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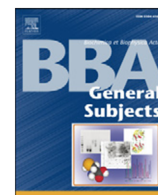

The PepT1-transportable soy tripeptide VPY reduces intestinal inflammation

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ABSTRACT

Background: Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal tract. The peptide transporter PepT1 is responsible for the intestinal uptake of dietary peptides, and its expression in the gastrointestinal tract is up-regulated during intestinal inflammation, indicating that PepT1 may be a promising target for IBD therapeutics.
Methods: The transport of soy-derived di- and tripeptides across Caco-2 intestinal epithelial cells was examined, and the anti-inflammatory effects of the transported peptide VPY were evaluated *in vitro* in Caco-2 and THP-1 macrophages, and *in vivo* in a mouse model of DSS-induced colitis.
Results: VPY inhibited the secretion of IL-8 and TNF-α, respectively, from Caco-2 and THP-1 cells. VPY transport and anti-inflammatory activity in Caco-2 cells was reduced in the presence of Gly-Sar, indicating this activity was mediated by PepT1. In mice, VPY treatment reduced DSS-induced colitis symptoms and weight loss, improved colon histology, reduced MPO activity, and decreased gene expression of the pro-inflammatory cytokines TNF-α, IL-6, IL-1β, IFN-γ and IL-17 in the colon.
Conclusions and general significance: VPY is a novel PepT1 substrate that can inhibit the production of pro-inflammatory mediators *in vitro* in intestinal epithelial and immune cells, and reduce the severity of colitis in mice by down-regulating the expression of pro-inflammatory cytokines in the colon, suggesting that VPY may be promising for the treatment of IBD.
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1. Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), is a chronic and relapsing inflammation of the gastrointestinal tract, believed to be caused by complex interactions between genetic, immunologic, microbial and environmental factors [1]. The breakdown of immune tolerance towards commensal enteric bacteria leads to ongoing activation of the intestinal immune system, including the enhanced recruitment of activated immune cells into the intestine where they release pro-inflammatory cytokines, causing tissue damage and perpetuating the inflammatory response [1,2]. Conventional treatment of IBD typically involves 5-aminosalisylates, corticosteroids or non-specific immunosuppressive drugs [2]. A number of biologic therapies targeting specific inflammatory cytokines or pathways have also been developed; however, these have been complicated by adverse side effects and limited efficacy, suggesting the need for further treatment options [3,4]. Consequently, there is growing interest in alternative or complementary therapies, such as dietary intervention, for the treatment of IBD [2].

The H^+-coupled peptide transporter PepT1, located on the apical membrane of enterocytes, is responsible for the absorption of dietary peptides in the intestine [5,6]. PepT1 has a broad substrate specificity including di- and tripeptides, as well as peptidomimetic drugs [7] and β-lactam antibiotics [8–10], but not free amino acids or peptides with more than 3 amino acid residues [6,10]. Under normal physiological conditions, PepT1 is expressed apically by epithelial cells of the small intestine. However, during inflammation, such as in IBD, the expression of PepT1 in the gastrointestinal tract is altered and it becomes up-regulated in the colon [11]. As such, it has been suggested that PepT1 may be a promising therapeutic target for IBD [12] and the tripeptide Lys-Pro-Val (KPV), from the C-terminal end of the α-melanocyte-stimulating hormone, was recently found to have PepT1-mediated anti-inflammatory activity *in vitro* and *in vivo* in mouse models of colitis [13].

The consumption of soy has been linked to numerous health benefits [14]. While many of these properties have been attributed to isoflavones and saponins, the enzymatic hydrolysis of soybean proteins has been shown to generate small peptides with biological activities such as

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anti-hypertensive [15,16], immunostimulatory [17], anti-oxidative [15], and anti-obesity properties [18]. We recently demonstrated that a soy-derived hydrolysate, enriched in di- and tripeptides, exerted anti-inflammatory activity *in vivo* in a porcine model of dextran sodium sulfate (DSS)-induced inflammation [19]. The orally administered soy peptides prevented DSS-induced changes in gut permeability and histology, and reduced myeloperoxidase (MPO) activity and the expression of inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-6, interferon (IFN)-γ, IL-1β, and IL-17A in the colon.

Soy-derived peptides have been shown to be readily absorbed *in vivo* [20], and may be a source of transportable bioactive peptides. In the present study, we examined the transepithelial transport of soy-derived di- and tripeptides across intestinal epithelial cells (IECs), and identified a transported tripeptide, VPY. VPY exerted anti-inflammatory activity *in vitro* in THP-1 macrophage cells as well as in IECs, which was mediated by the transporter PepT1. Moreover, VPY reduced colitis symptoms in mice, suggesting this peptide may be a novel therapeutic agent for the treatment intestinal inflammation.

2. Materials and methods

2.1. Peptides

Soy hydrolysate, prepared as previously described [18], was obtained from Fuji Oil Co. (Osaka, Japan), and contained 71% di- and tripeptides. Synthetic peptides VPY and QSP were purchased from GL Biochem Ltd. (Shanghai, China).

2.2. Cells and cell culture

Caco-2 cells (ATCC, Manassas, VA) were grown in minimal essential medium (MEM; Gibco/Life Technologies, Grand Island, NY) supplemented with 1 mM sodium pyruvate (Gibco), 1 mM non-essential amino acids (Gibco), 100 U/mL penicillin–streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 20% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT). HT-29 cells (ATCC) were grown in McCoy's 5A modified medium (Gibco) supplemented with 10 mM HEPES (Gibco), 100 U/mL penicillin–streptomycin, and 10% (v/v) FBS. THP-1 monocytes (RIKEN, Saitama, Japan) were grown in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/mL penicillin–streptomycin, 50 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 10% (v/v) FBS.

For transport experiments, Caco-2 cells were grown on BioCoat™ cell culture inserts coated with type I collagen (0.9 cm^2, 1.0 mm pore size; BD Biosciences, Bedford, MA). The cells were seeded at a density of 0.3×10^5 cells/well, and the culture medium was changed every second day until confluent monolayers with transepithelial electrical resistance (TEER) values greater than 500 $\Omega\cdot cm^2$ were obtained. TEER values were measured using a Millicell-ERS Volt-Ohm Meter (Millipore, Bedford, MA).

For *in vitro* inflammation experiments, Caco-2 and HT-29 cells (2.5×10^5 cells/well) were grown in 48-well tissue culture plates (Corning, Lowell, MA) until confluent monolayers were obtained. THP-1 cells (0.2×10^6 cells/well) were seeded into 48-well tissue culture plates (Corning) and cultured in the presence of 100 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 48 h as previously described [21], before use in *in vitro* inflammation experiments.

2.3. Peptide transport

Transport of peptides across Caco-2 monolayers was examined using an Ussing chamber (Model U-2500; Warner Instrument Corporation, Hamden, CT). Cell monolayers grown in transwell inserts were excised, rinsed with Hank's balanced salt solution (HBSS) and mounted into the transport chamber. HBSS, pH 6.4, containing 10 mM 2-(N-morpholino)ethanesulfonic acid (MES; Thermo Fisher Scientific, Waltham, MA) was added to the apical compartment and the basolateral compartment was filled with an equal volume of HBSS, pH 7.4, containing 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; Thermo Fisher). Soy hydrolysate (10 mg/mL) was added to the apical compartment and transport was allowed to proceed for 1 h. The temperature was maintained at 37 °C throughout the experiment and solutions on both sides of the membrane were bubbled continuously with a mixture of O_2:CO_2 (95:5). After 1 h, the solution from the basolateral compartment was applied to a Sep-Pak Plus C_{18} Cartridge (Waters, Milford, MA). The transported peptides were eluted with 35% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA; Sigma-Aldrich) and evaporated to dryness for HPLC analysis.

To measure transport of VPY, 1 mM of peptide was added to the apical compartment in the presence or absence of 10 mM glycyl-sarcosine (Gly-Sar) (Sigma-Aldrich). After 1 h of transport, the basolateral solution was prepared for HPLC analysis as described above. Uptake is expressed as $nmol/mL\cdot cm^2$ of VPY in the basolateral compartment.

2.4. HPLC and LC-MS/MS analysis of transported peptides

Peptides collected in the basolateral compartment were analyzed by reversed phase HPLC (Shimadzu LC10A; Shimadzu Co. Ltd., Kyoto, Japan) using a Cosmosil 5 C_{18}-AR-II column (∅4.6 mm × 250 mm; Nacalai Tesque, Kyoto, Japan). Dried samples were resuspended in 10% (v/v) acetonitrile containing 0.1% (v/v) TFA and applied to the column. Elution was carried out using a linear gradient of acetonitrile (10–70%, 70 min) containing 0.1% TFA at a flow rate of 1 mL/min. For peptide identification, the peaks were collected and evaporated before analysis by liquid chromatography–tandem mass spectrometry (LC-MS/MS). For LC-MS/MS, liquid chromatography was carried out using a XBridge™ BEH130 C_{18} (21 × 150 mm, 3.5 μm; Waters) column using 0.1% (v/v) formic acid as solvent A and 50% (v/v) methanol containing 0.1% formic acid as solvent B. A linear gradient from 0 to 100% B was run over 50 min at a flow rate of 0.2 mL/min. Mass spectrometric analysis was carried out using an Esquire6000 electrospray ionization (ESI)-ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in positive ionization mode.

2.5. Evaluation of anti-inflammatory activity in vitro

Caco-2 and HT-29 cell monolayers were rinsed with HBSS and incubated for 2 h with peptides at the indicated concentrations in Caco-2 culture medium containing 5% FBS, or HT-29 culture medium, as described in Section 2.2. To examine the role of PepT1 in anti-inflammatory activity, cells were treated with VPY (2 mM) along with Gly-Sar. After 2 h, TNF-α (2 ng/mL; Invitrogen/Life Technologies) was added and the cells were cultured for an additional 4 h. Culture supernatants were collected and IL-8 concentrations were measured by ELISA.

THP-1 cells, which had been treated for 48 h with PMA, were rinsed with HBSS and incubated for 2 h with VPY at the indicated concentrations. *Escherichia coli* O111:B4 lipopolysaccharide (LPS, 20 ng/mL; Sigma-Aldrich) was added and cells were cultured for an additional 6 h. Culture supernatants were collected and TNF-α concentrations were measured by ELISA.

2.6. WST-1 assay

Cell viability was measured using the WST-1 Cell Proliferation Reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.

2.7. Cytokine ELISAs

IL-8 concentrations were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtiter plates (Corning) were

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