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γ-Glutamyl cysteine and γ-glutamyl valine inhibit TNF-α signaling in intestinal epithelial cells and reduce inflammation in a mouse model of colitis *via* allosteric activation of the calcium-sensing receptor

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ABSTRACT

Background: The extracellular calcium-sensing receptor (CaSR) is distributed throughout the gastrointestinal tract, and its activation has been shown to promote intestinal homeostasis, suggesting that CaSR may be a promising target for novel therapies to prevent chronic intestinal inflammation such as inflammatory bowel disease (IBD). The γ-glutamyl dipeptides γ-glutamyl cysteine (γ-EC) and γ-glutamyl valine (γ-EV) are dietary flavor enhancing compounds, and have been shown to activate CaSR *via* allosteric ligand binding. The aim of this study was to examine the anti-inflammatory effects of γ-EC and γ-EV *in vitro* in intestinal epithelial cells and in a mouse model of intestinal inflammation.

Results: In vitro, treatment of Caco-2 cells with γ-EC and γ-EV resulted in the CaSR-mediated reduction of TNF-α-stimulated pro-inflammatory cytokines and chemokines including IL-8, IL-6, and IL-1β, and inhibited phosphorylation of JNK and IκBα, while increasing expression of IL-10. *In vivo*, using a mouse model of dextran sodium sulfate (DSS)-induced colitis, γ-EC and γ-EV treatment ameliorated DSS-induced clinical signs, weight loss, colon shortening and histological damage. Moreover, γ-EC and γ-EV reduced the expression of TNF-α, IL-6, INF-γ, IL-1β, and IL-17, and increased the expression of IL-10 in the colon, in a CaSR-dependent manner. The CaSR-mediated anti-inflammatory effects of γ-EC were abrogated in β-arrestin2 knock-down Caco-2 cells, and involvement of β-arrestin2 was found to inhibit TNF-α-dependent signaling *via* cross-talk with the TNF-α receptor (TNFR).

Conclusions: Thus CaSR activation by γ-EC and γ-EV can aid in maintaining intestinal homeostasis and reducing inflammation in chronic inflammatory conditions such as IBD.

1. Introduction

The calcium-sensing receptor (CaSR), a member of the G-protein-coupled receptor (GPCR) family, detects extracellular calcium ions and regulates the release of intracellular calcium, a secondary signaling molecule involved in intracellular signal transduction. CaSR is widely distributed in diverse cell types in various tissues including the parathyroid, brain, kidney, lung and bone marrow, where it regulates cellular activities including secretion, apoptosis, proliferation, differentiation and ion-channel activity [1,2]. CaSR is also present in the gastrointestinal tract, and is expressed on the apical and basolateral membranes of villous and crypt epithelial cells of the small intestine and colon, respectively, where it is involved in the regulation of various processes such as intestinal absorption, secretion and motility [3,4]. Studies have shown that CaSR plays a key role in maintaining and restoring intestinal homeostasis [5,6], and calcium-induced activation of CaSR was found to promote differentiation of colonic myofibroblasts and stimulate regeneration of the intestinal barrier [6], suggesting that CaSR may be a promising target for treating intestinal inflammation.

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic relapsing inflammation of the gastrointestinal tract [7]. Increased expression of inflammatory mediators during IBD results in the recruitment and activation of immune cells, including neutrophils and lymphocytes, perpetuating the inflammatory response. TNF-α plays a predominant role in IBD pathogenesis [8,9], and a number of anti-TNF-α therapies have been shown to successfully reduce pathology and morbidity in IBD patients [10].

GPCR agonists have been shown to exert anti-inflammatory effects and interfere in TNF-α-stimulated signaling pathways [11,12]. The use of exogenous ligands to modulate CaSR signaling is of great therapeutic

Abbreviations: CaSR, calcium-sensing receptor; GPCR, G-protein-coupled receptor; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; γ-EC, γ-glutamyl cysteine; γ-EV, γ-glutamyl valine; GRK, GPCR kinase; TRAF, TNF-receptor-associated factor; TNFR, TNF-α receptor; IEC, intestinal epithelial cell; MCP-1, monocyte chemoattractant protein-1; TAK1, TGF-β-activated kinase 1; TAB, TAK1 binding-protein; TLR, Toll-like receptor

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interest [13], and polyvalent CaSR agonists neomycin sulfate and spermine were recently found to reduce LPS-stimulated TNF-α secretion in mouse macrophage cells [14]. L-amino acids and γ-glutamyl peptides have been identified as CaSR agonists [15–17], however their role in modulating intestinal inflammation has not been addressed. The γ-glutamyl dipeptides γ-glutamyl cysteine (γ-EC) and γ-glutamyl valine (γ-EV) are dietary flavor enhancing compounds that have been isolated from various sources, including edible beans [18] and yeast extracts [19]. γ-Glutamyl dipeptides are also involved in glutathione (GSH) metabolism, which plays an important role in antioxidant defense, and γ-EC in particular is a crucial intermediate in GSH synthesis [20]. Both γ-EC and γ-EV have been shown to activate CaSR *via* allosteric ligand binding [17], and may therefore be useful for reducing inflammation and restoring intestinal homeostasis in IBD.

Upon ligand activation of GPCRs, signal transduction is regulated by β-arrestins, adaptor proteins that bind receptors phosphorylated by G protein-coupled receptor kinases (GRKs) [21]. β-arrestins couple GPCRs to various downstream signaling components including mitogen-activated protein kinases (MAPK) such as JNK, ERK 1/2, and p38 MAPK, as well as IκBα and TNF-receptor-associated factor (TRAF) 6 [22,23]. β-Arrestins can also mediate crosstalk between GPCR-induced signaling and other receptors, such as the TNF-α receptor (TNFR), and lead to the inhibition of inflammatory signaling pathways [11].

In the present study we demonstrated that γ-EC- and γ-EV-mediated activation of CaSR reduces inflammatory mediator expression *in vitro* in intestinal epithelial cells (IECs), by inhibiting JNK and IκBα phosphorylation, and improves clinical and histological parameters and reduces pro-inflammatory cytokine and chemokine responses in a mouse model of experimental colitis. Furthermore, we have shown that CaSR activation by γ-EC can prevent TNF-α-induced pro-inflammatory signaling *via* cross-talk with TNFR in a β-arrestin-dependent manner.

2. Materials and methods

2.1. Cell culture and treatment

Human colorectal adenocarcinoma-derived intestinal epithelial cells (Caco-2) (ATCC, Manassas, VA) were grown in DMEM/F12 (Gibco/Life Technologies, Grand Island, NY) supplemented with 1 mM sodium pyruvate (Gibco/Life Technologies), 20% FBS (HyClone, Logan, UT) and 50 U/mL penicillin–streptomycin (Gibco/Life Technologies). For treatment with CaSR agonists/antagonists, cells between passages 15–45 were seeded at a density of 1×10^5 cells/well in 24- or 48-well plates (Corning, Lowell, MA) and grown for 5–7 days. The CaSR agonists γ-EC and γ-EV, and the CaSR antagonist NPS-2143, were kindly provided by Ajinomoto Co., Ltd. (Kawasaki, Japan). Confluent cell monolayers were rinsed with Hank's buffered salt solution and treated with γ-EC or γ-EV in culture medium containing 5% FBS for 2 h. For dose determination experiments cells were treated with 0.01, 0.1, or 1 mM γ-EC or γ-EV; for all other experiments a concentration of 0.5 mM γ-EC or γ-EV was used. Cells were stimulated with recombinant human TNF-α (2 ng/mL) (Invitrogen/Life Technologies) to induce inflammation. To block CaSR activation, cells were pre-treated with NPS-2143 (1 μM) for 45 min before addition of γ-EC or γ-EV.

2.2. Animals

6–8-week-old female BALB/c mice (16–20 g) (Charles River Laboratories Inc., Montreal, Quebec) were group housed on a 12-h light–dark cycle and allowed unrestricted access to standard mouse chow and water. All animal studies were approved by the University of Guelph Animal Care Committee and carried out in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

2.3. Treatment of mice with CaSR agonists/antagonists and induction of DSS-induced colitis

Mice were administered γ-EC or γ-EV (50 or 150 mg/kg body weight in 100 μL of water) or vehicle, by oral gavage, starting on day 1 and continuing until day 14. On day 7, colitis was induced by the addition of 5% dextran sodium sulfate (DSS) (MW 36–50 kDa, MP Biomedicals, Solon, OH) to drinking water and continued until day 14. Negative control mice received water only, and positive control mice received DSS only. For NPS-2143 treatment, mice were injected i.v. with 1 mg/kg NPS-2143, dissolved in 20% 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO), as a 30 μL bolus into the tail vein. One hour after NPS-2143 injection mice were administered γ-EC or γ-EV (150 mg/kg) by oral gavage. Mice were euthanized on day 14. Colons were removed and measured, and tissue sections were flash frozen or stored in RNAlater® (Ambion/Life Technologies) for further analysis. A section of distal colon was fixed in 10% buffered formalin for histological analysis.

2.4. Clinical analysis of colitis

Mice were weighed daily, and data are expressed as mean percentage change relative to starting body weight. Mice were monitored daily for stool consistency, presence of blood in stool or bleeding and general appearance, and a clinical activity score (ranging from 0 to 7) was calculated as described by Maxwell et al. [24].

2.5. Histological analysis of colitis

Paraffin-embedded sections of distal colon were stained with hematoxylin and eosin (H&E) (Animal Health Laboratory, University of Guelph, Guelph, Ontario), and histological scoring to assess colonic tissue injury and inflammation was performed as described by Maxwell et al. [24].

2.6. Cytokine ELISAs

Measurement of IL-8 in Caco-2 culture supernatants was carried out as previously described [25]. Measurement of TNF-α and IL-6 concentrations in mouse colon tissues, tissues were homogenized in three volumes of ice-cold PBS containing 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin A (Sigma-Aldrich) using a Polytron® homogenizer (PT 1200, Kinematica Inc., Bohemia, NY) and centrifuged at 12,000 ×*g* for 10 min at 4 °C. Protein concentration was measured by DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). TNF-α and IL-6 ELISAs were carried out using anti-mouse IL-6 (MP5-20F3) or anti-mouse/rat TNF-α (TN3-19.12) and biotinylated anti-mouse IL-6 (32C11) or biotinylated anti-mouse TNF-α (C1150-14) (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

2.7. RNA isolation and real-time RT-PCR

Total RNA was extracted from cells using the Aurum™ Total RNA Mini Kit (Bio-Rad) according to the manufacturer's instructions. Mouse colon tissues were homogenized in TRIZOL® Reagent (Invitrogen/Life Technologies) according to the manufacturer's instructions, and total RNA was extracted from the aqueous phase using the Aurum Total RNA Mini Kit. RNA (1 μg) was reverse transcribed using a qScript™ cDNA Synthesis Kit (Quanta Biosciences, Inc., Gaithersburg, MD) and real-time quantitative PCR was carried out as previously described [26] using the primers listed in Supplemental Table S1. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [27] using GAPDH as the reference gene. Results are presented as fold expression change relative to negative control.