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PepT1-Mediated Tripeptide KPV Uptake Reduces Intestinal Inflammation

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Abstract

Background & Aims

KPV is a tripeptide (Lys-Pro-Val) which possesses anti-inflammatory properties however its mechanisms of action still remain unknown. PepT1 is a di/tripeptide transporter normally expressed in the small intestine and induced in colon during inflammatory bowel disease (IBD). The aim of this study was to 1) investigate whether KPV anti-inflammatory effect is PepT1-mediated in intestinal epithelial and immune cells, and 2) examine KPV anti-inflammatory effect in two models of mice colitis.

Methods

Human intestinal epithelial cells (Caco2-BBE and HT29-Cl.19A), and human T cells (Jurkat) were stimulated with pro-inflammatory cytokines in the presence or absence of KPV. KPV anti-inflammatory effect was assessed using a NF-κB luciferase gene reporter, western blot, real-time RT-PCR and ELISA. Uptake experiments were performed using cold KPV as a competitor for hPepT1 radiolabelled substrate or using [^{3}H]KPV to determine kinetic characteristics of KPV uptake. Anti-inflammatory effect of KPV was also investigated in DSS- and TNBS-induced colitis in mice. KPV was added to drinking water and inflammation was assessed at the histological level and by pro-inflammatory cytokine mRNA expression.

Results

Nanomolar concentrations of KPV inhibit the activation of NF-κB and MAP kinase inflammatory signaling pathways, and reduce pro-inflammatory cytokine secretion. We found that KPV acts via hPepT1 expressed in immune and intestinal epithelial cells. Furthermore, oral administration of KPV reduces the incidence of DSS-, and TNBS-induced colitis indicated by a decrease in pro-inflammatory cytokine expression.

Conclusion

This study indicates that KPV is transported into cells by PepT1 and might be a new therapeutic agent for IBD.

INTRODUCTION

One of the normal transport functions of gut epithelial cells is the absorption of small peptides from the diet by peptide transport activity (1). This is mediated via the H^+-coupled oligopeptide transporter (PepT1) which is located at the apical membrane of intestinal epithelial cells (IEC) and which cotransports peptides and H^+ (2). The specificity of hPepT1 is broad and includes many di- and tripeptides in addition to various peptide-derived drugs (3–8). PepT1 is mainly expressed in brush-border membranes of enterocytes in the small intestine, in proximal tubular cells of the S1 segment of the kidney, and in bile-duct epithelial cells (4, 5, 9–15). By contrast, in the colon, expression of PepT1 mRNA and protein is low (16) and sometimes cannot be detected (10, 15, 17). Although human PepT1 is not expressed in normal colonic epithelial cells (10, 16, 17), we detected its expression at the apical

membrane of epithelial cells in chronically inflamed colon (17). Interestingly, we have also shown that immune cells, such as macrophages, which are in close contact with the lamina propria of the intestine, also express PepT1 at their membranes (17, 18).

Since expression of colonic hPepT1 is up-regulated in IBD, its transport activity constitutes a potential new target for anti-inflammatory therapies. Furthermore, the importance of hPepT1 expression by immune cells during intestinal inflammation should be evaluated as it may be therapeutically advantageous to develop PepT1-mediated anti-inflammatory drugs. The tripeptide KPV (Lys-Pro-Val), which is the C-terminal sequence of α-melanocyte stimulating hormone (α-MSH), has anti-inflammatory activity (19–21) and, although the underlying mechanisms remain to be determined, it is known that KPV inhibits NF-κB activation, indicating inhibition of pro-inflammatory cytokine synthesis. In the present study, we examine the tripeptide KPV as a mediator of anti-inflammatory effects via PepT1 expressed in inflamed colonic epithelial and immune cells as well as its anti-inflammatory properties *in vivo* using murine models of colitis.

MATERIALS AND METHODS

Cell culture

Caco2-BBE and HT29-Cl.19A cells were grown in DMEM supplemented with 14 mM $NaHCO_3$, 10% FBS, and penicillin/streptomycin (Invitrogen, Grand Island, NY). Jurkat cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% FBS.

Reagents

See Supplemental Information.

Animals

Female C57BL/6 mice (8 weeks, 18–22g, Jackson Laboratories, Bar Harbor, ME) used for this study were group-housed under a controlled temperature (25°C) and photoperiod (12:12-hour light-dark cycle), and allowed unrestricted access to standard diet and tap water. Mice were allowed to acclimate to these conditions for at least 7 days before inclusion in experiments.

Induction of colitis

Colitis was induced by the addition of 3% (w/v) dextran sodium sulfate (DSS; molecular weight 40,000 Da; ICN Biochemicals, Aurora, OH) to the drinking water or by colonic injection of 150 mg/kg body weight of trinitrobenzene sulfonic acid (TNBS; Sigma) dissolved in 50% ethanol. Colonic inflammation was assessed 8 days after DSS treatment or 48 hours after TNBS administration. N=10 mice/group.

Myeloperoxidase (MPO) activity in the colon

See Supplemental Information.

Dual-Luciferase reporter assay

See supplemental Information.

Western blot analysis

See Supplemental Information.

Uptake experiments

Caco2-BBE cells were grown on filters for 15 days (area = $1cm^2$; pore size 0.4µm; Transwell-Clear polyester membranes, Costar VWR, Suwanee, GA), washed and stabilized in $HBSS^+$-10 mM HEPES (pH 7.4) in the basolateral compartment and 10 mM MES (pH 6.2) in the apical compartment for 15 minutes at 37°C. The apical compartment was loaded for 15 minutes at room temperature with $HBSS^+$-10 mM MES (pH 6.2) containing 20 nM $[^3H]$KPV ± 20 mM Glycine-Leucine, or 20 µM $[^{14}C]$Glycine-Sarcosine ± 100 µM KPV, or 20 µM $[^{14}C]$Glycine-Sarcosine ± 100 µM Glycine-Leucine, or 20 nM $[^{14}C]$Glycine-Sarcosine ± 20 mM Glycine-Leucine. Cells were then washed in ice-cold PBS, and cell-associated radioactivity was determined by liquid scintillation counting in a β-counter.

For Jurkat cells, 5.10^6 cells were used per assay. Cells were washed twice with $HBSS^+$-10 mM MES (pH 6.2), stabilized for 15 minutes at 37°C, and incubated for 1 hour at room temperature in the same buffer containing different concentrations of $[^3H]$KPV ± 20 mM Glycine-Leucine. Afterwards, cells were washed in ice-cold PBS and total radioactivity was determined. Specific uptakes were calculated as follow: (uptake of radiolabel peptide)-(uptake of radiolabel peptide + Glycine-Leucine).

cAMP measurement

See Supplemental Information.

RNA extraction and real-time RT-PCR

Total RNA was extracted from cells or colon using the TRIzol reagent (Invitrogen) and reverse transcribed using the RETROscript® System (Ambion Inc, Austin, Tx). The real-time iCycler sequence detection system (Bio-Rad) was used for the real-time RT-PCR. Briefly, 10 ng of cDNA was amplified at 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute using 10 µM of gene-specific primers (Table 1, Supplementary data) and the iQ SYBR Green Suppermix (Bio-Rad). The GAPDH or 36B4 expression levels were used as housekeeping genes, and fold-induction was calculated using the Ct method as follow: $\Delta C_T = (Ct_{Target} - Ct_{housekeeping})$treatment $- (Ct_{Target} - Ct_{housekeeping})$nontreatment, and the final data were derived from $2^{-\Delta CT}$.

Detection of MCRs in cells

PCR of cDNA for each of the melanocortin receptors was conducted by a seminested approach with forward and reverse primers in the first PCR and inner forward (infw) and rev primers in the subsequent PCR as previously described (22). Primers are shown in Table 2, Supplementary data. PCR products were cloned in pGEM®-T Easy Vectors (Promega), amplified and sequenced.

Statistical analysis

All evaluations were performed using SigmaPlot (SPSS, Chicago, IL) and InStat v3.06 (GraphPad, San Diego, CA) softwares, with data reported as means ± SEM. Multiple groups were compared by ANOVA, using Tukey's post-hoc test. *P values < 0.05 were considered statistically significant.

RESULTS

KPV decreases inflammatory responses in Caco2-BBE cells stimulated by IL-1β

Many cell types, including IEC, express NF-κB, which is a transcriptional factor activated in response to immune and pro-inflammatory signals. NF-κB is known to be involved in the up-regulation of several immunomodulatory genes including interleukin-8 (IL-8) (23, 24). After transient transfection of IEC

Caco2-BBE with an NF-κB-dependent luciferase reporter plasmid, IL-1β treatment led to a ~6-fold increase in luciferase activity compared to untreated cells (Figure 1A). However, co-incubation of Caco2-BBE cells with KPV (10 nM) and IL-1β significantly decreased the IL-1β-induced luciferase activity (Figure 1A). To confirm that KPV decreases NF-κB activation, IκB-α degradation and phosphorylation which can account for NF-κB activation, were assessed by immunoblot analyses in Caco2-BBE cells treated with IL-1β ± KPV. We found high levels of IκB-α degradation 20 minutes after IL-1β stimulation while, in the presence of KPV, IκB-α degradation was reduced at this time (Figure 1B). IκB-α level then returned to the baseline level after 180 minutes of IL-1β stimulation while, in the presence of KPV, IκB-α baseline levels were reached within 90 minutes of stimulation (Figure 1B). Furthermore, IκB-α was still phosphorylated after 45 minutes of IL1-β stimulation but not in the presence of KPV (Figure 1C). Collectively, our results show that KPV delays NF-κB activation and also shortened the delay of IκB-α recovery, suggesting that KPV decreases the duration of NF-κB activation. KPV-mediated decrease of NF-κB activity was also confirmed by EMSA (Supplementary results, Figure 1).

Figure 1. KPV decreases inflammatory response in Caco2-BBE cells stimulated by IL-1.

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Caco2-BBE cells were untreated (control), or stimulated with 2 ng/ml IL-1β ± 10 nM KPV. A) Luciferase assay. Cells were transfected with a NF-κB-luciferase promoter construct and stimulated for 8 hours. Data were normalized by Renilla activity and expressed as relative luciferase activity. B, C) Western blot analyses of the time course of IκB-α degradation and phosphorylation in stimulated cells, using antibodies against IκB-α (B) and phospho-IκB-α (C), respectively. Bar graphs represent the densitometric quantification of IκB-α at time points 20 and 90 minutes. Values represent means ± SEM of 4 blots from 4 independent experiments. D) Western blot analyses of the time course of ERK1/2, JNK, and p38 kinases phosphorylation in stimulated cells, using respective anti-phospho-protein antibodies. Bar graphs represent the densitometric quantification of phospho-protein at indicated times. Values represent means ± SEM of 4 blots from 4 independent experiments. E) Real-time RT-PCR assay for the detection of IL-8 mRNA after 3 hours of stimulation. Values represent means ± SEM of 3 determinations. F) ELISA experiment for the determination of IL-8 concentrations

in the cell culture medium after 3 or 5 hours of stimulation. Values represent means ± SEM of three determinations. *$P < 0.05$ (A, B, E, F); **$P < 0.005$ (B, D).

Since mitogen-activated protein kinases (MAPK) can also play an important role in inflammation (24), we tested, by immunoblot analysis, the effect of KPV on MAPK phosphorylation and, therefore, activation. Figure 1D shows that IL-1β induces rapid phosphorylation of ERK1/2, JNK and p38 in Caco2-BBE cells. However, co-treatment with KPV strongly decreased IL-1β-induced MAPK phosphorylation and, therefore, their activation (Figure 1D).

It is known that MAPK and NF-κB pathways activation in IEC induces the production of pro-inflammatory cytokines that have a role in the recruitment of immune cells such as IL-8 (25). To examine whether KPV affects IL-8 expression and secretion by Caco2-BBE cells, IL-8 mRNA and protein levels were assessed by real-time RT-PCR and ELISA. We found that IL-1β induced a ~200-fold increase of IL-8 mRNA after 3 hours of stimulation in comparison with untreated cells (Figure 1E). In the presence of KPV, however, the IL-1β-induced increase of IL-8 mRNA was significantly reduced (by ~35%) (Figure 1E). Correlatively, the increase of IL-8 concentration in the culture medium of Caco2-BBE cells treated with IL-1β for 3 or 5 hours was significantly decreased by co-incubation with KPV (Figure 1F).

Together, these results show that KPV reduces NF-κB and MAPK activation which constitute the classical signaling pathways involved in cytokine secretion by inflamed IEC.

The anti-inflammatory effect of KPV is hPepT1-mediated in intestinal epithelial cells

KPV constitutes the three C-terminal amino acids of α-MSH which binds the melanocortin receptors (MCRs). We found by RT-PCR that Caco2-BBE cells express two of the five MCR isoforms: MC3R and MC5R (Figure 2A). Therefore, we cannot exclude the possibility that KPV acts via these receptors. Since MCR activation induces an increase of intracellular cyclic adenosine monophosphate ($cAMP_i$), we assessed $cAMP_i$ levels in Caco2-BBE cells after KPV treatment. ELISA results showed that $cAMP_i$ levels were not increased after stimulation by KPV, indicating that KPV does not act via these receptors (Figure 2B). Moreover, we also found that these receptors may not be functional in IEC since treatment of Caco2-BBE cells with 10 nM and 100 µM α-MSH did not affect $cAMP_i$ levels (Figure 2B). To confirm that MCRs are not functional in Caco2-BBE cells, cells were stimulated by IL-1β in the presence or absence of α-MSH, and IκB-α degradation was assessed by immunoblot analyses. Our results showed

that when administered at either a low (10 nM; Figure 2C, D) or high dose (100 μM; Figure 2E, F), α-MSH, unlike KPV (Figure 1B, C), did not significantly alter the kinetics of IL-1β-induced IκB-α degradation. This confirms that MCRs expressed in IEC do not mediate KPV inhibitory effect on inflammatory signaling pathways stimulated by IL-1β. In previous studies, it has been hypothesized that a stereochemical analogue of KPV, Lys-D-Pro-Val, can be an antagonist of IL-1β receptors (26). Therefore we examined the effects of KPV in the human colonic cell line HT29-Cl.19A which does not express hPepT1 (17). We found that treatment of HT29-Cl.19A cells with IL-1β increased NF-κB-dependent luciferase activity (Figure 3A), induced IκB-α degradation (Figure 3B) and increased IL-8 mRNA expression (Figure 3D), indicating that HT29-Cl.19A cells express functional IL-1β receptors. However co-treatment of HT29-Cl.19A cells with KPV did not decrease NF-κB-dependent luciferase activity and IκB-α degradation induced by IL-1β treatment (Figure 3A, B). Furthermore, Figure 3C shows that, in the presence of KPV, IκB-α basal level was not reached as fast as we found in Caco2-BBE cells (Figure 1C) after degradation induced by IL-1β treatment. Finally, no inhibitory effect of KPV on IL-1β-induced increased IL-8 mRNA expression was observed in HT29-Cl.19A cells (Figure 3D).

Figure 2. The anti-inflammatory effect of KPV is not associated with MCRs in Caco2-BBE cells.

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A) RT-PCR analysis of MC3R and MC5R expression in Caco2-BBE cells. B) ELISA assay for intracellular cAMP levels in Caco2-BBE cells after different times of stimulation with 10 nM KPV, 10 nM, 100 µM α-MSH, or 10 µM forskolin (FSK, positive control). Values represent means ± SEM of 3 determinations. ***$P < 0.001$ *vs* untreated cells (Control). C, D, E, F) Western blot analyses of the time course of IκB-α degradation in Caco2-BBE cells stimulated with 2 ng/ml IL-1β ± 10 nM α-MSH (C, D) or 2 ng/ml IL-1β ± 100 µM α-MSH (E, F), using anti-IκB-α antibodies. Bar graphs represent densitometric quantification of IκB-α at time points 20 and 90 minutes. Values represent means ± SEM of 4 blots from 4 independent experiments.

Figure 3. The anti-inflammatory effect of KPV is associated with hPepT1 expression.

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Cells were untreated (control), or stimulated with 2 ng/ml ± 10 nM KPV. A) Luciferase assay. HT29-Cl.19A cells were transfected with a NF-κB-luciferase promoter construct and stimulated for 8 hours. Data were normalized by Renilla activity and expressed as relative luciferase activity. Values represent means ± SEM of 3 determinations. B, C) Western blot analyses of the time course of IκB-α degradation in stimulated HT29-Cl.19A cells, using anti-IκB-α antibodies. D) Real-time RT-PCR assay for the detection of IL-8 mRNA in HT29-Cl.19A cells after 3 hours of stimulation. E, F) Luciferase assays. Caco2-BBE cells (E) or HT29-Cl.19A cells stably transfected with hPepT1 (F) were transfected with a NF-κB-luciferase promoter construct. Cells were then stimulated with IL-1β ±10 nM KPV± 20 mM Gly-Leu for 8 hours. Data were normalized by Renilla activity and expressed as relative luciferase activity. Values represent means ± SEM of 3 determinations. $*P < 0.05$ (E); $**P < 0.005$ (F).

Together, these results indicate that the anti-inflammatory effect of KPV is not mediated via IL-1β receptors but may involve the transporter hPepT1.

To confirm the dependence of KPV anti-inflammatory effect on hPepT1 expression, Caco2-BBE cells were transfected with a NF-κB-dependent luciferase reporter plasmid and stimulated with IL-1β alone or IL-1β + KPV in the presence or absence of Glycine-Leucine (Gly-Leu) which is a commonly used substrate for hPepT1. Figure 3E shows that unlike KPV, Gly-Leu did not affect IL-1β-induced activation

of NF-κB demonstrating the specificity of the KPV effect. However, KPV-mediated decrease of IL-1β-induced activation of NF-κB was completely reversed by the addition of Gly-Leu. This result suggests that KPV effect on NF-κB activation is dependent on hPepT1. To further confirm this result, we used HT29-Cl.19A cells previously stably transfected with hPepT1 or empty vector (17). These cells were transiently transfected with a NF-κB-dependent luciferase reporter plasmid and treated with the abovementioned stimuli. We found that KPV reduces NF-κB activation in HT29-Cl.19A cells expressing hPepT1 (Figure 3F) while addition of Gly-Leu abolished this KPV-mediated effect (Figure 3F). In contrast, KPV did not decrease IL-1β-induced NF-κB luciferase activity in HT29-Cl.19A cells stably transfected with empty vector (See Supplemental results, Figure 2). Together, these results confirm that KPV anti-inflammatory effect is hPepT1-mediated.

We therefore investigated whether KPV can be transported by hPepT1 into Caco2-BBE cells. We first assessed the inhibitory effect of KPV *vs* Gly-Leu on hPepT1-mediated transport of [^{14}C]Glycine-Sarcosine (Gly-Sar) which is a commonly used hPepT1 substrate. One hundred µM KPV inhibited [^{14}C]Gly-Sar uptake more efficiently than 100 µM Gly-Leu (~45% inhibition by KPV *vs* ~25% by Gly-Leu) (Figure 4A). This indicates that hPepT1 has a higher affinity for KPV than for Gly-Leu. To further confirm that hPepT1 transports KPV, uptake experiments were performed using [^{3}H]KPV. We show that, in contrast to [^{14}C]Gly-Sar, nanomolar concentrations of [^{3}H]KPV were efficiently transported by hPepT1 (Figure 4B). These results were confirmed by kinetic experiments showing that hPepT1 had a low Km of ~160 µM for KPV (Figure 4C).

Figure 4. hPepT1 transports KPV into Caco2-BBE cells.

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A) Uptake of 20 µM [^{14}C]Gly-Sar alone or in combination with 100 µM cold KPV or 100 µM cold Gly-Leu by Caco2-BBE cells. Values represent means ± SEM of 3 determinations. B) Specific uptake of 20 nM [^{14}C]Gly-Sar or 20 nM [^{3}H]KPV by Caco2-BBE cells. Values represent means ± SEM of 3 determinations. C) Kinetic parameters of [^{3}H]KPV specific uptake in Caco2-BBE cells. Values represent means ± SEM of 3 determinations. *$P < 0.05$ (A); ***$P < 0.001$ (A, B).

Together, these results indicate that the anti-inflammatory effect of KPV is not due to its interaction with the IL-1β receptor, but is mediated after transport by hPepT1 into cells where it accumulates and inactivates inflammatory pathways.

hPepT1-mediated KPV transport decreases inflammatory responses in TNF-α-stimulated Jurkat cells

Since the immune system plays a crucial role in IBD and is in close contact with IEC, we investigated the anti-inflammatory effect of KPV in the human T cell line Jurkat. Cells were stimulated with tumor necrosis factor-α (TNF-α) in the presence or absence of KPV and IκB-α degradation was assessed by immunoblot. We found that after 15 minutes of TNF-α treatment, in the presence of KPV, IκB-α protein level was higher compared with that in cells treated with TNF-α alone (Figure 5A), suggesting a partial inhibitory effect of KPV on TNF-α-induced IκB-αdegradation.

Figure 5. hPepT1-mediated KPV transport decreases inflammatory responses in Jurkat cells stimulated by TNF-α.

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Jurkat cells were untreated (control) or treated with 10 ng/ml TNF-α with or without pre-incubation with 10 nM KPV (A, B) or 10 nM α-MSH (D, E) for 30 minutes. A) Western blot analysis of IκB-α degradation after 15 and 30 minutes of stimulation with TNF-α following 30 minutes incubation with KPV, using anti-IκB-α antibodies. Bar graph represents the densitometric quantification of IκB-α after 15 minutes of stimulation. Values represent means ± SEM of 4 blots from independent experiments. B) Real-time RT-PCR assay for the detection of IL-8 mRNA after 6 hours of stimulation with TNF-α. Values represent means ± SEM of 3 determinations. C) RT-PCR analysis of MCRs expression in Jurkat cells. D) ELISA assay for intracellular cAMP levels in Jurkat cells after 10 and 30 minutes of stimulation with 10 nM

KPV, 10 nM α-MSH, or 10 µM forskolin (FSK, positive control). Values represent means ± SEM of 3 determinations. E) Western blot analysis of IκB-α degradation after 15 and 30 minutes of stimulation with TNF-α following 30 minutes incubation with 10 nM α-MSH, using anti-IκB-α antibodies. H) Kinetic parameters of [^{3}H]KPV specific uptake in Jurkat cells. Values represent means ± SEM of 3 determinations. *$P < 0.05$ (B); **$P < 0.005$ (A); ***$P < 0.001$ *vs* control (D).

The anti-inflammatory effect of KPV in Jurkat cells was confirmed by real-time RT-PCR. After 6 hours of stimulation, TNF-α induced a ~5-fold increase of IL-8 mRNA which was significantly reduced in the presence of KPV (Figure 5B). Using RT-PCR, we found that Jurkat cells express MC2,3,4,5R (Figure 5C). However, ELISA results showed that $cAMP_i$ levels were not increased after KPV stimulation (Figure 5D), indicating that KPV does not act via these receptors. Moreover, as found in Caco2-BBE cells (Figure 2B), α-MSH did not affect $cAMP_i$ levels (Figure 5D), suggesting that these MCRs may not be functional. This was confirmed by immunoblot analysis of IκB-α degradation in Jurkat cells stimulated with TNF-α ± α-MSH, which showed that α-MSH has no inhibitory effect on TNF-α-induced IκB-α degradation (Figure 5E).

We then investigated whether hPepT1 transports KPV into Jurkat cells. After confirmation of hPepT1 expression at mRNA and protein levels (Supplementary results, Figure 3), uptake kinetic experiments were performed. The results demonstrated that hPepT1 transports KPV with a Km of ~700 µM (Figure 5F).

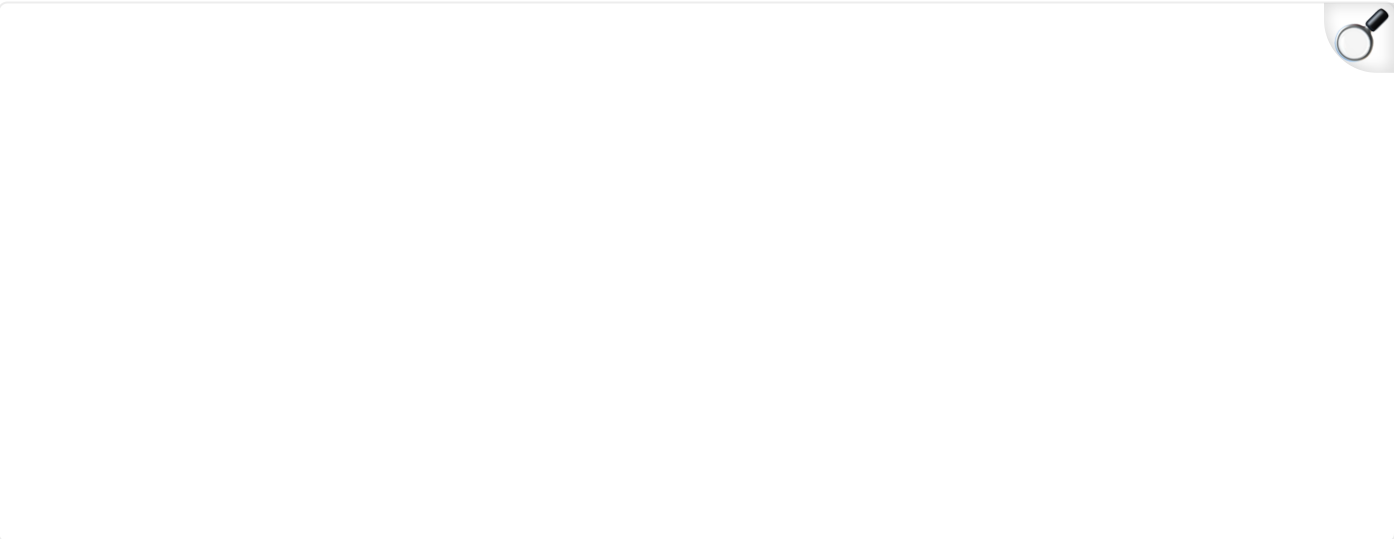
Together, these results show for the first time that i) Jurkat cells express a functional transporter hPepT1 and ii) intracellular accumulation of KPV suppresses activation of inflammatory signaling pathway in immune cells.

KPV decreases intestinal inflammatory response in vivo

Many experimental animal models have been used for the study of human IBD (27). Here we investigated KPV anti-inflammatory effect on dextran sulfate sodium (DSS)- and trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. The dose used in our study (100 µM KPV) was based on previous publications using α-MSH to treat experimental colitis (28, 29). We first investigated the anti-inflammatory effect of KPV in DSS-treated mice. Animals received water ± 3% DSS ± KPV for 8 days. DSS treatment resulted in a characteristic loss of body weight that started after 4 days of treatment (Figure 6A). Administration of KPV significantly reduced weight loss at day eight compared with mice

that received DSS alone (Figure 6A). Colonic myeloperoxidase (MPO) activity was measured as an indicator of the extent of neutrophil infiltration. We found that DSS-induced increase of MPO activity was significantly decreased by ~50% by the addition of KPV in the drinking water (Figure 6B). The anti-inflammatory effect of KPV was confirmed at the histological level using H&E-stained colonic sections. DSS induced cell wall damage, interstitial edema, and a general increase in the number of inflammatory cells in the lamina propria (Figure 6C). However mice that received both DSS and KPV showed a markedly reduced intestinal inflammation compared with DSS-treated mice (Figure 6C). Finally, KPV prevented other inflammatory changes such as increase of colon weight and decrease of colon length (Figure 6D, E). The administration of KPV alone had no effect on the basal MPO levels and other inflammatory parameters in the colonic mucosa (Figure 6).

Figure 6. KPV decreases DSS-induced intestinal inflammation.



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A) Body weight assessment in DSS-treated C57BL/6 mice. Mice were given water alone ( ), water + 100 μM KPV (○), 3% DSS alone (▼), or 3% DSS + 100 μM KPV (▼) (5 mice/group) and body weight was assessed over time. Results are expressed as percent weight loss over time. *$P < 0.05$ *vs* 3% DSS-treated mice (▼). B) Determination of MPO enzymatic activity in the colon. Results are expressed as mUnits of MPO activity per μg protein and represent means ± SEM of 5 determinations. C) Histological assessment of DSS-induced colitis using H&E-stained colonic sections. Pictures were magnified 40 times and 60 times. D) Assessment of weight (D) and length (E) of mouse colon. After mouse sacrifice, colons were removed; their length and weight were then measured. Results represent means ± SEM of 5 determinations. *$P < 0.05$ (B, D, E).

The expression of pro-inflammatory cytokines is known to be involved in intestinal inflammation. As expected, real-time RT-PCR experiments showed that DSS treatment increased mRNA levels of various pro-inflammatory cytokines (IL-6, IL-12, IFN-γ, IL-1β) in mouse colon (Figure 7). Interestingly, KPV treatment decreased the expression of these cytokines and this effect was significant for IL-6 and IL-12 (Figure 7A, B). Since inflammation is a balance between pro- and anti-inflammatory cytokines, we also assessed the effect of KPV on the main anti-inflammatory cytokine IL-10. We found that KPV did not change IL-10 mRNA expression in mouse colon (data not shown) suggesting that KPV acts by decreasing pro-inflammatory cytokines rather than increasing anti-inflammatory cytokines.

Figure 7. KPV decreases DSS-induced pro-inflammatory cytokines mRNA in mouse colon.

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After treatment of mice with the different abovementioned conditions, real-time RT-PCR was used to quantify mRNA levels of cytokines IL-6 (A), IL-12 (B), IFN-γ (C) and IL-1β (D). Values represent means ± SEM of 3 determinations. *$P < 0.05$; **$P < 0.05$; ***$P < 0.001$.

We then investigated the anti-inflammatory effect of KPV in TNBS-induced mouse colitis model 48 hours after its administration. Addition of KPV in the drinking water significantly reduced weight loss at day one and two compared with mice that received TNBS alone (Figure 8A). TNBS-induced increase

of MPO activity was significantly inhibited by ~30% by the addition of KPV (Figure 8B). Furthermore, KPV prevented other inflammatory changes such as decrease of colon length (Supplementary results, Figure 4). Finally, the KPV anti-inflammatory effect was confirmed using real-time RT-PCR. We found that KPV significantly reduced TNBS-induced IL-1β, IL-6, TNF-α and IFN-γ mRNA levels in mouse colon (Figure 8C, D, E, F).

Figure 8. KPV decreases TNBS-induced intestinal inflammation.

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A) Body weight assessment in TNBS-treated C57BL/6 mice. Mice were administrated intrarectal EtOH and oral water ( ); intrarectal EtOH and oral KPV (100 µM) (○); intrarectal TNBS (150 mg/kg) and oral water (▼); and intrarectal TNBS (150 mg/kg) and oral KPV (100 µM) (▼) (10 mice/group). Mice body weight was assessed 1 and 2 days after treatment. Results are expressed as percent weight. *$P < 0.05$ vs TNBS/water (▼). B) Determination of MPO enzymatic activity in the colon. Results are expressed as mUnits of MPO activity per µg protein and represent means ± SEM of 10 determinations. C, D, E, F) Quantification of mRNA levels of cytokines IL-1β, IL-6, TNF-α and IFN-γ by real-time RT-PCR after the different

abovementioned treatments of mice. Values represent means ± SEM of 10 determinations. *P < 0.05 (A, B, C, D, E, F).

Together, these results demonstrate that orally delivered KPV decreases the severity of DSS- and TNBS-induced colitis in mice.

DISCUSSION

α-MSH and other melanocortin peptides are potent anti-inflammatory agents and have been shown to be effective in many diseases (30). Here, we demonstrate that the tripeptide KPV, which is the C-terminal sequence of α-MSH, has an anti-inflammatory effect *in vitro* and *in vivo*. We show that the anti-inflammatory effect of KPV is not melanocortin receptor-mediated but PepT1-mediated. The finding that MCRs are not involved is supported by the results of a previous study showing that the anti-inflammatory and polymorphonuclear leucocytes anti-migratory activities of KPV are retained in mice that have a nonfunctional MC1R (31). Furthermore, it was recently demonstrated that KPV does not bind to MC1,3,5R (32) and does not compete with α-MSH (20), indicating a non-MCR mechanism. Using human intestinal epithelial and immune cell lines, we demonstrated that hPepT1 transports KPV and that subsequent increased intracellular level of KPV decreases the activation of NF-κB and MAPK inflammatory signaling pathways and finally reduces IL-8 secretion. Interestingly, we found, in Caco2-BBE cells, that hPepT1 has a high affinity for KPV (Km ~160 μM) that allows low doses of KPV to be efficiently targeted to the intracellular compartment. To our knowledge, this Km is among the lowest Km_s reported for hPepT1. For example Gly-Sar, which is the most commonly used PepT1 substrate, has a Km ≥ 1 mM in Caco2-BBE cells (33). Similar results were found in Jurkat cells. Indeed the Km is ~700 μM and only one study reported kinetic experiments in immune cells showing that the Km of hPepT1 for its substrates Gly-Sar and fMLP were ~2 mM (18).

Up-regulated expression of colonic hPepT1 in intestinal inflammation could allow oral delivery of small peptides into inflamed colonic cells. Such transport activity may therefore provide a good target for the development of anti-inflammatory therapies.

Our *in vivo* experiments showed that orally administered KPV significantly decreased inflammation in DSS- and TNBS-induced colitis. KPV reduced loss of body weight, colonic MPO activity, and markedly decreased histological signs of inflammation and pro-inflammatory cytokines mRNA levels. This work constitutes the first report of KPV-mediated reduction of colitis. Our *in vitro* experiments suggested that

this anti-inflammatory role of KPV results from inhibition of pro-inflammatory mechanisms in both IEC and immune cells.

The higher dose of KPV (100 μM) used in our in vivo studies was based on previous studies using α-MSH to treat experimental colitis (28, 29) and was chosen to increase the chances of KPV to reach mouse colon. Since our in vitro studies showed that PepT1 has a very high affinity for KPV, it is very likely that KPV is transported into inflamed colonic cells even if it is present at lower concentrations. It is therefore reasonable to hypothesize that orally administrated KPV is taken up by small intestine and inflamed colonic cells expressing PepT1, thereafter inhibiting epithelial inflammatory responses, including cytokine secretion. The inhibition of chemoattractants expression by colonic epithelial cells reduces the transport of neutrophils through the underlying matrix, as well as across the epithelium.

KPV can also reach the lamina propria through both the transcellular and the paracellular pathways, where it can interact directly with immune cells. We previously showed that human monocytes express a functional hPepT1 protein (18). Our work demonstrates for the first time that the human Jurkat T cell line also expresses a functional hPepT1 protein able to transport KPV into the cytosol where it can accumulate and inhibit inflammatory signaling pathways and subsequent cytokine secretion. PepT1 expression in immune cells provides the opportunity to deliver small peptides into cells that are actively involved in intestinal inflammation. Therefore, immune cells may participate in the reduction of colitis through KPV-mediated inhibition of immune responses.

Together our results show that i) KPV reduces the two most important intracellular signaling pathways in the pathogenesis of inflammatory bowel diseases: the NF-κB and MAPK cascade pathways as well as the subsequent synthesis of pro-inflammatory cytokines, ii) the anti-inflammatory effect of KPV is mediated through the transporter PepT1, and iii) oral delivery of KPV reduces the severity of DSS- and TNBS-induced colitis in mice.

These results indicate that targeting KPV transport into both epithelial and immune cells may reduce the overall level of pro-inflammatory cytokine production by mucosal and immune cells and therefore raise the use of KPV as an attractive therapeutic strategy against IBD.

Supplementary Material

01

NIHMS36963-supplement-01.pdf (112.3KB, pdf)

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Footnotes

No conflicts of interest exist

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Associated Data

This section collects any data citations, data availability statements, or supplementary materials included in this article.

Supplementary Materials

01

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