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PepT1-Mediated Tripeptide KPV Uptake Reduces Intestinal Inflammation

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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 the KPV anti-inflammatory effect is PepT1-mediated in intestinal epithelian and immune cells, and 2) examine the anti-inflammatory effects in two models of mice colitis.** ***Methods:*** **Human intestinal epithelial cells Caco2-BBE, HT29-Cl.19A, and human T cells (Jurkat) were stimulated with pro-inflammatory cytokines in the present 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 PepT1 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 histologic level and by proinflammatory 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 PepT1 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.** ***Conclusions:*** **This study indicates tht KPV is transported into cells by PepT1 and might be a new therapeutic agent for IBD.**

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 (IECs) and that cotransports peptides and H^+.[2] The specificity of hPepT1 is broad and includes many dipeptides and tripeptides in addition to various peptide-derived drugs.[3–8] PepT1 is expressed mainly 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 messenger RNA (mRNA) and protein is low[16] and sometimes cannot be detected.[10,15,17] Although human PepT1 (hPepT1) 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 also have shown that immune cells, such as macrophages, which are in close contact with the lamina propria of the intestine, also express hPepT1 at their membranes.[17,18]

Because expression of colonic hPepT1 is up-regulated in inflammatory bowel disease (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 because it may be therapeutically advantageous to develop hPepT1-mediated anti-inflammatory drugs. The tripeptide Lys-Pro-Val (KPV), 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 nuclear factor-κB (NF-κB) activation, indicating inhibition of proinflammatory cytokine synthesis. In the present study, we examined 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.

Abbreviations used in this paper: $cAMP_i$, intracellular cyclic adenosine monophosphate; DSS, dextran sodium sulfate; ELISA, enzyme-linked immunosorbent assay; Gly-Leu, glycine-leucine; Gly-Sar, glycine-sarcosine; IEC, intestinal epithelial cell; IL, interleukin; KPV, Lys-Pro-Val; MAPK, mitogen-activated protein kinase; MCR, melanocortin receptor; MPO, myeloperoxidase; α−MSH, α-melanocyte–stimulating hormone; NF-κB, nuclear factor-κB; PepT1, H^+-coupled oligopeptide transporter 1; RT-PCR, reverse-transcription polymerase chain reaction; TNF-α, tumor necrosis factor-α.

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Materials and Methods

Cell Culture

Caco2-BBE and HT29-Cl.19A cells were grown in Dulbecco's modified Eagle medium supplemented with 14 mmol/L NaHCO3, 10% fetal bovine serum, and penicillin/streptomycin (Invitrogen, Grand Island, NY). Jurkat cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum.

Reagents

See supplementary information (see supplementary material online at www.gastrojournal.org).

Animals

Female C57BL/6 mice (8 wk, 18–22 g; 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% (wt/vol) dextran sodium sulfate (DSS) (molecular weight 40,000 daltons; ICN Biochemicals, Aurora, OH) to the drinking water or by colonic injection of 150 mg/kg body weight of trinitrobenzene sulfonic acid (TNBS; Sigma, St. Louis, MO) dissolved in 50% ethanol. Colonic inflammation was assessed 8 days after DSS treatment or 48 hours after TNBS administration. N = 10 mice/group.

Myeloperoxidase Activity in the Colon

See supplementary information (see supplementary material online at www.gastrojournal.org).

Dual-Luciferase Reporter Assay

See supplementary information (see supplementary material online at www.gastrojournal.org).

Western Blot Analysis

See supplementary information (see supplementary material online at www.gastrojournal.org).

Uptake Experiments

Caco2-BBE cells were grown on filters for 15 days (area, 1 cm^2; pore size, 0.4 μm; Transwell-Clear polyester membranes; Costar VWR, Suwanee, GA), washed, and stabilized in Hank's balanced salt solution $(HBSS)^+$-10 mmol/L HEPES (pH 7.4) in the basolateral compartment and 10 mmol/L 2-[N-Morpholino]ethanesulphonic acid (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 mmol/L MES (pH 6.2) containing 20 nmol/L [^{3}H]KPV ± 20 mmol/L glycine-leucine, or 20 μmol/L [^{14}C]glycine-sarcosine ± 100 μmol/L KPV, or 20 μmol/L [^{14}C]glycine-sarcosine ± 100 μmol/L glycine-leucine, or 20 nmol/L [^{14}C]glycine-sarcosine ± 20 mmol/L glycine-leucine. Cells then were washed in ice-cold phosphate-buffered saline (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 mmol/L 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 [^{3}H]KPV ± 20 mmol/L glycine-leucine. Afterward, cells were washed in ice-cold PBS and total radioactivity was determined.

Specific uptakes were calculated as follows: (uptake of radiolabel peptide) − (uptake of radiolabel peptide + glycine-leucine).

Cyclic Adenosine Monophosphate Measurement

See supplementary information (see supplementary material online at www.gastrojournal.org).

RNA Extraction and Real-Time Reverse-Transcription Polymerase Chain Reaction

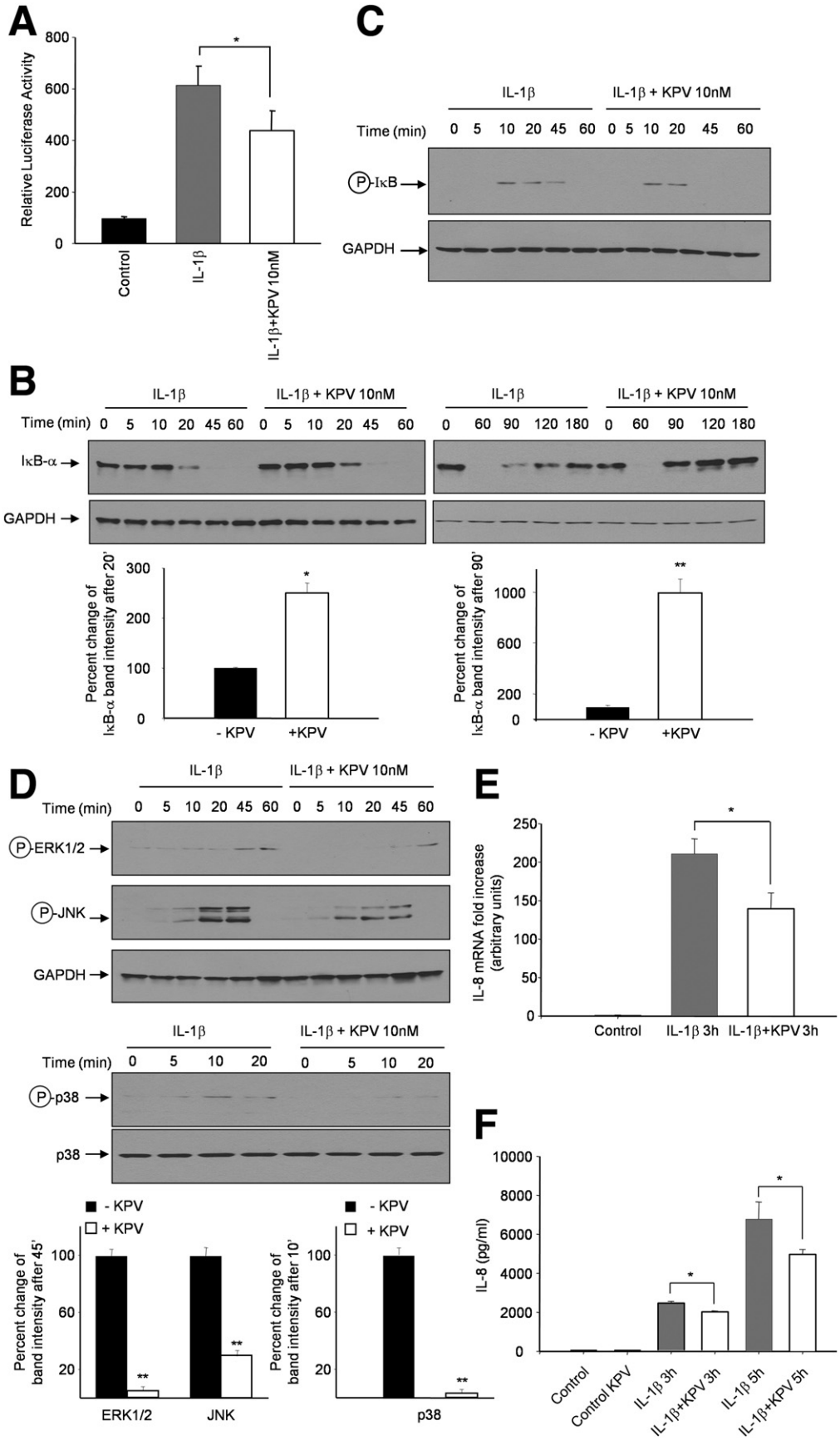
Total RNA was extracted from cells or colon using the TRIzol reagent (Invitrogen) and reverse transcribed using the RETROscript System (Ambion Inc, Austin, TX). The real-time iCycler sequence detection system (Bio-Rad, Hercules, CA) was used for real-time reverse-transcription polymerase chain reaction (RT-PCR). Briefly, 10 ng of complementary DNA (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 μmol/L of gene-specific primers (supplementary Table 1; see supplementary material online at www.gastrojournal.org) and the iQ SYBR Green Supermix (Bio-Rad). The glyceraldehyde-3-phosphate dehydrogenase or 36B4 expression levels were used as housekeeping genes, and fold-induction was calculated using the cycle threshold (Ct) method as follows: $\Delta\Delta CT = (Ct_{Target} - Ct_{housekeeping})$ treatment − $(Ct_{Target} - Ct_{housekeeping})$ nontreatment, and the final data were derived from $2^{-\Delta\Delta CT}$.

Detection of Melanocortin Receptors in Cells

PCR of cDNA for each of the melanocortin receptors (MCRs) was conducted by a seminested approach with forward and reverse primers in the first PCR and inner forward and reverse primers in the subsequent PCR as previously described.[22] Primers are shown in supplementary Table 2 (see supplementary material online at www.gastrojournal.org). PCR products were cloned in pGEM-T Easy Vectors (Promega, Madison, WI), amplified, and sequenced.

Statistical Analysis

All evaluations were performed using SigmaPlot (SPSS, Chicago, IL) and InStat v3.06 (GraphPad, San


A
Relative Luciferase Activity
800
600
400
200
0
*
Control
IL-1β
IL-1β+KPV 10nM
C
IL-1β
IL-1β + KPV 10nM
Time (min) 0 5 10 20 45 60 0 5 10 20 45 60
P-IκB
GAPDH
B
IL-1β
IL-1β + KPV 10nM
Time (min) 0 5 10 20 45 60 0 5 10 20 45 60
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IκB-α
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Percent change of IκB-α band intensity after 20'
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IL-1β+KPV 5h

Diego, CA) software, with data reported as means ± SEM. Multiple groups were compared by analysis of variance, using the Tukey post hoc test. *P* values less than .05 were considered statistically significant.

Results

KPV Decreases Inflammatory Responses in Caco2-BBE Cells Stimulated by Interleukin-1β

Many cell types, including IECs, express NF-κB, which is a transcriptional factor activated in response to immune and proinflammatory 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 an approximate 6-fold increase in luciferase activity compared with untreated cells (Figure 1*A*). However, co-incubation of Caco2-BBE cells with KPV (10 nmol/L) and IL-1β significantly decreased the IL-1β–induced luciferase activity (Figure 1*A*). 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 whereas, in the presence of KPV, IκB-α degradation was reduced at this time (Figure 1*B*). The IκB-α level then returned to the baseline level after 180 minutes of IL-1β stimulation whereas, in the presence of KPV, IκB-α baseline levels were reached within 90 minutes of stimulation (Figure 1*B*). Furthermore, IκB-α still was phosphorylated after 45 minutes of IL-1β stimulation, but not in the presence of KPV (Figure 1*C*). Collectively, our results show that KPV delays NF-κB activation and also shortens the delay of IκB-α recovery, suggesting that KPV decreases the duration of NF-κB activation. KPV-mediated decrease of NF-κB activity also was confirmed by electrophoretic mobility shift assay (EMSA) (Figure 1, supplementary Results section; see supplementary material online at www.gastrojournal.org).

Because mitogen-activated protein kinases (MAPKs) also can play an important role in inflammation,[24] we tested, by immunoblot analysis, the effect of KPV on MAPK phosphorylation and, therefore, activation. Figure 1*D* shows that IL-1β induces rapid phosphorylation of extracellularly regulated kinase (ERK1/2), c-Jun NH2-terminal kinase (JNK) and p38 in Caco2-BBE cells. However, cotreatment with KPV strongly decreased IL-1β–induced MAPK phosphorylation and, therefore, their activation (Figure 1*D*).

It is known that MAPK and NF-κB pathway activations in IECs induces the production of proinflammatory 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 enzyme-linked immunosorbent assay (ELISA). We found that IL-1β induced an approximately 200-fold increase of IL-8 mRNA after 3 hours of stimulation in comparison with untreated cells (Figure 1*E*). In the presence of KPV, however, the IL-1β–induced increase of IL-8 mRNA was reduced significantly (by ~35%) (Figure 1*E*). 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 decreased significantly by co-incubation with KPV (Figure 1*F*).

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

The Anti-Inflammatory Effect of KPV Is hPepT1-Mediated in IECs

KPV constitutes the 3 C-terminal amino acids of α-MSH that bind the MCRs. We found by RT-PCR that Caco2-BBE cells express 2 of the 5 MCR isoforms: MC3R and MC5R (Figure 2*A*). Therefore, we cannot exclude the possibility that KPV acts via these receptors. Because 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 2*B*). Moreover, we also found that these receptors may not be functional in IECs because

BASIC–ALIMENTARY TRACT

Figure 1. KPV decreases inflammatory response in Caco2-BBE cells stimulated by IL-1β. Caco2-BBE cells were untreated (control), or stimulated with 2 ng/mL IL-1β ± 10 nmol/L 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* and *C*) Western blot analyses of the time course of IκB-α degradation and phosphorylation in stimulated cells, using antibodies against (*B*) IκB-α and (*C*) phospho–IκB-α, respectively. Bar graphs represent the densitometric quantification of IκB-α at time points of 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. ■, -KPV; □, +KPV. (*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 3 determinations. (*A*, *B*, *E*, and *F*) $^*P < .05$; (*B* and *D*) $^{**}P < .005$.

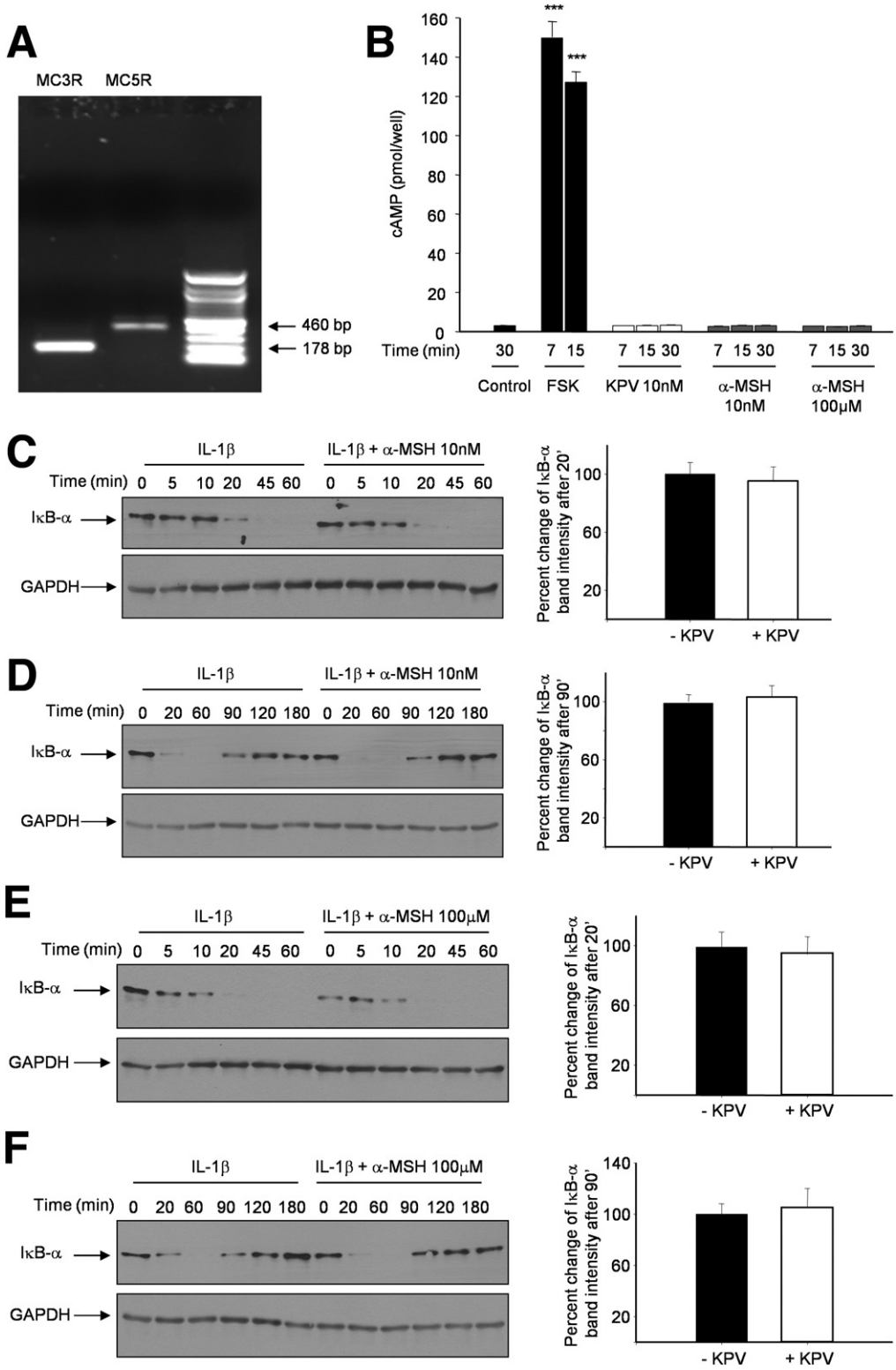

Figure 2. The anti-inflammatory effect of KPV is not associated with MCRs in Caco2-BBE cells. (*A*) RT-PCR analysis of MC3R and MC5R expression in Caco2-BBE cells. (*B*) ELISA assay for intracellular cAMP levels in Caco2-BBE cells after different times of stimulation with 10 nmol/L KPV, 10 nmol/L, α-MSH 100 μmol/L α-MSH, or 10 μmol/L forskolin (FSK, positive control). Values represent means ± SEM of 3 determinations. ***$P < .001$ vs untreated cells (control). (*C–F*) Western blot analyses of the time course of IκB-α degradation in Caco2-BBE cells stimulated with (*C* and *D*) 2 ng/mL IL-1β ± 10 nmol/L α-MSH or (*E* and *F*) 2 ng/mL IL-1β ± 100 μmol/L α-MSH, using anti–IκB-α antibodies. Bar graphs represent densitometric quantification of IκB-α at time points of 20 and 90 minutes. Values represent means ± SEM of 4 blots from 4 independent experiments.

treatment of Caco2-BBE cells with 10 nmol/L and 100 μmol/L of α-MSH did not affect $cAMP_i$ levels (Figure 2*B*). 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 nmol/L; Figure 2*C* and *D*) or high dose (100 μmol/L; Figure 2*E* and *F*), α-MSH, unlike KPV (Figure 1*B* and *C*), did not significantly alter the kinetics of IL-1β–induced IκB-α degradation. This confirms that MCRs expressed in IECs do not mediate the KPV inhibitory effect on inflammatory sig-

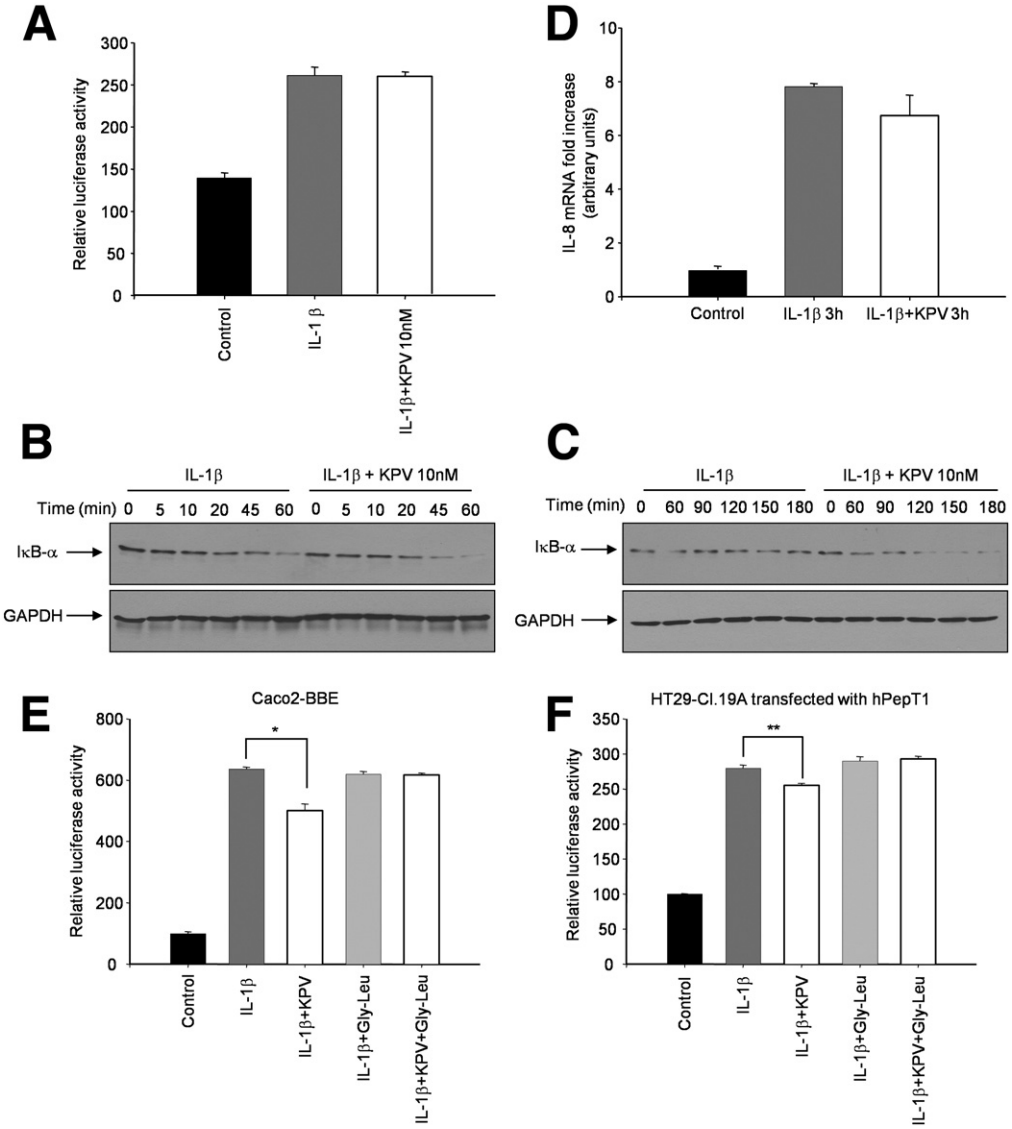

Figure 3. The anti-inflammatory effect of KPV is associated with hPepT1 expression. Cells were untreated (control), or stimulated with 2 ng/mL ± 10 nmol/L KPV. (*A*) Luciferase assay. HT29-Cl.19A cells were transfected with a NF-κB–luciferase promoter construct and stimulated for 8 hours. Data were normalized by Renilla activity and expressed as relative luciferase activity. Values represent means ± SEM of 3 determinations. (*B* and *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* and *F*) Luciferase assays. (*E*) Caco2-BBE cells or (*F*) HT29-Cl.19A cells stably transfected with hPepT1 were transfected with a NF-κB–luciferase promoter construct. Cells then were stimulated with IL-1β ± 10 nmol/L KPV± 20 mmol/L Gly-Leu for 8 hours. Data were normalized by Renilla activity and expressed as relative luciferase activity. Values represent means ± SEM of 3 determinations. (*E*) $^{*}P < .05$; (*F*) $^{**}P < .005$.

naling 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 3*A*), induced IκB-α degradation (Figure 3*B*), and increased IL-8 mRNA expression (Figure 3*D*), indicating that HT29-Cl.19A cells express functional IL-1β receptors. However, cotreatment 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 3*A* and *B*). Furthermore, Figure 3*C* shows that, in the presence of KPV, IκB-α basal level was not reached as fast as we found in Caco2-BBE cells (Figure 1*C*) 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 3*D*).

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 3*E* shows that unlike KPV, Gly-Leu did not affect IL-1β–induced activation of NF-κB, showing the specificity of the KPV effect. However, KPV-mediated decrease of IL-1β–induced activation of NF-κB was reversed completely 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 transfected transiently with a NF-κB–dependent luciferase reporter plasmid and treated with the earlier-mentioned stimuli. We found that KPV reduces NF-κB activation in HT29-Cl.19A cells expressing hPepT1 (Figure 3*F*), whereas the addition of Gly-Leu abolished this KPV-mediated effect (Figure 3*F*). In contrast, KPV did not decrease IL-1β–induced NF-κB luciferase activity in HT29-Cl.19A cells stably transfected with empty vector (Figure 2, supplementary Results section; see supplementary material online at www.gastrojournal.org). 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. A total of 100 μmol/L KPV inhibited [^{14}C]Gly-Sar uptake more efficiently than 100 μmol/L Gly-Leu (~45% inhibition by KPV vs ~25% by Gly-Leu) (Figure 4*A*). 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 transported efficiently by hPepT1 (Figure 4*B*). These results were confirmed by kinetic experiments showing that hPepT1 had a low Michaelis-Menton constant (Km) of approximately 160 μmol/L for KPV (Figure 4*C*).

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

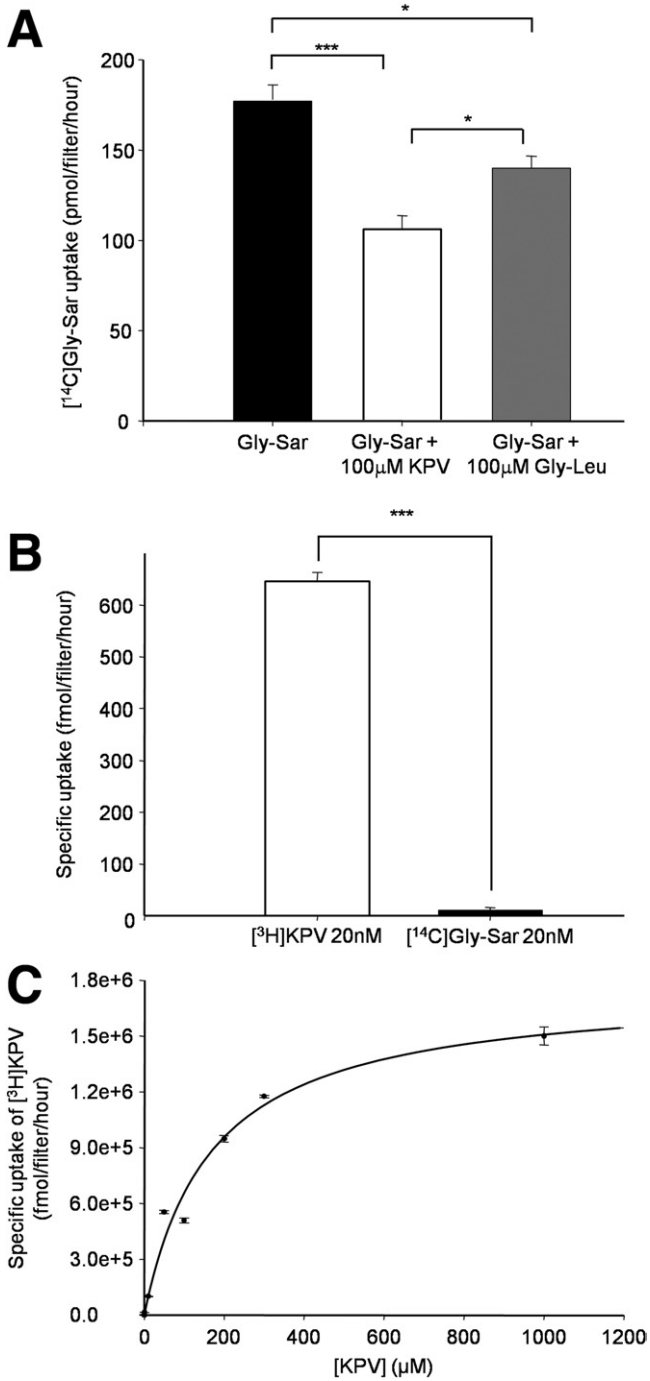

Figure 4. hPepT1 transports KPV into Caco2-BBE cells. (*A*) Uptake of 20 μmol/L [^{14}C]Gly-Sar alone or in combination with 100 μmol/L cold KPV or 100 μmol/L cold Gly-Leu by Caco2-BBE cells. Values represent means ± SEM of 3 determinations. (*B*) Specific uptake of 20 nmol/L [^{14}C]Gly-Sar or 20 nmol/L [^{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. (*A*) $^{*}P < .05$; (*A* and *B*) $^{***}P < .001$.

hPepT1-Mediated KPV Transport Decreases Inflammatory Responses in Tumor Necrosis Factor-α Stimulated Jurkat Cells

Because the immune system plays a crucial role in IBD and is in close contact with IECs, 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 5*A*), suggesting a partial inhibitory effect of KPV on TNF-α–induced IκB-α degradation.

The anti-inflammatory effect of KPV in Jurkat cells was confirmed by real-time RT-PCR. After 6 hours of stimulation, TNF-α induced an approximately 5-fold increase of IL-8 mRNA, which was reduced significantly in the presence of KPV (Figure 5*B*). By using RT-PCR, we found that Jurkat cells express MC2,3,4,5R (Figure 5*C*). However, ELISA results showed that $cAMP_i$ levels were not increased after KPV stimulation (Figure 5*D*), indicating that KPV does not act

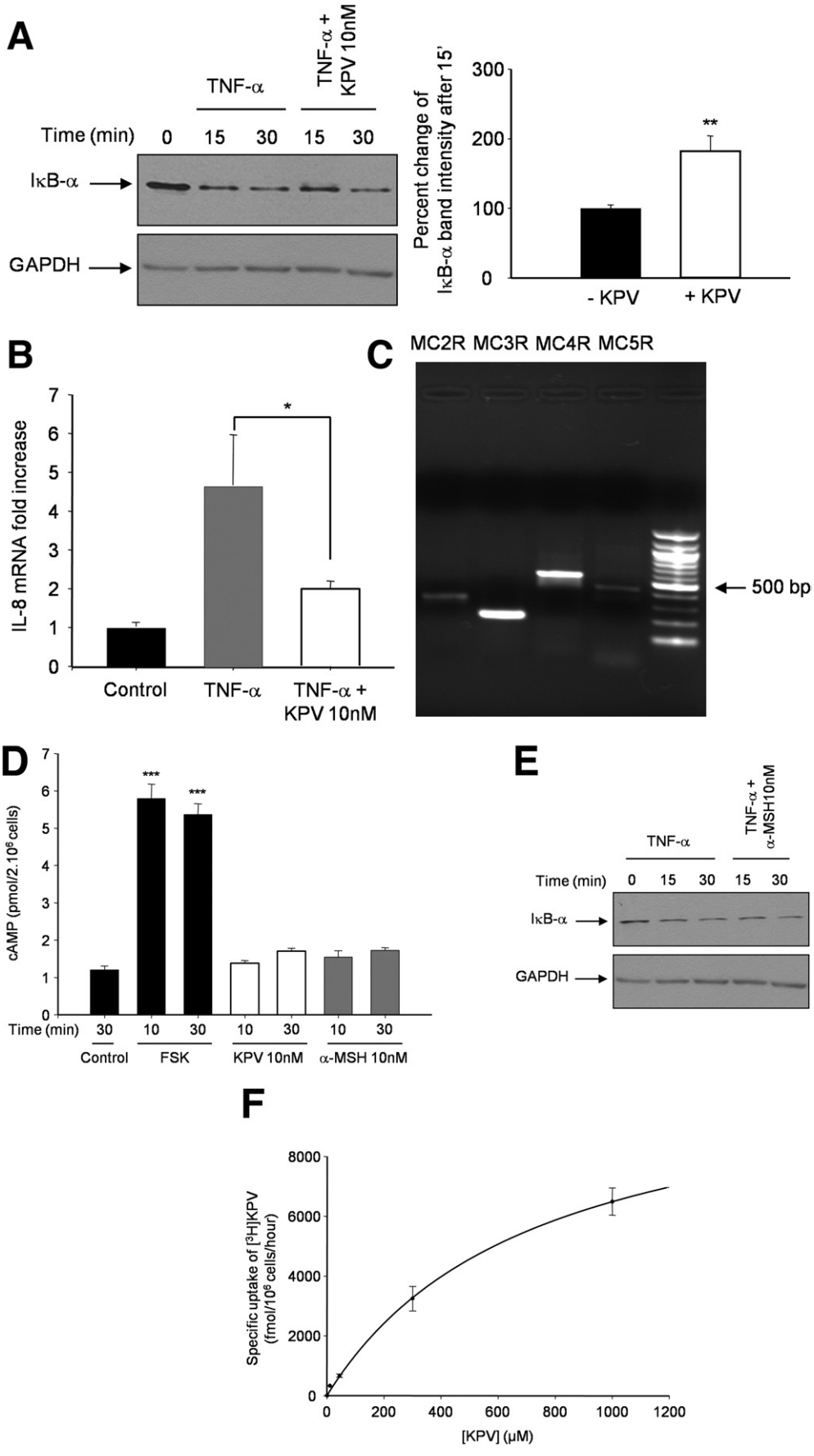

Figure 5. hPepT1-mediated KPV transport decreases inflammatory responses in Jurkat cells stimulated by TNF-α. Jurkat cells were untreated (control) or treated with 10 ng/mL TNF-α with or without pre-incubation with (*A* and *B*) 10 nmol/L KPV or (*D* and *E*) 10 nmol/L α-MSH for 30 minutes. (*A*) Western blot analysis of IκB-α degradation after 15 and 30 minutes of stimulation with TNF-α after 30 minutes of 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 nmol/L KPV, 10 nmol/L α-MSH, or 10 μmol/L 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 after 30 minutes of incubation with 10 nmol/L 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. (*A*) $^{**}P < .005$; (*B*) $^{*}P < .05$; (*D*) $^{***}P < .001$ vs control.

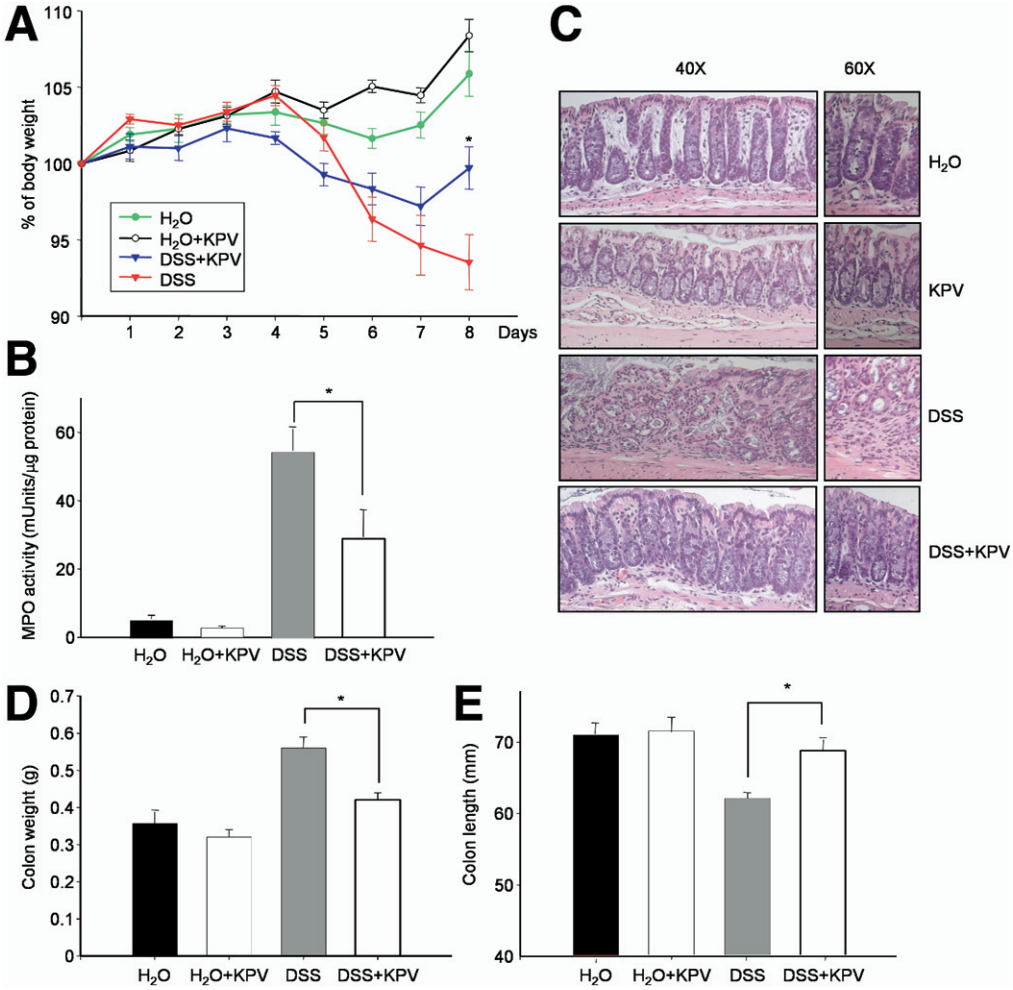

Figure 6. KPV decreases DSS-induced intestinal inflammation. (*A*) Body weight assessment in DSS-treated C57BL/6 mice. Mice were given water alone (green circle), water + 100 μmol/L KPV (○), 3% DSS alone (red triangle), or 3% DSS + 100 μmol/L KPV (blue triangle) (5 mice/group) and body weight was assessed over time. Results are expressed as percentage of weight loss over time. *$P < .05$ vs 3% DSS-treated mice (red triangle). (*B*) Determination of MPO enzymatic activity in the colon. Results are expressed as MPO mUnits per μg protein and represent means ± SEM of 5 determinations. (*C*) Histologic assessment of DSS-induced colitis using H&E-stained colonic sections. Pictures were magnified 40 and 60 times. Assessment of (*D*) weight and (*E*) length of mouse colon. After the mice died, colons were removed; their length and weight then were measured. Results represent means ± SEM of 5 determinations. (*B*, *D*, and *E*) *$P < .05$.

via these receptors. Moreover, as found in Caco2-BBE cells (Figure 2*B*), α-MSH did not affect $cAMP_i$ levels (Figure 5*D*), 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 5*E*).

We then investigated whether hPepT1 transports KPV into Jurkat cells. After confirmation of hPepT1 expression at mRNA and protein levels (Figure 3, supplementary Results section; see supplementary material online at www.gastrojournal.org), uptake kinetic experiments were performed. The results showed that hPepT1 transports KPV with a Km of approximately 700 μmol/L (Figure 5*F*).

Together, these results show that Jurkat cells express a functional transporter hPepT1 and that 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 DSS- and TNBS-induced colitis in mice. The dose used in our study (100 μmol/L 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 6*A*). Administration of KPV significantly reduced weight loss at day 8 compared with mice that received DSS alone (Figure 6*A*). 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 decreased significantly by approximately 50% by the addition of KPV in the drinking water (Figure 6*B*). The anti-inflammatory effect of KPV was confirmed at the histologic 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 6*C*). However, mice that received both DSS and KPV showed a markedly reduced intestinal inflammation compared with DSS-

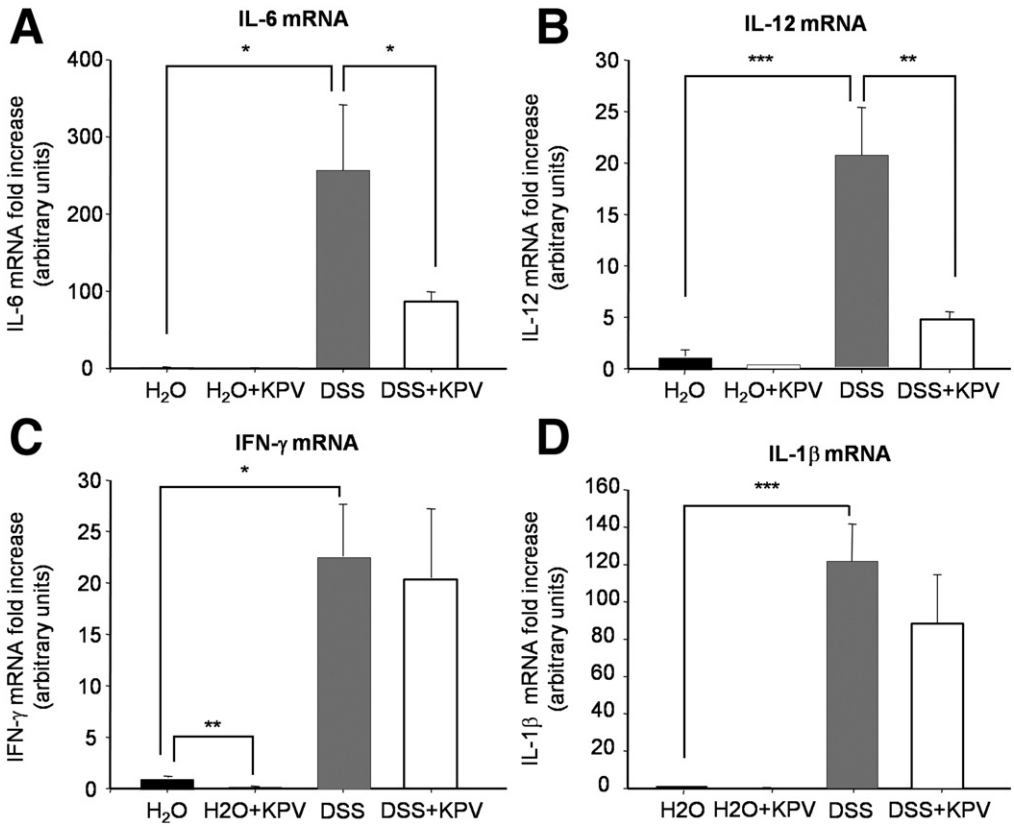

Figure 7. KPV decreases DSS-induced proinflammatory cytokines mRNA in mouse colon. After treatment of mice with the different earlier-mentioned conditions, real-time RT-PCR was used to quantify mRNA levels of cytokines (*A*) IL-6, (*B*) IL-12, (*C*) IFN-γ, and (*D*) IL-1β. Values represent means ± SEM of 3 determinations. $^{*}P < .05$; $^{**}P < .05$; $^{***}P < .001$.

treated mice (Figure 6*C*). Finally, KPV prevented other inflammatory changes such as increase of colon weight and decrease of colon length (Figure 6*D* and *E*). The administration of KPV alone had no effect on the basal MPO levels and other inflammatory parameters in the colonic mucosa (Figure 6).

The expression of proinflammatory 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 proinflammatory cytokines (IL-6, IL-12, IFN-γ, and 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 7*A* and *B*). Because inflammation is a balance between proinflammatory 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 proinflammatory cytokines rather than increasing anti-inflammatory cytokines.

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 days 1 and 2 compared with mice that received TNBS alone (Figure 8*A*). TNBS-induced increase of MPO activity was inhibited significantly by approximately 30% by the addition of KPV (Figure 8*B*). Furthermore, KPV prevented other inflammatory changes such as decrease of colon length (Figure 4, supplementary Results section; see supplementary material online at www.gastrojournal.org). 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 8*C*–*F*).

Together, these results show 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 show 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 MCR-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 leukocytes antimigratory activities of KPV are retained in mice that have a nonfunctional MC1R.[31] Furthermore, it recently was shown that KPV does not bind to MC1,3,5R[32] and does not compete with α-MSH,[20] indicating a non-MCR mechanism. By using human IECs and immune cell lines, we showed that

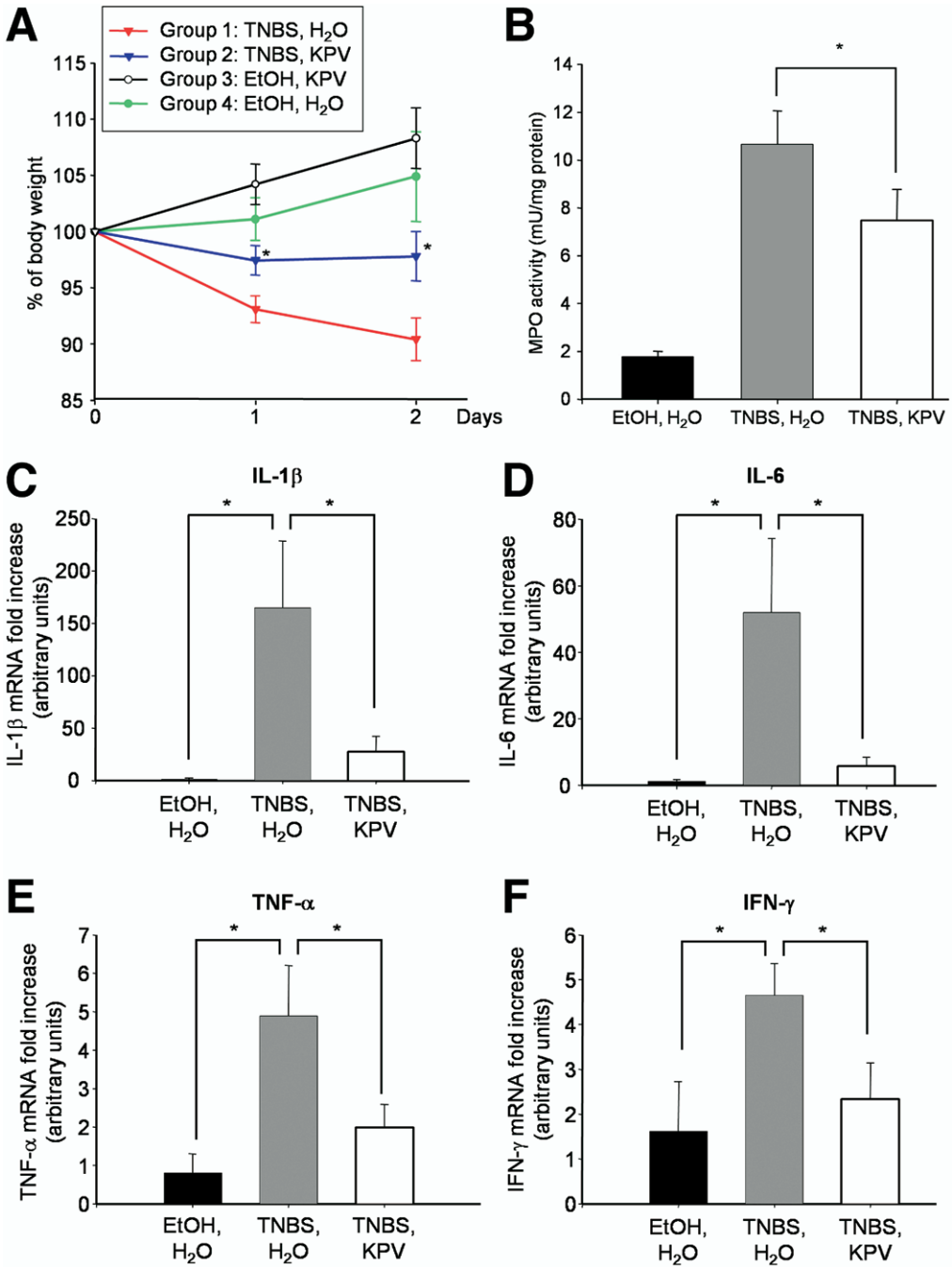

Figure 8. KPV decreases TNBS-induced intestinal inflammation. (*A*) Body weight assessment in TNBS-treated C57BL/6 mice. Mice were administrated intrarectal EtOH and oral water (green circle); intrarectal EtOH and oral KPV (100 μmol/L) (○); intrarectal TNBS (150 mg/kg) and oral water (red triangle); and intrarectal TNBS (150 mg/kg) and oral KPV (100 μmol/L) (blue triangle) (10 mice/group). Mice body weight was assessed 1 and 2 days after treatment. Results are expressed as percentage of weight. *$P < .05$ vs TNBS/water (red triangle). (*B*) Determination of MPO enzymatic activity in the colon. Results are expressed as MPO mUnits per μg protein and represent means ± SEM of 10 determinations. (*C–F*) Quantification of mRNA levels of cytokines IL-1β, IL-6, TNF-α, and IFN-γ by real-time RT-PCR after the different earlier-mentioned treatments of mice. Values represent means ± SEM of 10 determinations. (*A–F*) *$P < .05$.

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 μmol/L) 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 of 1 mmol/L or greater in Caco2-BBE cells.[33] Similar results were found in Jurkat cells. Indeed, the Km is approximately 700 μmol/L and only one study reported kinetic experiments in immune cells showing that the Km of hPepT1 for its substrates Gly-Sar and formyl-methionyl-leucyl-phenylalanine (fMLP) were approximately 2 mmol/L.[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 histologic signs of inflammation and proinflammatory cytokine mRNA levels. This work constitutes a report of KPV-mediated reduction of colitis. Our in vitro experiments suggested that this anti-inflammatory role of KPV results from inhibition of proinflammatory mechanisms in both IECs and immune cells.

The higher dose of KPV (100 μmol/L) 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. Because 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 chemoattractant expression by colonic epithelial cells reduces the transport of neutrophils through the underlying matrix, as well as across the epithelium.

KPV also can 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 shows 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 (1) KPV reduces the 2 most important intracellular signaling pathways in the pathogenesis of IBDs: the NF-κB and MAPK cascade pathways as well as the subsequent synthesis of proinflammatory cytokines; (2) the anti-inflammatory effect of KPV is mediated through the transporter PepT1; and (3) 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 proinflammatory cytokine production by mucosal and immune cells and therefore raise the use of KPV as an attractive therapeutic strategy against IBD.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi:10.1053/j.gastro.2007.10.026.

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