 ± 2 °C). All cages were floored with sawdust, and bedding was replaced on a regular basis. The animals were allowed to acclimate to the environment for at least 7 d. Rats undergoing in vivo treatments were randomly chosen and allocated into individual cages before initiating the study, with the remaining rats caged together (4 rats/cage) in close proximity to allow experimental animals to see and smell their companions. Rats had free access to water and food when they were not under testing. All animals were handled and trained for at least 1 wk to minimize the possible stress of the drug administration procedure.

**Gastrointestinal motility test**

A repeated measures protocol was designed for in vivo study, so that each rat, at one-week intervals, received the following treatments either subcutaneously (sc) or intraperitoneally (ip): vehicle (1 mL/kg), granisetron (25, 50, 75 μg/kg/sc soon before testing), ZnPPIX (50 μg/kg/ip, 60 min before testing), hemin (50 μmol/L/kg/ip 24 h before testing), ZnPPIX (50 μg/kg/ip, 60 min before granisetron) with granisetron (25, 50, 75 μg/kg/sc), or hemin (50 μmol/L/kg/ip 24 h before granisetron) with granisetron (25, 50, 75 μg/kg/sc). The timing and dosing for ZnPPIX and hemin were carefully chosen to obtain the greatest level of HO inhibition or induction, respectively[9,22,23]. In a pilot study, we observed that the average time to first defecation in vehicle-treated rats was between 80-110 min (median 105 min; interquartile range 90-110; full range 80-180). Based on these preliminary findings, the observation cut-off time was set at 180 min. In the late afternoon preceding the test day, rats were fasted with free access to water. On the test day, animals were weighed and then allowed to free feed for 20 min. The amount of food eaten and the weight of the fed rats were calculated.

Following drug administration, each rat was monitored every 10 min for 180 min, and the time to first defecation was assumed as an index of whole-gut transit[24,25].

**Tensiometric studies**

After induction of general anesthesia (pentobarbital 80 mg/kg ip), rats were killed by cervical dislocation. A 3-cm section of proximal colon (1 cm from the ileocecal sphincter), obtained through a midline incision of the abdomen, was immediately placed in a cooled organ bath (20 mL) filled with modified Krebs’ solution (pH = 7.4) of the following composition (mmol/L): NaCl 113, KCl 4.8, MgSO₄ 1.2, CaCl₂ (H₂O) 2.2, NaH₂PO₄ 1.2, NaHCO₃ 25, glucose 5.5, and ascorbic acid 5.5. The specimen was then cleaned and rinsed, and a circular ring (0.5-cm length) was mounted in an organ bath (20 mL) filled with modified Krebs’ solution, maintained at 37 °C and gassed with a mixture of 95% O₂ and 5% CO₂. One end of the circular ring was connected to a metal rod, while the other end was attached to a strain gauge transducer (FORT 25, WPI, Sarasota, FL, United States). Isometric tension was measured by the PowerLab data acquisition system and recorded using Chart 5.5.5 (ADIn-
struments, Castle Hill, Australia). The colon ring was allowed to equilibrate for at least 30 min prior to the experiment. An initial load of 0.5 g tension was applied to the preparation.

The neurogenic contractile response was measured by applying a transmural stimulation (Electrical Field Stimulation, EFS) at frequencies of 3, 5, and 10 Hz (14 V, 1 ms pulse, trains lasting 10 s) through two parallel platinum electrodes connected to a stimulator (Digital Stimulator, LE 12106, Letica, Ugo Basile, Italy). The EFS results in an immediate relaxation, followed at the end of EFS by a so-called off-contraction. This contractile response is indicative of a nervous reflex that is abolished by tetrodotoxin and reduced by atropine and tachykinin antagonists. Activation of enteric nerves by EFS mimics the in vivo conditions in which neurotransmitters are released by motor neurons to the neuroeffector apparatus; the interaction between the interstitial cells of Cajal, neurons, glial cells and smooth muscle cells generates contraction.

The myogenic contractile response was explored by calculating the extent of contraction induced by acetylcholine (ACh, 0.1-100 µmol/L).

Both neurogenic and myogenic contractile responses were measured after incubation with the following agents alone or in combination: granisetron hydrochloride (3 µmol/L, 15 min), ZnPPIX (10 µmol/L, 60 min), L-NNA (100 µmol/L, 20 min), and CORM-3 (100, 200, 400 µmol/L). For the last compound, CORM-3, a water-soluble Ru-containing compound releasing one mole of CO per mole, the effect was evaluated within 10 min from administration to avoid its spontaneous breakdown.

The neurogenic contractile responses were expressed as a percentage of three consecutive contractile responses to EFS (10 Hz, 14 V, 1 ms pulse, trains lasting 10 s) recorded and averaged before drug administration.

The myogenic contractile responses to ACh (0.1-100 µmol/L) were expressed as a percentage of tension values elicited by the highest ACh concentration (100 µmol/L) before drug administration.

The activity of ZnPPIX and CORM-3 (indicative of a specific CO-dependent effect) on neurogenic contractile response was measured in the absence and in the presence of atropine (3 µmol/L).

Drugs and chemicals

The following drugs were used: atropine sulphate and granisetron hydrochloride dissolved in saline (Sigma Chemical Co., St. Louis, Missouri, United States). Zinc protoporphyrin IX and hemin were dissolved in 0.1 N NaOH and equilibrated to a pH of 7.4 with HCl (Sigma Chemical Co., St. Louis, Missouri, United States). Tri-carboxyl Chloro(glycinato)ruthenium (II) (CORM-3) and N’-nitro-L-Arginine (L-NNA) were dissolved in distilled water (Sigma Chemical Co., St. Louis, Missouri, United States). In in vivo studies, vehicle-treated rats received the same amount of vehicle as did drug-treated animals. In in vitro experiments, vehicle-treated preparations were exposed to the same amount of vehicle as drug-treated preparations.

Statistical analysis

For in vivo study, Friedman’s ANOVA for repeated measures followed by a post hoc test was performed. For in vitro study, two-way ANOVA for repeated measures (treatment effect, frequencies or concentrations effect and interaction effect, with frequency or concentrations as repeated measure) was performed. When the interaction effect was significant, a one-way ANOVA at each frequency or concentration was performed with pre-planned multiple comparison tests for each treatment vs vehicle.

The results are presented as individual observations (n = 8) for each in vivo treatment; results are expressed as the mean ± SD of 6-8 preparations for each in vitro treatment. Statistical analysis was performed by the biomedical statistician Dr. Margherita Fanelli (coauthor) using SPSS software (version 20.0). A P value < 0.05 was considered to indicate statistical significance.

RESULTS

In vivo study

Effect of granisetron, ZnPPIX and hemin on the time to first defecation: The average amount of food eaten before drug administration was 5 g. After 20 min of free access to food, the body weight increased by approximately 8 g in all animals.

Consistent with results obtained in our previous study, acute administration of granisetron increased the time to first defecation. Interestingly, the delay to first defecation was dose-dependent, with no significant effect measured for the lowest dose of granisetron used (25 µg/kg) and with a substantial increase in the time to first defecation observed in animals administered higher doses of granisetron; in this respect, both 50 and 75 µg/kg of granisetron were equally effective (Friedman’s test = 13, P = 0.005, post hoc: granisetron 25 µg/kg vs vehicle, P = 0.132; granisetron 50 µg/kg vs vehicle, P = 0.045; granisetron 75 µg/kg vs vehicle: P = 0.045) (Figure 1). A preliminary comparison of the amount of food eaten before vehicle or drug administration showed no statistically significant differences among treatments (Friedman’s test = 0.958, P = 0.811).

Although ZnPPIX (50 µg/kg) alone did not modify the time to first defecation, co-administration of ZnPPIX (50 µg/kg) with granisetron (25, 50, 75 µg/kg) was able to counteract the constipating effect of granisetron: Friedman’s test = 10.486, P = 0.033; post hoc comparisons: ZnPPIX vs vehicle: P = 1; granisetron 25 µg/kg with ZnPPIX vs vehicle: P = 1; granisetron 50 µg/kg with ZnPPIX vs vehicle: P = 1;
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treatment with granisetron (G) significantly increased the time to first defecation at doses of 50 and 75 µg/kg. Friedman’s test = 13 P = 0.005, post-hoc: G 25 µg/kg vs vehicle, P = 0.132; G 50 µg/kg vs vehicle, P = 0.045; G 75 µg/kg vs vehicle, P = 0.045. Each point represents an individual observation.

On the other hand, hemin (50 µmol/L/kg) did not affect the time to first defecation. Co-administration of hemin (50 µmol/L/kg) with granisetron (G) (50, 75 µg/kg) resulted in an increased time to first defecation. Friedman’s test = 20.364 P = 0.000; post-hoc comparisons: hemin vs vehicle: P = 1; G 25 µg/kg + hemin vs vehicle: P = 0.108; G 50 µg/kg + hemin vs vehicle: P = 0.028; G 75 µg/kg + hemin vs vehicle: P = 0.004. Each point represents an individual observation.

Effects of granisetron on EFS-induced and ACh-induced contractile response of colon preparations: Incubation of colon specimens with granisetron did not significantly modify the contractile response to EFS obtained in vehicle-treated samples (F_{treatments} = 1.26, df = 1/9, P = 0.29; F_{frequencies} = 22.50, df = 2/18, P = 0.001; F_{treatments x frequencies} = 1.79, df = 2/18, P = 0.21) (Figure 4A). Interestingly, a trend to increase the contractile effect induced by ACh (0.1-100 µmol/L) was measured in samples incubated with granisetron, although no statistical significance was measured with respect to vehicle-treated samples (F_{treatments} = 3.48, df = 1/9, P = 0.09; F_{concentrations} = 21.35, df = 3/27, P < 0.0001; F_{treatments x concentrations} = 0.08, df = 3/27, P = 0.85) (Figure 4B).

Effects of ZnPPIX on EFS-induced and ACh-induced contractile response of colon preparations: When compared to vehicle-treated preparations, a significant decrease in the contractile response to EFS was observed in specimens incubated with ZnPPIX (10 µmol/L, 60 min) (F_{treatments} = 8.78, df = 1/9, P = 0.016; F_{frequencies} = 50.33, df = 2/18, P < 0.0001; F_{treatments x frequencies} = 1.79, df = 2/18, P = 0.21) (Figure 5A). Interestingly, the ZnPPIX-mediated effect on EFS was abolished by concomitant incubation with atropine (3 µmol/L, 20 min) (F_{treatments} = 1.44, df = 1/11, P = 0.25; F_{frequencies} = 37.66, df = 2/22, P < 0.0001; F_{treatments x frequencies} = 2.74, df = 2/22, P = 0.09), therefore suggesting that ZnPPIX may exert its effects by inhibiting the EFS-mediated release of endogenous ACh (Figure 5B). However, ZnPPIX did not affect the contractile case, a preliminary comparison of the amount of food eaten before vehicle or drug administration showed no statistically significant differences among treatments (Friedman’s test = 2.205, P = 0.698).

In vitro studies

Effects of granisetron on EFS-induced and ACh-induced contractile response of colon preparations: Incubation of colon specimens with granisetron did not significantly modify the contractile response to EFS obtained in vehicle-treated samples (F_{treatments} = 1.26, df = 1/9, P = 0.29; F_{frequencies} = 22.50, df = 2/18, P = 0.001; F_{treatments x frequencies} = 1.79, df = 2/18, P = 0.21) (Figure 4A). Interestingly, a trend to increase the contractile effect induced by ACh (0.1-100 µmol/L) was measured in samples incubated with granisetron, although no statistical significance was measured with respect to vehicle-treated samples (F_{treatments} = 3.48, df = 1/9, P = 0.09; F_{concentrations} = 21.35, df = 3/27, P < 0.0001; F_{treatments x concentrations} = 0.08, df = 3/27, P = 0.85) (Figure 4B).
response to exogenous ACh (0.1-100 µmol/L) compared to vehicle (F\text{treatments} = 0.006, df = 1/9, P = 0.94; F\text{concentrations} = 36.89, df = 3/27, P < 0.0001; F\text{treatments x concentrations} = 0.84, df = 3/27, P = 0.45) (Figure 5C).

Effects of CORM-3 on EFS-induced and ACh-induced contractile response of colon preparations: Assessment of the EFS-induced contractile response after CORM-3 (100-400 µmol/L) administration shows that CORM-3 (400 µmol/L) significantly increased the EFS-induced contractile response compared to vehicle at 10 Hz (F\text{treatments} = 2.75, df = 3/20, P = 0.07; F\text{frequencies} = 55.38, df = 2/40, P < 0.0001; F\text{treatments x frequencies} = 4.36, df = 6/40, P = 0.02; at 10 Hz: CORM-3 (400 µmol/L) vs vehicle: *P = 0.01) (Figure 6A).

When repeated after 20-min incubation with atropine (3 µmol/L, 20 min), the increased EFS-induced contractile response by CORM-3 (400 µmol/L) administration was abolished: F\text{treatments} = 3.06, df = 3/20, P = 0.052; F\text{frequencies} = 50.05, df = 2/40, P < 0.0001; F\text{treatments x frequencies} = 1.14, df = 6/40, P = 0.36. Consistent with the results obtained with ZnPPIX, these observations suggest that CORM-3 may enhance the EFS-induced release of endogenous ACh (Figure 6B).

Analysis performed to determine the effect of CORM-3 administration (100-400 µmol/L) on the contractile response to exogenous ACh (0.1-100 µmol/L) showed that incubation with CORM-3 (400 µmol/L) increases the contractile response to the highest ACh concentration (100 µmol/L) compared to vehicle-treated samples (F\text{treatments} = 2.28, df = 3/22, P = 0.11; F\text{concentrations} = 36.22, df = 3/66, P < 0.0001; F\text{treatments x concentrations} = 3.49, df = 9/66, P = 0.02; for ACh 100 µmol/L: CORM-3 (400 µmol/L) vs vehicle: P = 0.012) (Figure 6C).

Effects of co-administration of granisetron with ZnPPIX or CORM-3 on EFS-induced and ACh-induced contractile response of colon preparations: When co-administered with granisetron (3 µmol/L, 15 min), incubation with ZnPPIX (10 µmol/L, 60 min) did not significantly modify the EFS-induced contraction compared to vehicle-treated samples (F\text{treatments} = 0.43, df = 1/8, P = 0.53; F\text{frequencies} = 55.35, df = 2/16, P < 0.0001; F\text{treatments x frequencies} = 1.66, df = 2/16, P = 0.22) (Figure 7A). Because incubation with ZnPPIX alone decreased the contractile response to EFS (Figure 5A), it is plausible to infer that co-administration of granisetron was responsible for the abolished effects of ZnPPIX on EFS-induced colon contraction.

Co-administration of ZnPPIX (10 µmol/L, 60 min) and granisetron (3 µmol/L, 15 min) did not modify the myogenic contractile response to ACh (F\text{treatments} = 0.22, df = 1/8, P = 0.65; F\text{concentrations} = 39.19, df = 3/24, P < 0.0001; F\text{treatments x concentrations} = 4.06, df = 3/24, P = 0.02) (Figure 7B).

When the effects of CORM-3 (100-400 µmol/L) on the EFS-induced contractile response were analyzed in combination with granisetron (3 µmol/L, 15 min), the results showed that coincubation of CORM-3 (400 µmol/L) and granisetron significantly increased the EFS-induced contractile response when compared to vehicle-treated samples at 5 and 10 Hz (F\text{treatments} = 5.47, df = 3/19, P < 0.01; F\text{frequencies} = 55.40, df = 2/38, P < 0.0001; F\text{treatments x frequencies} = 3.05, df = 6/38, P = 0.04; granisetron (3 µmol/L, 15 min) and CORM-3 (400 µmol/L) vs vehicle at 5 Hz: P = 0.007 and at 10 Hz: P = 0.001 (Figure 7C).

Interestingly, when compared to vehicle-treated samples, the concomitant incubation of CORM-3 (400 µmol/L) with granisetron significantly increased the myogenic response to ACh at 10 and 100 µmol/L (F\text{treatments} = 7.40, df = 3/19, P = 0.002; F\text{concentrations} = 61.69, df = 3/57, P < 0.0001; F\text{treatments x concentrations} = 3.55, df = 9/57, P = 0.027; at ACh 10 µmol/L: P = 0.001 and at ACh 100 µmol/L: P = 0.001) (Figure 7D).

Effects of co-administration of granisetron, ZnPPIX, L-NNA and granisetron, CORM-3, L-NNA on EFS-induced and ACh-induced contractile response of colon preparations: When co-adminis-
treatment of granisetron (3 \( \mu \text{mol/L}\), 15 min) and ZnPPIX (10 \( \mu \text{mol/L}\), 60 min) significantly reduced the electrical field stimulation (EFS)-induced contractile response compared to vehicle. ANOVA results: \( F_{\text{treatments}} = 4.89, df = 1/9, P = 0.016; F_{\text{frequencies}} = 24.89, df = 2/18, P < 0.0001; F_{\text{treatments} \times \text{frequencies}} = 0.84, df = 3/27, P = 0.45. Values are expressed as the mean ± SD of 6-8 experiments.

Figure 5 Effects of in vitro treatment with zinc protoporphyrin on rat colon contractile response to electrical field stimulation, without and with atropine, and to acetylcholine. A: Incubation with zinc protoporphyrin (ZnPPIX) (10 \( \mu \text{mol/L}\), 60 min) significantly reduced the electrical field stimulation (EFS)-induced contractile response compared to vehicle. ANOVA results: \( F_{\text{treatments}} = 2.75, df = 3/20, P = 0.07; F_{\text{frequencies}} = 5.38, df = 2/18, P < 0.0001; F_{\text{treatments} \times \text{frequencies}} = 4.36, df = 6/40, P = 0.002. At 10 Hz: C (400 \( \mu \text{mol/L}\)) vs vehicle: \( P = 0.01\); B: Co-incubation of ZnPPIX (10 \( \mu \text{mol/L}\), 60 min) with atropine (3 \( \mu \text{mol/L}\), 20 min) abolished the effect of ZnPPIX alone. ANOVA results: \( F_{\text{treatments}} = 1.44, df = 1/11, P = 0.25; F_{\text{frequencies}} = 1.79, df = 2/18, P = 0.21\); C: Incubation with ZnPPIX (10 \( \mu \text{mol/L}\), 60 min) had no effect on contractile response to atropine (At) (0.1-100 \( \mu \text{mol/L}\)) compared to vehicle. ANOVA results: \( F_{\text{treatments}} = 0.008, df = 1/9, P = 0.94; F_{\text{frequencies}} = 36.89, df = 3/27, P < 0.0001; F_{\text{treatments} \times \text{frequencies}} = 0.84, df = 3/27, P = 0.45. Values are expressed as the mean ± SD of 6-8 experiments.

Figure 6 Effects of in vitro treatment with CORM-3 on rat colon contractile response to electrical field stimulation, without and with atropine, and to acetylcholine. A: Incubation with CORM-3 (C) (400 \( \mu \text{mol/L}\)) significantly increased the electrical field stimulation (EFS)-induced contractile response compared to vehicle at 10 Hz. ANOVA results: \( F_{\text{treatments}} = 2.75, df = 3/20, P = 0.07; F_{\text{frequencies}} = 55.38, df = 2/18, P < 0.0001; F_{\text{treatments} \times \text{frequencies}} = 4.36, df = 6/40, P = 0.002. At 10 Hz: C (400 \( \mu \text{mol/L}\)) vs vehicle: \( P = 0.01\); B: Co-incubation of C (100-400 \( \mu \text{mol/L}\)) with atropine (At) (3 \( \mu \text{mol/L}\), 20 min) abolished the effect of C when administered alone. ANOVA results: \( F_{\text{treatments}} = 3.06, df = 3/20, P = 0.052; F_{\text{frequencies}} = 50.05, df = 2/18, P < 0.0001; F_{\text{treatments} \times \text{frequencies}} = 1.14, df = 6/40, P = 0.36; C: Incubation with C (400 \( \mu \text{mol/L}\)) increased the contractile response to acetylcholine (ACh) (100 \( \mu \text{mol/L}\)) compared to vehicle. ANOVA results: \( F_{\text{treatments}} = 2.28, df = 3/22, P = 0.11; F_{\text{frequencies}} = 86.22, df = 3/66, P < 0.0001; F_{\text{treatments} \times \text{frequencies}} = 3.48, df = 9/66, P = 0.02. For ACh 100 \( \mu \text{mol/L}\)-C (400 \( \mu \text{mol/L}\)) vs vehicle: \( P = 0.012\). Values are expressed as the mean ± SD of 6-8 experiments.
Figure 7 Effects of in vitro treatment with granisetron and zinc protoporphyrin and with granisetron and CORM-3 on rat colon contractile response to electrical field stimulation (EFS) and to acetylcholine (ACh). A: Co-incubation with granisetron (G) (3 μmol/L, 15 min) and zinc protoporphyrin (ZnPPIX) (10 μmol/L, 60 min) did not significantly modify the EFS-induced contraction compared to vehicle. ANOVA results: F_{treatments} = 0.43, df = 1/8, P = 0.53; F_{concentrations} = 55.35, df = 2/16, P < 0.0001; F_{treatments x concentrations} = 1.66, df = 2/16, P = 2; B: Co-incubation with G (3 μmol/L, 15 min) and ZnPPIX (10 μmol/L, 60 min) did not modify the myogenic contractile response to acetylcholine (ACh) (0.1-100 μmol/L) compared to vehicle. ANOVA results: F_{treatments} = 0.22, df = 1/8, P = 0.65; F_{concentrations} = 39.19, df = 3/24, P < 0.0001; F_{treatments x concentrations} = 4.06, df = 3/24, P = 0.02; C: Co-incubation with G (3 μmol/L, 15 min) and CORM-3 (C) (400 μmol/L) increased the contractile response to EFS at 5 and 10 Hz compared to vehicle. ANOVA results: F_{treatments} = 5.47, df = 3/19, P < 0.01; F_{concentrations} = 55.40, df = 2/38, P < 0.0001; F_{treatments x concentrations} = 3.05, df = 6/38, P = 0.04. G (3 μmol/L, 15 min) and C (400 μmol/L) vs vehicle at 5 Hz: ^P = 0.007 and at 10 Hz: ^P = 0.001; D: Co-incubation of G (3 μmol/L, 15 min) and C (400 μmol/L) increased the contractile response at ACh 10 and 100 μmol/L compared to vehicle. ANOVA results: F_{treatments} = 7.40, df = 3/19, P = 0.002; F_{concentrations} = 61.69, df = 3/37, P < 0.0001; F_{treatments x concentrations} = 5.35, df = 9/51, P = 0.027. G (3 μmol/L, 15 min) and C (400 μmol/L) compared to vehicle at ACh 10 μmol/L: ^P = 0.001 and at ACh 100 μmol/L: ^P = 0.001. Values are expressed as the mean ± SD of 6-8 experiments.

**DISCUSSION**

This study was planned to clarify the mechanisms underlying the potential contribution of the HO/CO pathway in the constipating effects of granisetron in rats. In a previous report, we found that inhibition of HO or increased expression of HO-1 in rat duodenum was able to influence the granisetron effects on the EFS-dependent response[9]. These findings provided a first evidence that the HO/CO pathway may play a...
role in the constipating activity of granisetron. However, because constipation is more closely related to abnormalities of colon motility, rather than in the duodenum,[16-19], we planned to focus directly on the colon contractile responses. Moreover, in our previous study, the role of the HO/CO pathway on rat duodenum was evaluated under NANC conditions[9] to avoid the overwhelming effects of the main neurotransmitters at the gastrointestinal level, namely ACh and noradrenaline (NA). However, neurogenic gastrointestinal motility is strictly dependent on ACh and NA-mediated effects, and the functional relevance of NANC neurotransmission in vivo is still largely unknown[100]. Thus, in this work, the assessment of colon neurogenic response to granisetron was investigated under conditions directly resembling the existing intestinal environment.

Consistent with literature data reporting constipation in patients treated with granisetron as an anti-emetic therapy,[11,15], we observed an increased time to first defecation, a recognized indicator of whole-gut transit,[24,35], after acute administration of granisetron in rats. Granisetron-induced constipation was abolished by in vivo co-administration with ZnPPIX (HO inhibitor), whereas co-administration of hemin (HO-1 inducer) did not decrease the delayed time to first defecation observed in granisetron-treated rats. These data support an active role of the HO/CO system in the constipating effect of granisetron[9]. Interestingly, neither ZnPPIX nor hemin was able to affect rat gastrointestinal motility when administered alone in vivo. This is not surprising because the HO/CO pathway is likely to be a fine-tuning mechanism whose activity may enhance or limit the extension of major signals involved in the integrated control of colon motility.

Consistent with this view, and with studies reporting a substantial effect of 5-HT3 antagonists only in the presence of high levels of 5-HT, either exogenously administered or endogenously released from enterochromaffin cells (for example, by mucosal pressure, distortion and/or chemical stimuli)[31-33], granisetron administration in vitro did not significantly inhibit the contractile response to EFS and showed a borderline trend to increase the contraction mediated by ACh (P = 0.09). Interestingly, colon contractile responses to
EFS were decreased in vitro by incubation with ZnPPIX alone. Because ZnPPIX inhibits the HO-mediated production of CO, it is plausible to infer that the EFS-dependent contraction is mediated, at least in part, by CO. This hypothesis is consistent with studies reporting an almost completely abolished inhibitory response to EFS in jejunal smooth muscle strips of mice with targeted genomic deletion of HO-2. Concomitantly, in these animals, an exogenous administration of CO restores the EFS response.

CO appears to have a facilitatory effect on EFS-mediated ACh release, as suggested by the impaired ACh release observed in frog neuromuscular junctions under ZnPPIX incubation. Analogous behavior was observed in our study in which the impaired contractile response to EFS obtained under ZnPPIX was restored by concomitant incubation with the muscarinic antagonist atropine. This finding, together with the lack of any effect of ZnPPIX on the myogenic contractile response to exogenous ACh, implies that a phasic CO production is required for physiological ACh release in rat colon.

The potential role of CO on granisetron effects, investigated in vivo by co-administration of hemin, was mimicked in vitro by co-administration of CORM-3, a CO-releasing molecule able to replicate the effects of HO-1 stimulation with hemin. At the highest dose used (400 µmol/L) CORM-3 significantly increases the contractile response to both EFS (10 Hz) and exogenous ACh (100 µmol/L). These findings suggest that one mechanism by which CO may enhance the contractile response in rat colon is by facilitating the release of endogenous ACh. In addition, CO may indirectly potentiate the ACh contractile effects, as proposed by Lim et al., by concurrently activating L-type calcium channels in human intestinal smooth muscle via a nitric oxide (NO)-dependent mechanism. The binding of NO to guanylyl cyclase with subsequent changes in cAMP and intracellular Ca²⁺ levels will eventually lead to activation of the "contractile apparatus".

When granisetron and CORM-3 were co-administered, the colon's contractile responses to both EFS and ACh were further increased, suggesting a synergistic effect between these two substances. Similarly, when granisetron and ZnPPIX were co-administered, the effects of ZnPPIX alone were lost. Although the exact mechanism of granisetron and HO/CO system interplay remains to be clearly established, some explanations may be proposed: one is that, as suggested by the bell-shaped curve for in vivo response, granisetron may behave as a partial agonist at the concentrations used for the present in vitro and in vivo studies. In this case, the activation of 5-HT₃ receptors followed by subsequent increased release of ACh may have overcome the inhibition of ACh release secondary to ZnPPIX. Concomitantly, acting as a partial 5-HT₃ agonist, granisetron may synergistically potentiate CORM-3 effects by increasing calcium influx.

Because the activation of L-type Ca²⁺ channels operated by CO is a NO-dependent mechanism, inhibition of NO production is expected to decrease the CORM-3-mediated effects. Indeed, in the presence of NO synthase inhibitor L-NNA, the potentiating effect of CORM-3-mediated effects. Indeed, in the presence of NO synthase inhibitor L-NNA, the potentiating effect of CORM-3 on granisetron activity was lost, confirming the necessary role of NO for the observed activities.

Because of the nature of the study, the following limitations must be considered. First, we cannot conclusively exclude that the colon response to granisetron/ZnPPIX treatment might be related to changes in the serotonergic system; nevertheless, the results obtained strongly suggest that the constipating effect of granisetron is only indirectly affected by ZnPPIX, which acts through reduction of EFS-induced acetylcholine release. Second, it is not clear whether the alleviation of granisetron-induced constipation might affect the antiemetic potential of this drug; studies directly evaluating this parameter would require a specific animal model and a completely different experimental approach, both of which are unavailable at this time. However, our per-
ception is that alleviation of granisetron-induced constipation does not interfere with its antiemetic activity because this last effect relates to granisetron’s ability to reach the CNS. In this regard, it has been reported that ZnPPIX does not cross the blood-brain barrier \[1,41\]. Thus, it is plausible that the effects of ZnPPIX to reduce granisetron-induced constipation are related to peripheral mechanisms not involving the CTZ. Third, gastrointestinal transit (GIT) was measured by observing the time to first defecation after food ingestion; although intragastric administration of a non-absorbable, colored marker is considered the reference method to measure GIT, additional gavage administration would increase stress in animals and potentially affect the parameter evaluated. In our study, we considered the delayed GIT in rats treated with granisetron (compared to rats treated with vehicle) as a positive control to evaluate the effects of ZnPPIX and CORM-3 on the “time to first defecation” after food ingestion.

In conclusion, findings from the present study may shed light on the involvement of the HO/CO pathway in the neurogenic and myogenic contractile responses in rat colon and propose potential mechanisms underlying the interaction of granisetron and CO on colon motility (Figure 10).

Considering that granisetron is mainly used to prevent chemotherapy-induced nausea and vomiting in cancer patients and that increased expression of HO-1 has been observed in several cancer types \[42\], our findings suggest that HO inhibitors may be a reasonable therapeutic approach to reduce the unwanted constipating effects of granisetron.

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COMMENTS

Background

In recent decades, the role played by carbon monoxide (CO) in several biochemical processes has been increasingly recognized. Once considered only for its lethal effects, the therapeutic use of CO has been proposed after the discovery of its potential "positive" functions. Ion channels have been shown to be, among others, the target of CO; thus, it is possible that CO may modulate the effects of other signals by acting directly on the same target or indirectly on the shared pool of secondary messengers. A similar modulating activity of CO might also be plausible toward specific drugs.

Research frontiers

In a previous report, authors observed the involvement of the heme oxygenase (HO)/CO pathway in granisetron-mediated effects on duodenal motility.
Innovations and breakthroughs
Findings from the present study may shed light on the involvement of the HO/CO pathway in the neurogenic and myogenic contractile responses in rat colon and propose potential mechanisms underlying the interaction of granisetron and CO on colon motility.

Applications
Considering that granisetron is mainly used to prevent chemotherapy-induced nausea and vomiting in cancer patients and that increased expression of HO-1 has been observed in several cancer types, the authors findings suggest that HO inhibitors may be a reasonable therapeutic approach to reduce the unwanted constipating effects of granisetron.

Terminology
Electrical field stimulation allows measurement of the neurogenic contractile response. In rat colon preparations, the electrical field stimulation (EFS) induces an immediate relaxation of specimens followed, at the end of EFS, by a contraction called off-contraction. This contractile response is indicative of a nervous reflex. Moreover, activation of enteric nerves by electrical field stimulation mimics the in vivo conditions because neurotransmitters are released by motor neurons to the neuroeffector apparatus in which interstitial cells of Cajal, neurons, glial cells and smooth muscle cells interact and induce contraction.

Peer-review
The authors present interesting data about HO/CO pathway and granisetron. The authors report detailed data and proposed potential mechanisms underlying the interaction of granisetron and CO. Overall, it is an important study, and should be considered for publication.

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