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Basic Study

Calcium/calcimimetic *via* calcium-sensing receptor ameliorates cholera toxin-induced secretory diarrhea in mice

Tang LQ *et al*. Calcium/calcimimetic ameliorates secretory diarrhea

Abstract

BACKGROUND

Enterotoxins produce diarrhea through direct epithelial action and indirectly *via* activating the enteric nervous system. Calcium-sensing receptor (CaSR) inhibits both actions. The latter has been well documented *in vitro*, but not *in vivo*. The hypothesis to be tested was that activating CaSR inhibits diarrhea *in vivo*.

AIM

To determine if agonists of CaSR ameliorate secretory diarrhea evoked by cholera toxin (CTX) in mice.

METHODS

CTX was given orally to C57BL/6 mice to induce diarrhea. Calcium and calcimimetic R568 were used to activate CaSR. To maximize their local intestinal actions, calcium was administered luminally *via* oral rehydration solution (ORS) whereas R568 applied serosally using an intraperitoneal route. To verify that their actions resulted from the intestine, effects were also examined on *cre-lox* intestine-specific CaSR knockouts. Diarrhea outcome was measured either biochemically through monitoring changes in fecal Cl^- or clinically by assessing stool consistency and weight losses.

RESULTS

CTX induced secretory diarrhea, as evidenced by increases in fecal Cl^-, stool consistency, and weight losses following CTX exposure, but did not alter CaSR, neither in content nor in function. Accordingly, calcium and R568 were each able to ameliorate diarrhea when applied to diseased intestines. The intestinal CaSR involvement is suggested by gene knockout experiments where antidiarrheal actions of R568 in wild-type mice were not observed in knockouts, in neither $^{villin}Cre/Casr^{flox/flox}$ lacking epithelial CaSR, nor $^{nestin}Cre/Casr^{flox/flox}$ lacking neuronal CaSR.

CONCLUSION

Treatment of acute secretory diarrheas remains a global challenge. Despite advances in diarrhea research, few advancements have been made in the realm of diarrhea therapeutics, and ORS therapy has remained the standard of care although it does not halt the losses of intestinal fluid and ions caused by pathogens. There is no cost-effective therapeutic for diarrhea. This and other studies suggest that adding calcium to ORS or using calcimimetic to activate intestinal CaSR might represent a novel approach for treating secretory diarrheal diseases.

Key Words: Cholera; Enteric nervous system; Secretory diarrhea; Oral rehydration solution; Calcium-sensing receptor; Gene knockout

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Core Tip: Treatment of diarrhea remains a global challenge. Enterotoxins produce diarrhea through direct epithelial action and indirectly *via* activating the enteric nervous system. Using *in vitro* models in isolated tissues, we have previously shown that calcium-sensing receptor (CaSR) inhibits both actions. In the present study, using a mouse model of secretory diarrhea in conjunction with a tissue-specific knockout approach, we show that calcium or calcimimetic *via* CaSR ameliorates cholera toxin-induced secretory diarrhea *in vivo*. This study suggests that adding calcium to oral rehydration solution or using calcimimetic to activate intestinal CaSR might represent a new approach for treating secretory diarrheal diseases.

INTRODUCTION

Acute infectious diarrhea remains among the top causes of morbidity and deaths in children throughout the world[1,2]. According to United Nations Children's

Fund/World Health Organization[3], approximately 9 million children (about half the population of New York) under 5 years died in 2008. 40% of these deaths were due to two diseases: Pneumonia and diarrhea. Diarrhea remains the second leading cause of death in children younger than 5 years globally accounting for one in every five child deaths - around 1·5 million a year - more than acquired immune deficiency syndrome, malaria, and measles combined. Importantly, most of the morbidity and mortality is not due to infection but dehydration. Accordingly, reducing the fluid loss from acute diarrhea offers a major opportunity for improving child health globally.

Enterotoxins produce diarrhea through direct epithelial action and indirectly *via* activating the enteric nervous system (ENS)[4]. For example, cholera induces diarrhea through the generation of cholera toxin (CTX) from *V. cholera*. CTX binds to the enterocyte, leading to ADP-ribosylation of the G_s α-subunit. This constitutively activates membrane-bound adenylyl cyclase of enterocytes and elevates cyclic adenosine monophosphate (cAMP) in the cell. Elevation of cAMP stimulates protein kinase A and phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) channel as well as the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), causing secretion of Cl^- and water. Elevated levels of cAMP also inhibit Cl^- and water absorption mediated by the Cl^-/HCO_3^- exchange and Na^+/H^+ exchange[4,5]. In addition to direct epithelial action, CTX elicits neuronal secretory reflexes through binding of the toxin to enterochromaffin cells of the mucosa, leading to the production of 5-hydroxytryptamine, activation of ENS and release of neurotransmitters (*e.g.*, vasoactive intestinal peptide and acetylcholine) that stimulate and amplify fluid secretion, leading to dehydration and rapid body weight losses[4,6,7].

Importantly, the extracellular calcium-sensing receptor (CaSR)[8] is present on cells of both pathways[5,9,10] and, when activated *in vitro*, blocks both diarrhea-causing pathways evoked by CTX and other diarrhea-causing enterotoxins/secretagogues. For example, using a microperfused colonic crypt technique, it has been shown that calcium, calcimimetics or polyamines that activate the CaSR can act on intestinal epithelium and reverse CTX/forskolin-induced fluid secretion using a signal

transduction pathway that promotes cyclic nucleotide destruction[11-13]. Using the Ussing chamber technique, it has been shown that the effects of CTX/forskolin and CaSR agonists on electrolyte secretion by the intestine can also be attributed to opposing actions of enterotoxins/secretagogues and CaSR on the activity of the ENS[14,15]. These results suggest that targeting intestinal CaSR might represent a previously undescribed novel approach for treating secretory diarrheal diseases[5,9,10,16-18]. However, so far, all the experiments that suggest CaSR modulates dual-pathway secretion by the intestine have been performed *in vitro* in isolated tissues. Neither the functionality of the CaSR receptors *in vivo* nor the anti-diarrheal potential of CaSR agonists in live animals have been documented, although the latter is necessary before clinical trials in humans are performed.

In this study, we tested the hypothesis that calcium/calcimimetic *via* CaSR ameliorates secretory diarrhea *in viv*o in mice. A CTX mouse model of secretory diarrhea was employed and effects of CaSR agonists on biochemical (*i.e.*, changes in fecal Cl^-) and clinical outcomes (*i.e.*, changes in stool consistency and body weight losses) of secretory diarrheal disease were assessed. We selected the CTX mouse model because we had employed it as a model for our previous *in vitro* studies. Also, it has been widely used to provide proof of concept of whether an antidiarrheal agent is therapeutic or not[19-22]. In addition to testing calcium, we also examined effects of the calcimimetic R568, a pharmacological allosteric CaSR agonist[23]. To maximize their local intestinal actions, we delivered agonists in such a way that calcium was administered orally by adding it to oral rehydration solution (ORS) whereas R568 was applied serosally using an intraperitoneal route, as previously described[15]. To verify that their actions resulted from intestinal tissues and not a non-specific off-target action, effects were also measured on intestine-specific CaSR knockouts. We show for the first time that targeting intestinal CaSR with calcium or calcimimetic is efficacious in reducing CTX-evoked secretory diarrhea *in vivo* in live animals and that this occurs through receptor-mediated reduction of both the neurally and non-neuronally mediated secretory responses. A portion of this work was presented in an abstract in the Global

Health Forum of 5th World Congress of Pediatric Gastroenterology, Hepatology and Nutrition[24].

MATERIALS AND METHODS

Animals

Experiments were performed using male/female C57BL/6 mice (wild-type and *Casr* mutants). Mice lacking CaSR expression in intestinal epithelial cells ($^{villin}Cre/Casr^{flox/flox}$ mice) and mice lacking CaSR expression in intestinal neurons ($^{nestin}Cre/Casr^{flox/flox}$ mice) and their wild-type littermates were bred and maintained in-house at the University of Florida Communicore Animal Facility. Mutant mice were generated as previously described[25,26]. Briefly, CaSR flox/flox mice were bred with transgenic mice expressing *Cre* recombinase under the control of the villin 1 or nestin promoter and genotyped prior to all experiments after an approximate 20-30 generations. Mice were used at 5-10 wk of age and weight of 17-23 g in accordance with the Animal Welfare Act and the Public Health Policy on Humane Care. Animals were fed and maintained on regular chow (Harlan) with free access to water before the experiment. To maximally protect the welfare of the animal, we used numbers of animals in each experiment group as minimal and small as scientifically or statistically allowed. Thus, depending on variation of the data obtained and/or availability of the animals tested, number of 5-11 animals were employed, although in some dose-dependence studies number of 2-3 were also used. This was so because these were the minimal numbers that were required for statistical significance using one-way ANOVA and $P < 0.05$ as determined in a pilot experiment. To minimize the effects of subjective bias in allocating animals, we treated controls, interventions, wild type, and mutants in the same manner in the same days by the same investigators. The animal protocols were designed to minimize pain or discomfort to the animals. After completion of the experiment, animals were sacrificed with standard CO_2 inhalation and by cervical dislocation. The use of animals as well as the protocols for CTX treatment and colon tissue isolation was approved by

the Institutional Animal Care and Use Committee (IACUC# 201807567) at University of Florida.

CTX mouse model of secretory diarrhea

Two protocols were used to induce diarrhea: Protocol 1 is long and was used to compare effects of with *vs* without oral calcium, a poorly absorbed mineral agonist of CaSR[27-29]; whereas protocol 2 is short and was used to compare effects of with *vs* without R568, a quickly absorbable small-molecule agonist of the receptor[30].

Protocol 1: Animals were first fasted overnight for 16 h before they were gavaged, intragastrically, with 200 μL 7% $NaHCO_3$ buffer containing 20 μg CTX or vehicle per mouse to induce diarrhea. After CTX gavage, animals were fasted for additional 90 min before they were allowed access to regular chow to avoid food interference on toxin binding and action. Afterwards, animals were divided into two groups: Group 1 received drinking bottles containing ORS only whereas group 2 received drinking bottles containing ORS supplemented with 5 mmol/L calcium. This calcium concentration was selected because it is the lowest concentration of calcium that generated maximal CaSR-activation effects[11,12]. Diarrhea was monitored and was either semi-quantitated, clinically, according to stool consistency [0, normal feces (solid); 1, moist feces (semi-solid); 2, mild diarrhea (loose); and 3, severe diarrhea (watery)[31]] or quantitated, biochemically, according to fecal Cl^- content. Degree of dehydration was measured by diarrhea-associated body weight losses[32]. In this study, the onset of diarrhea is defined as the appearance of the first diarrheic stool with stool consistency scored one or higher, as described[31].

Protocol 2: Animals were pretreated and treated as in protocol 1 except for the following: (1) The calcimimetic R568 (diluted in 100 μL normal saline) was administered serosally at time when diarrhea was induced. R568 was administered serosally using an *i.p.* rather than *o.s.* route in an attempt to minimize the unwanted systemic effects while

maximizing the desired local intestinal action as described[15]. Neither anesthesia nor analgesia were used; (2) 1.5 h post CTX treatment animals were allowed to drink ORS without calcium; and (3) Animals were sacrificed 3.5 h post CTX treatment before watery stool was seen. Pilot studies showed that diarrhea started to occur about 0.5 h post CTX gavage and reached a peak plateau about 0.5 h later[33] but no appearance of diarrheic stool was seen until 3.5 h post CTX treatment. Three and half-hours later, animals were killed, fluid accumulated in the intestine was removed and weighed, changes compared, and is expressed as mg/mg intestine.

Fecal Cl^- measurement

Feces were collected in pre-weighed Eppendorf tubes. To avoid variations from freeze-thaw cycle and bacterial overgrowth from storage, all fecal samples were collected freshly, processed gently, and measured promptly as described[34]. In brief, following collection, samples were weighed and diluted appropriately in deionized water so that the content of Cl^- in each sample fell within the linear range of the standard curve. Diluted samples were homogenized gently but thoroughly through pipetting before subjected to centrifugation at 14000 g for 10 min. During this process, sonication was not used to minimize release of intracellular contents. The resulting supernatants were collected, and Cl^- contents measured with an ion-selective electrode by potentiometric titration (Model LIS-146CLCM-XS system, JENCO Electronics, Ltd). The results were calculated according to the standard curve, and are expressed as mole/L, where 1 Liter of feces ≈ 1 kg of feces. Previous studies have shown that these methods caused no or minimal variations in fecal (Cl^-)[34-36].

CaSR western blot

Isolation and preparation of intestinal homogenates and lysates and western blot analysis of CaSR protein were performed as described[12] with an affinity-purified mouse monoclonal antibody (5C10, ADD) raised against a 22-amino acid peptide corresponding to amino acid residues 214-235 of human CaSR (Abcam, Cambridge,

MA). The CaSR protein signals were normalized by heat shock protein 90 (HSP90) as a loading control and are expressed as CaSR/HSP90 protein signal ratios[37].

Chemicals, antibodies, and solutions

CTX was obtained from Sigma, and 5 mg/mL stock solutions were prepared in water, R568 from Tocris Bioscience (Ellisville, MI), and 20 mg/mL stock solutions were prepared in 15% 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals International, Natick, MA), calcium chloride from Sigma, and rabbit polyclonal antibody for HSP90 from Santa Cruz (Dallas, TX). ORS was prepared freshly containing (in mmol/L) 75 Na^+, 20 K^+, 65 Cl^-, 10 citrate and 75 glucose with total osmolarity of 257 mOsm/kg H_2O.

Statistical analysis

The statistical methods of this study were reviewed by Dr. Han-Zhi Gao, PhD, member of the Biostatistics Service from the Clinical and Translational Science Institute of the University of Florida. Data from all animals were included in the analysis. Values are given as means ± standard error of mean. The normality of variables was checked; the data for intestinal fluid accumulations exhibited a skewed distribution and were therefore Log transformed. After log transformation, the data became normally distributed. Statistical comparisons between two means were performed by Student's *t*-test, whereas comparisons among multiple means were by one-way ANOVA with Tukey's *post hoc* tests. Both tests were performed either using Microsoft Excel 2016 for Windows or using GraphPad Prism version 6.07 for Windows (GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant.

RESULTS

Effects of calcimimetic

Our first set of experiments were performed with the calcimimetic R568 used in conjunction with intestinal CaSR-specific knockouts to demonstrate the functionality of the intestinal CaSR *in vivo* and to prove that the intestine but no other tissues was

targeted by the agent. In these experiments, the diarrhea-provoking CTX or vehicle was given intragastrically (*i.g.*) whereas the anti-diarrheal R568 or vehicle was administered intraperitoneally (*i.p.)* to avoid interference between the two agents. To be quantitated accurately, diarrhea was induced in such a way that all the secreted fluid was contained inside the lumen of the intestine without loss to the outside of the body. Before they were sacrificed, animals from all 4 groups did not exhibit any noticeable adverse effects. Data from all animals were included in the analysis.

CTX induces secretory diarrhea but does not alter the expression of CaSR in the intestine

In non-stimulated vehicle-gavaged wild type mice, the fluid accumulated in the intestine was 0.20 ± 0.02 mg/mg intestine. Intragastric gavage of CTX caused diarrhea, as evidenced by increased intestinal fluid accumulation in a dose-dependent fashion (Figure 1). At the EC_{50} of 0.5 mg/kg approximately, the amount of fluid accumulated in the intestine was 1.46 ± 0.15 mg/mg intestine, which is about 7 times more fluid accumulated in intestines of non-CTX controls (Table 1).

Many conditions like carcinogenesis reduce CaSR in the intestine[38]. To assess if this occurred to CTX-treated intestines, we studied the CaSR protein expression by western blots (Figure 1). Although CTX caused diarrhea, the toxin did not seem to alter intestinal CaSR expression. The CaSR/HSP90 protein signal ratios were control 0.50 ± 0.02 (5) *vs.* CTX 0.53 ± 0.03 (5), $P > 0.05$.

R568 reverses CTX-induced diarrhea

Having known that CaSR protein expression was unaltered, we then studied the CaSR function by assessing the ability of the calcimimetic R568 to reverse CTX-induced diarrhea *in vivo* (Figure 2). For this, the EC_{50} dose 0.5 mg/kg of CTX was intragastrically gavaged to induce moderate severity of diarrhea while different doses of R568 were administered intraperitoneally. Three and half hours later, animals were sacrificed, intestines removed, fluid accumulated within the intestines measured (Figure 2A). CTX

induced diarrhea (Figure 2B). When R568 was applied to the CTX-induced diseased intestine, it ameliorated diarrhea and reduced the fluid evoked by CTX (Figure 2B). The degree of the fluid reduction was dependent on the amount of the drug applied. Fluid accumulation was reduced by 50% approximately when the near maximally effective doses of 30-50 mg/kg of R568 were applied (Figure 2C) (ANOVA test; $P < 0.05$). In non-CTX vehicle gavaged mice, R568 generated no or only minimally yet statistically insignificant inhibitory effects (Figure 2C) (ANOVA test; $P > 0.05$). These results indicate that not only the content but also the function of CaSR remain unaltered in diseased intestines, consistent with the *in vitro* findings[13,15].

R568 reverses CTX-induced diarrhea via intestinal CaSR

To show that the intestinal CaSR was indeed targeted and was not simply due to a non-specific off-target action, additional studies on intestinal tissue-specific CaSR knockout mice (*i.e.*, $^{villin}Cre/Casr^{flox/flox}$ and $^{nestin}Cre/Casr^{flox/flox}$) were performed and effects compared (Table 1, Figures 2D and E). Under non CTX vehicle-stimulated basal conditions, the fluid accumulated in the intestine was larger in $^{villin}Cre/Casr^{flox/flox}$ mice (0.59 ± 0.13 mg/mg intestine, $P < 0.05$ *vs* wild type mice) (Table 1), and was unchanged in $^{nestin}Cre/Casr^{flox/flox}$ mice (0.17 ± 0.02 mg/mg intestine, $P > 0.05$ *vs* wild type mice) (Table 1). Addition of CTX (0.5 mg/kg) caused diarrhea, as evidenced by significantly increased fluid accumulation in intestines of these mice (Table 1), although the diarrhea was less severe in knockouts than in wild type (Table 1) due to activation of compensatory mechanisms. Importantly, administration of R568, at all tested concentrations, caused no inhibition on CTX-induced diarrhea, in neither $^{villin}Cre/Casr^{flox/flox}$ mice (ANOVA test; $P > 0.05$) (Figure 2D) nor $^{nestin}Cre/Casr^{flox/flox}$ mice (ANOVA test; $P > 0.05$) (Figure 2E), confirming that the anti-diarrheic action was indeed exerted largely, if not exclusively, *via* CaSR in the intestinal tissues. R568 alone had no effect, in neither $^{villin}Cre/Casr^{flox/flox}$ mice (ANOVA test; $P > 0.05$) (Figure 2D) nor $^{nestin}Cre/Casr^{flox/flox}$ mice (ANOVA test; $P > 0.05$) (Figure 2E) despite the presence of

diarrhea in $^{villin}Cre/Casr^{flox/flox}$ mice, further confirming that the CaSR is required for the calcimimetic to exert its anti-diarrheic action.

Effects of calcium

After performing the proof-of-concept studies using the calcimimetic R568 and verifying the functionality of intestinal CaSR, we tested whether targeting CaSR with calcium, an inexpensive widely available child-friendly mineral, was antidiarrheal *in vivo*. We tested this by adding calcium to ORS and investigating whether it helped reduce the severity of diarrhea and enhance the rate of rehydration by ORS. In these experiments, animals were first intragastrically gavaged with CTX or vehicle. Ninety minutes later, they were allowed to drink ORS with calcium or ORS alone, and development and progression of diarrhea were then monitored, both biochemically through changes in fecal Cl^- content and clinically by assessing changes in stool consistency and diarrhea-associated body weight losses (Figure 3A).

Adding calcium to ORS reduces CTX-induced Cl^- losses from the intestine

In secretory diarrhea, active Cl^- secretion and inhibited Cl^- absorption is the primary driving force for water moving from the blood to the lumen of the intestine[39]. Thus, to assess whether calcium supplemented ORS (ORS + Ca) is better than ORS alone in reducing intestinal Cl^- loss from diarrhea, we first measured and compared changes in fecal Cl^- concentration (Cl^-). Figure 3B shows the changes in fecal (Cl^-) at day 1, day 4, and day 6 post CTX gavage. Day 1 represents acute stage of diarrhea, day 4 recovery stage, and day 6 post-recovery. Consistent with enterotoxin-induced intestinal Cl^- loss, mice in both groups displayed a significantly higher mean fecal (Cl^-) upon CTX exposure ($P < 0.01$, day 1 post CTX *vs* day 1 Control). However, compared to the high fecal (Cl^-) in CTX:ORS-treated mice, a lower fecal (Cl^-) was noted in mice receiving CTX:((ORS + Ca) treatment (ANOVA test $P < 0.05$; Student's *t* test *P* values at day 1, 4 and 6 = 0.56, 0.07 and 0.08 *vs* respective non-Ca controls). Moreover, relative to mice on CTX:ORS treatment, mice on CTX:(ORS + Ca) recovered from diarrhea-associated Cl^-

losses significantly faster. Thus, while the CTX:ORS group fecal Cl^- losses had remained significantly elevated above baseline until day 6 post CTX gavage, in CTX:(ORS + Ca) group, a close to normal fecal (Cl^-) had been observed at day 4 post CTX treatment (Figure 3B). Calcium had no or only minimal effect on non-CTX vehicle-treated mice (one-way ANOVA test; $P > 0.05$). These results suggest that ORS + Ca is better than ORS alone in reducing diarrhea-associated intestinal Cl^- losses.

Adding calcium to ORS reduces severity and duration of diarrhea caused by CTX

Reducing intestinal Cl^- loss suggests the possibility of reducing diarrhea and dehydration. Thus, we compared the onset, severity and recovery of diarrhea/dehydration induced by CTX in ORS + Ca *vs* ORS groups. First comparison was made regarding the onset of diarrhea (*i.e.*, the time from CTX gavage to the appearance of the first diarrheic stool). Since it would take some time for calcium to produce a clinically visible antidiarrheal action, calcium may or may not influence the onset of diarrhea. Before CTX gavage, all mice displayed normal solid stool. Following CTX gavage, mice receiving ORS had the first diarrheic stool at 4.5 ± 1.7 h, whereas mice receiving ORS + Ca developed diarrhea at 4.3 ± 1.8 h. No statistically significant difference was noted ($P = 0.69$).

We then compared the changes in stool consistency over time. Similarly, adding calcium reduced stool consistency score in CTX-treated (ANOVA test; $P < 0.05$) but not in non-CTX control (ANOVA test; $P > 0.05$). The result is summarized in Figure 3C. Specifically, in ORS group, CTX significantly increased stool consistency scores in day 1, day 2 and day 3 but not in day 4 compared to non-CTX control, whereas in ORS + Ca group, CTX significantly increased stool consistency scores only in day 1 and day 2 but not in day 3 and day 4. Thus, while the ORS group stool consistency score had remained significantly elevated above baseline until day 4 post CTX gavage, in ORS + Ca group, a normal stool consistency score had been observed 1 d earlier at day 3 post CTX treatment. These results suggest that ORS + Ca is better than ORS alone in reducing diarrhea.

Considering that the scoring of stool consistency used is only semi-quantitative and has large performance-dependent variations, we compared the body weight changes before and after disease induction, a quantitative way of measuring the severity of diarrhea and degree of dehydration[32]. We chose to monitor body weight instead of monitoring 24-h stool volume because we had technical difficulties in accurately collecting stool and quantifying 24-h stool volume. Figure 3D shows body weight changes over time in mice in ORS + Ca group *vs* ORS only group along with their non-CTX controls. In response to CTX challenge, mice in both groups lost significant weight, particularly in day 1 and day 2. However, mice receiving ORS + Ca lost significantly less weight and recovered significantly sooner than mice receiving ORS only (ANOVA test; $P < 0.01$). Thus, while mice in ORS group continued to lose weight to a statistically significant degree until after day 4 post CTX, mice in ORS + Ca group had achieved a close to normal body weight at day 3 post CTX treatment (Figure 3D). The estimated time at which mice returned to their initial weight was 3.5 d in Ca-ORS group and 4.5 d in ORS only group, which is 22% faster in ORS + Ca group. These results suggest that adding calcium to ORS reduces the severity of dehydration, hastens its recovery, and accelerates the rate of rehydration by ORS.

DISCUSSION

This first *in vivo* study proves that targeting intestinal CaSR with calcium or calcimimetic is efficacious in reducing CTX-evoked secretory diarrhea and that this occurs through receptor-mediated reduction of both the neurally (*i.e.,* nestin-expressing enteric neuron) and non-neuronally (*i.e.,* villin-expressing epithelial cell) mediated Cl- secretory responses. A schematic diagram illustrating how CTX induces and calcium/calcimimetic inhibits these two Cl- secretory responses is depicted in Figure 4.

We showed that CTX induced secretory diarrhea in mice as previously reported[19-22]. This was evidenced by increased fecal Cl^- and water content/stool consistency and weight losses following CTX induction. Importantly, although it altered intestinal fluid balance and caused diarrhea, CTX did not seem to alter CaSR content or function.

Accordingly, when applied to diseased intestines, calcium and calcimimetic were each able to ameliorate diarrhea. Intestinal CaSR involvement is further supported by gene knockout experiments in which the antidiarrheal activity of CaSR agonists observed in wild-type mice was not noted in knockouts. Neither the $^{villin}Cre/Casr^{flox/flox}$ mice that lack epithelial CaSR nor the $^{nestin}Cre/Casr^{flox/flox}$ mice that lack neuronal CaSR experienced amelioration of diarrhea with CaSR agonists.

Interestingly, while both CaSR knockouts responded to CTX stimulation, their responses were less prominent than their wild types. The reason is unknown and is related to the down regulation of NKCC1 and CFTR in these animals[32]. NKCC1 and CFTR are two ion transporters required for the intestine to generate an effective diarrheal response to secretagogues[40,41].

Additionally, differences in the phenotype of two intestinal CaSR knockouts under basal conditions are noted. While $^{villin}Cre/Casr^{flox/flox}$ mice developed spontaneous diarrhea, as evidenced by mild but significant increased fluid accumulation in unstimulated intestines of these mice, $^{nestin}Cre/Casr^{flox/flox}$ mice did not, as evidenced by no significant increased fluid accumulation in unstimulated intestines of these mice (Table 1). The reason is unknown but may be related to the fasting condition used and differences in roles and functions these epithelial and neuronal CaSR receptors may play in the control of intestinal functions. The primary function of the gastrointestinal (GI) tract is to digest food and absorb nutrients. To aid digestion, the GI tract secretes a large amount of fluid to mix the food components and to lubricate the surface of the lumen. It is estimated that following the ingestion of a meal, intestinal secretion can be increased eightfold[42]. Upon completion of digestion and extraction of nutrients, intestinal secretion stops. While studies suggest that mechanical sensors in the ENS have a significant role in triggering the meal-evoked secretion[6], there is evidence that chemical sensors (*e.g.*, CaSR) on epithelium and enteric neurons have a key role in terminating this process. The latter do so through their ability to sense nutrients released as a result of digestion[25] (also, a recent review by Tang *et al*[10]). Consistent with active regulation of intestinal secretion by the CaSR, under the no-food-no-nutrient

fasting condition used in the present study, the neuronal CaSR would have been silent and, as a result, no intestinal phenotype would be expected to have occurred in $^{nestin}Cre/Casr^{flox/flox}$ mice (Table 1).

The finding of spontaneous diarrhea in $^{villin}Cre/Casr^{flox/flox}$ mice is notable (Table 1). This indicates that unlike the neuronal CaSR, the epithelial CaSR does remain active, at least to some degree, under a no-food-no-nutrient fasting condition. This is not surprising given the multiple roles and functions the CaSR plays in GI biology[43]. In addition to its established function as a nutrient sensor regulating fluid secretion and absorption during food digestion, the CaSR on the epithelium is also a fundamental mechanism for sensing and regulating the ionic and nutrient compositions of extracellular milieu surrounding the epithelium of the entire GI tract[10]. Thus, at basal no-food no-nutrient fasting state, this epithelial CaSR may perform other tasks, for example, monitoring the Ca^{2+} surrounding the epithelium.

According to the present model of intestinal Ca^{2+} transport[44], the Ca^{2+} ion cycles between the leaking crypt, which secretes Ca^{2+} *via* a passive paracellular pathway, and the electrically tight villous/surface epithelium, which absorbs back Ca^{2+} *via* an active transcellular transport mechanism. Interestingly, under basal conditions fluid also cycles in a similar fashion between the crypt, which secretes, and the villous/surface epithelium, which absorbs[45]. The purpose of this fluid cycling is to lubricate the surface of the lumen, to prevent the crypt lumen from obstructing, and to defend the crypt from invasion by lumen bacteria. It is likely that the CaSR located on the apical and basolateral membranes of enterocytes may be sensing Ca^{2+} constantly, thereby monitoring and controlling these processes.

CONCLUSION

The most notable observation of the present study is that calcium and calcimimetic both significantly ameliorated CTX-induced secretory diarrhea. The latter has important therapeutic value. Treatment of acute secretory diarrheas remains a global challenge. Despite advances in diarrhea research, few advancements have been made in the realm

of diarrhea therapeutics, and ORS therapy has remained the standard of care even though it does not stop the losses of intestinal fluid and ions caused by pathogens. There is no cost-effective therapeutic for diarrhea. This study suggests that adding calcium to ORS or using calcimimetic to activate intestinal CaSR might represent a novel approach for treating secretory diarrheal diseases. Limitations of this study include: (1) The present study was an animal but not human study; (2) No data on oral calcimimetic was obtained; and (3) Neither local nor systemic adverse effects were documented, despite the fact that some animals in study protocol 1 appeared to be sick, particularly at day 2 following the exposure of the disease-causing CTX. Better designed animal studies and randomized clinical trials in humans are warranted.

ARTICLE HIGHLIGHTS

Research background

Treatment of diarrhea such as cholera remains a global challenge. Cholera toxin (CTX) produces diarrhea through direct epithelial action and indirectly *via* activating the enteric nervous system. Calcium-sensing receptor (CaSR) is present in both tissues and, when activated, inhibits both actions. The latter has been well documented *in vitro*, but not *in vivo*. Thus, the present study was testing whether activating intestinal epithelial or neuronal CaSR inhibits diarrhea *in vivo*.

Research motivation

Acute infectious diarrhea remains among the top causes of morbidity and deaths in the world. Most of the morbidity and mortality is not due to infection but dehydration. Accordingly, how to effectively reduce the fluid loss from acute diarrhea offers a major opportunity for improving global health.

Research objectives

The objective of the present study was to determine if agonists of CaSR ameliorate secretory diarrhea evoked by CTX in wild type mice and the two knockout mice, that is

the $^{villin}Cre/Casr^{flox/flox}$ mice that lack epithelial CaSR and the $^{nestin}Cre/Casr^{flox/flox}$ mice that lack neuronal CaSR.

Research methods

To realize the objectives, CTX was administered orally to C57BL/6 mice to induce secretory diarrhea while calcium and calcimimetic R568 were employed to activate CaSR. To maximize their local intestinal actions, calcium was administered luminally *via* oral rehydration solution (ORS) whereas R568 applied serosally using an intraperitoneal route. To verify that their actions resulted from the intestinal epithelium and enteric neurons, effects were also examined on the two cre-lox intestine-specific CaSR knockouts. Diarrhea outcome was measured either biochemically through monitoring changes in fecal Cl- or clinically by assessing stool consistency and weight losses.

Research results

CTX induced secretory diarrhea, as evidenced by increases in fecal Cl-, stool consistency, and weight losses following CTX exposure. Calcium and R568 were each able to ameliorate the CTX-induced secretory diarrhea in wild-type mice, but not in knockouts, in neither $^{villin}Cre/Casr^{flox/flox}$ lacking epithelial CaSR, nor $^{nestin}Cre/Casr^{flox/flox}$ lacking neuronal CaSR.

Research conclusions

The new theory that the present study proposes is that activating intestinal epithelial or neuronal CaSR is able to inhibit secretory diarrhea *in vivo*. The new methods that this study proposed is that adding calcium to ORS or using calcimimetic to activate the intestinal CaSR might represent a novel approach for treating secretory diarrheal diseases in humans.

Research perspectives

Future research should be directed to conduct randomized clinical trials utilizing calcium or calcimimetics to treat cholera and other secretory diarrheal diseases in humans.

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