Taurocholate uptake by Caco-2 cells is inhibited by pro-inflammatory cytokines and butyrate

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Research Article

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Abstract

Inflammatory bowel disease (IBD) is a group of chronic and life-threatening inflammatory diseases of the gastrointestinal tract. The active intestinal absorption of bile salts is reduced in IBD, resulting in higher luminal concentrations of these agents that contribute to the pathophysiology of IBD-associated diarrhea. Butyrate is a short-chain fatty acid produced by colonic bacterial fermentation of dietary fibers. Butyrate utilization is impaired in the intestinal inflamed mucosa of IBD patients. The aim of this work is to investigate the link between IBD and bile acid absorption, by testing the effect of the pro-inflammatory cytokines TNF-α and IFN-γ and butyrate upon $^3$H-taurocholate uptake by Caco-2 cells. The results obtained show that the proinflammatory cytokines TNF-α and IFN-γ inhibit Na$^+$-independent, non-ASCT-mediated $^3$H-TC uptake by Caco-2 cells. The inhibitory effect of these cytokines on $^3$H-taurocholate uptake is PI3K- and JAK2-mediated. Moreover, butyrate in high but physiological concentrations was also found to inhibit $^3$H-TC uptake and showed an additive effect with IFN-γ in reducing $^3$H-taurocholate uptake. So, an interaction between fiber-derived butyrate and inflammatory cytokines appears to exist in relation to bile salt intestinal uptake in IBD, which may participate in the link between fiber intake and IBD.

Introduction

Inflammatory bowel disease (IBD) is a group of chronic and life-threatening diseases of the gastrointestinal tract characterized by episodes of intestinal inflammation, with Crohn's disease and ulcerative colitis being the most significant. The incidence and prevalence of IBD markedly increased over the second half of the 20th century, and since the beginning of the 21st century, IBD has been considered one of the most prevalent gastrointestinal diseases with accelerating incidence in newly industrialized countries [1]. Although the etiology and pathogenesis of inflammatory bowel disease (IBD) is multifactorial, cytokine secretion, which is important in the regulation of normal gastrointestinal immune responses, appears to be dysregulated in IBD, with increased levels of proinflammatory cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α).

Bile acids (BA), classically known for their roles in facilitating the digestion and absorption of dietary lipids, are also appreciated as having important roles in regulating many aspects of mammalian physiology, both within and outside the intestinal tract. BA regulate the expression of multiple genes in the intestine by acting on both nuclear receptors (the farnesoid X receptors (FXR)) and cell membrane-located receptors (TGR5). Besides FXR, BA are also able to activate other nuclear receptors (the pregnane X receptor (PXR)), the vitamin D receptor (VDR) and the glucocorticoid receptor (GR)) [2]. At the intestinal tract, BA as signaling molecules are known to play an important role in regulating epithelial transport and barrier function, which are associated with the pathogenesis of various intestinal conditions, including IBD [2].

Several lines of evidence have clearly established a link between BA and IBD. First, secondary BA present pro-inflammatory characteristics and chronically high physiological levels of these compounds constitute risk factors for colonic inflammation [3]. Second, bile acid signaling is altered in IBD [4]. Third, genetic
variants of FXR have been linked to IBD [5]. Fourth, pharmacological activation of FXR [6] and TGR5 [7] protect against chemically-induced animal models of colitis and, accordingly, FXR KO [8] and GPR5 KO [9] mice have an increased colonic inflammatory phenotype. Finally, epidemiologic, clinical, and experimental data have uncovered an important association between dietary habits and IBD [10] and BA, through modulation of gut microbiota, appear to link a high-fat diet and IBD [11].

Active BA absorption occurs predominantly in the distal ileum via the apical sodium-dependent bile acid transporter (ASBT), encoded by the apical sodium-dependent BA transporter gene (SLC10A2) [12]. A reduction in ASBT activity results in BA malabsorption with a consequent increase in colonic BA levels [12]. Interestingly, ASBT function and expression appear to be reduced in IBD, resulting in higher luminal concentrations of BA that contribute to the pathophysiology of the associated diarrhea [13–15]. The mechanism involved in the link between IBD and a reduced ASBT activity remains not fully understood [16].

Butyrate (BT) is a short-chain fatty acid produced by colonic bacterial fermentation of dietary fibers. BT plays an important role in maintaining the health and integrity of the colonic mucosa. Specifically, this compound is the primary energy source of the colonic epithelium, promotes mucosal immune cell activity and the integrity of the intestinal epithelium, thus contributing to the intestinal epithelial barrier, and possesses anticarcinogenic and antioxidant effects [17, 18]. Increased dietary fiber intake and SCFA are also associated with anti-inflammatory effects [3]. Of interest, BT utilization is impaired in the intestinal inflamed mucosa of IBD patients [19] and one of the mechanisms involved consists in a defect in epithelial BT uptake [20], resulting in higher luminal concentrations of this SCFA.

In this work, we decided to investigate the interaction between IBD and intestinal epithelial BA uptake. For this, we investigated the effect of inflammatory mediators - the pro-inflammatory cytokines TNF-α and IFN-γ - and of BT upon the apical uptake of 3H-taurocholate by intestinal epithelial (Caco-2) cells.

**Methods**

**Caco-2 cell culture**

The Caco-2 cell line was obtained from ATCC (Manassas, VA, USA) and was used between passage number 9–38. The cells were maintained in a humidified atmosphere of 5% CO₂–95% air and were grown in MEM medium containing 5.55 mM glucose supplemented with 25 mM HEPES, 26.2 mM sodium bicarbonate, 15% heat-inactivated FBS and 1% antibiotic/antimycotic solution. Culture medium was renewed every 2–3 days, and the culture was split (1:3) every 7 days. For the experiments, cells were seeded on 24-well culture dishes and used 10–15 days after the initial seeding (100% confluence).

**Quantification Of H-taurocolate (H-tc) Uptake**

The culture medium was made free of fetal bovine serum for 24h before the experiments. For the uptake experiments, culture medium was discarded, and the cells were washed with 300 µl buffer (containing, in
mM: 125 NaCl, 4.8 KCl, 1.2 MgSO₄, 1.2 CaCl₂, 1.6 KH₂PO₄, 0.4 K₂HPO₄, 5.55 glucose and 20 HEPES (pH 7.4)) at 37°C. Then, cell monolayers were pre-incubated for 20 min in buffer at 37°C. Uptake was then initiated by the addition of 200 µl buffer at 37°C containing ^3^H-TC 1 µM (except in kinetics experiments). Incubation was stopped after 3 min (except in time-course experiments) by removing the incubation medium, placing the cells on ice, and rinsing them with 0.50 ml ice-cold buffer. Cells were then solubilized with 300 µl 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) and placed at 4°C overnight. Intracellular radioactivity was measured by liquid scintillation counting (LKB Wallac 1209 Rackbeta, Turku, Finland). Results were normalized for total protein content (Bradford method).

To test the effect of compounds on ^3^H-TC uptake, these drugs were present for 1h, 3h or 24h before the experiments and were also present during the pre-incubation and incubation periods. To test the Na⁺-dependence of ^3^H-TC uptake, sodium chloride of the preincubation and incubation buffers was replaced by an equimolar amount of choline chloride.

**Rt-qpcr**

Total RNA was extracted from Caco-2 cells treated for 24h with TNF-α, IFN-γ and/or BT (10 mM) and RT-qPCR was carried out as described[21]. hASBT was amplified with gene-specific primers (forward primer: 5'-GCC CCA AAA AGC AAA GAT CA-3'; reverse primer:5'-GCT ATG AGC ACA ATG AGG ATG G-3')[22]. Samples were analyzed in duplicate and the amount of hASBT mRNA was normalized to the amount of mRNA of housekeeping gene human β-actin. The primer pair for β-actin was: 5'-AGA GCC TCG CCT TTG CCG AT-3' (forward) and 5'-CCA TCA CGC CCT GGT GCC T-3' (reverse). Cycling conditions for hASBT amplification were as follows: denaturation (95°C for 10 min), amplification and quantification [95°C for 10 s, annealing temperature (AT = 58°C) for 10 s, with a single fluorescence measurement at the end of the 72°C for 10 s segment] repeated 45 times, followed by a melting curve program [(AT + 10)°C for 10 s and 75°C with a heating rate of 0.1°C/s and continuous fluorescence measurement] and a cooling step to 37°C for 30 s. Cycling conditions for human β-actin amplification were as follows: denaturation (95°C for 5 min), amplification and quantification (95°C for 10 s, AT (65°C) for 10 s, with a single fluorescence measurement at the end of the 72°C for 10 s segment) repeated 45 times, followed by a melting curve program [(AT + 10)°C for 10 s and 75°C with a heating rate of 0.1°C/s and continuous fluorescence measurement] and a cooling step to 37°C for 30 s. Data were collected using the LightCycler 96 SW 1.1 analysis software (Roche, Mannheim, Germany), and results were analyzed by the comparative Ct (ΔΔCT) method [23]. β-actin mRNA expression levels were not affected by the treatments (data not shown).

**Protein Determination**

The protein content of cell monolayers was determined as described by Bradford [24], using human serum albumin as standard.

**Materials**
All chemicals were obtained from standard commercial suppliers and were of analytical-grade quality. \( ^3 \text{H(G)]-taurocholic acid (}^3 \text{H-DG); specific activity 5–10 Ci mmol}^{-1} \) (American Radiolabeled Chemicals, St Louis, MO, USA), antibiotic/antimycotic solution (100 U ml\(^{-1} \) penicillin; 100 mg ml\(^{-1} \) streptomycin and 0.25 mg ml\(^{-1} \) amphotericin B), MEM medium (catalogue #M-0643, Sigma), HEPES (N-2-hydroxyethylpiperazine-N\(^\prime\)-2-ethanesulfonic acid), trypsin–EDTA (ethylenediamine tetraacetic acid) solution, sodium butyrate, choline chloride, human interferon-\( \gamma \), human TNF-\( \alpha \), cholic acid, taurocholic acid, deoxycholic acid, chenodeoxycholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid, ursodeoxycholic acid, fludarabine, LY 294002 (Sigma, St Louis, MO, USA); DMSO (dimethylsulphoxide), Triton X-100 (Merck, Darmstadt, Germany); fetal bovine serum (Invitrogen Corporation, Carlsbad, CA, USA).

The drugs to be tested were dissolved in DMSO (bile acids, LY 294002, fludarabine, sodium butyrate) or 0.1% BSA in PBS (IFN-\( \gamma \) and TNF-\( \alpha \)), the final concentration of these solvents being 1% in the buffer or 0.1% in the culture medium. Controls for these drugs were run in the presence of the solvent. None of these solvents significantly affected \(^3 \text{H-TG uptake (data not shown). When tested, compounds were present in culture medium without fetal bovine serum. In transport experiments, compounds were also present in the buffer (preincubation and incubation periods).}

**Calculation And Statistics**

For the analysis of the time-course of \(^3 \text{H-TC uptake, the parameters of the following equation were fitted to the experimental data by a nonlinear regression analysis.} \( K_{\text{in}} \) is given in pmol/mg protein min\(^{-1} \) and \( K_{\text{out}} \) in min\(^{-1} \).

\[
A(t) = \frac{K_{\text{in}}}{K_{\text{out}}} \times (1 - e^{-K_{\text{out}} \times t})
\]

In this equation, \( A(t) \) represents the accumulation of \(^3 \text{H-TC at time } t, K_{\text{in}} \) and \( K_{\text{out}} \) the rate constants for inward and outward transport, respectively, and \( t \) the incubation time. \( A_{\text{max}} \) is defined as the accumulation at steady state (\( t \to \infty \)).

Arithmetic means are given with SEM, and geometric means are given with 95% confidence limits. The value of \( n \) indicates the number of replicates of at least 3 different experiments. Statistical significance of the difference between two groups was evaluated by Student’s t-test; statistical analysis of the difference between various groups was evaluated by the analysis of variance (ANOVA) test, followed by the Student-Newman-Keuls test. Differences were considered to be significant when \( P < 0.05 \).

**Results**

**Uptake of \(^3 \text{H-TC is time-dependent}**
The time-dependence of $^3$H-TC uptake was first investigated. Total $^3$H-TC uptake by Caco-2 cells increased with time, being linear with time in the first 3 min of incubation (Fig. 1). Therefore, in subsequent experiments, initial rates of uptake were measured by incubating cells for 3 min with $^3$H-TC. In the absence of Na$^+$, uptake of $^3$H-TC was markedly reduced (Fig. 1).

Comparison of the time-course of $^3$H-TC uptake in the presence and absence of Na$^+$ shows that $^3$H-TC uptake by Caco-2 cells includes both a Na$^+$-dependent and a Na$^+$-independent component, with a greater contribution of the Na$^+$-independent component (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>$k_{in}$ (pmol mg protein$^{-1}$ min$^{-1}$)</th>
<th>$k_{out}$ (min$^{-1}$)</th>
<th>$A_{max}$ (pmol mg prot$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total uptake</td>
<td>1.123 ± 0.052</td>
<td>0.079 ± 0.005</td>
<td>14.15 ± 0.33</td>
</tr>
<tr>
<td>Na$^+$-independent uptake</td>
<td>0.471 ± 0.051</td>
<td>0.048 ± 0.008</td>
<td>9.8 ± 0.74</td>
</tr>
<tr>
<td>Na$^+$-dependent uptake</td>
<td>0.843 ± 0.107</td>
<td>0.167 ± 0.025</td>
<td>5.05 ± 0.21</td>
</tr>
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</table>

Uptake Of $^3$H-TC Uptake Is Saturable

The kinetics of $^3$H-TC uptake (total, Na$^+$-dependent and Na$^+$-independent) was next determined. Analysis of the curves showed that both total and the Na$^+$-dependent component of $^3$H-TC uptake (1–1000 µM) uptake are saturable (Fig. 2; Table 2). In contrast, the Na$^+$-independent component of $^3$H-TC uptake is nonsaturable.

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol mg prot$^{-1}$ 3 min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total uptake</td>
<td>683.6 ± 187.4</td>
<td>69.8 ± 10.2</td>
</tr>
<tr>
<td>Na$^+$-dependent uptake</td>
<td>191.8 ± 103.2</td>
<td>13.8 ± 2.5</td>
</tr>
</tbody>
</table>

Uptake Of $^3$H-TC Uptake Is Inhibited By Deoxycholic Acid And Chenodeoxycholic Acid

Next, we tested the effect of a 24h exposure to several distinct bile acids on $^3$H-TC uptake. Total and Na$^+$-independent uptake of $^3$H-TC were significantly reduced by deoxycholic acid and chenodeoxycholic acid.
Additionally, cholic acid increased Na\(^+\)-independent uptake and decreased Na\(^+\)-dependent \(^3\)H-TC uptake, but it had no effect on total uptake (Fig. 3).

**Tnf-α And Ifn-γ Inhibit H-tc Uptake**

The effect of a 24h exposure to increasing concentrations of two pro-inflammatory cytokines (TNF-α and IFN-γ) upon \(^3\)H-TC uptake was next evaluated. At a concentration of 30 ng/ml and above, both cytokines were able to reduce total \(^3\)H-TC uptake (Fig. 4). The inhibitory effect of IFN-γ was the result of an inhibition of Na\(^+\)-independent uptake, whereas TNF-α was found to decrease both Na\(^+\)-dependent and Na\(^+\)-independent \(^3\)H-TC uptake (Fig. 4). The inhibitory effect of the two cytokines was already observed after a 1h-exposure time (results not shown).

The inhibitory effect of TNF-α and IFN-γ upon \(^3\)H-TC total and Na\(^+\)-independent apical uptake is mediated by the PI3K and the JAK/STAT1 signaling pathways

As shown in Fig. 5, the inhibitory effect of TNF-α and IFN-γ upon total and Na\(^+\)-independent \(^3\)H-TC uptake was abolished in the presence of the PI3K inhibitor (LY 294002). This suggests that the inhibitory effect of these cytokines upon total and Na\(^+\)-independent \(^3\)H-TC uptake by Caco-2 cells is mediated by the PI3K intracellular signaling pathway (Fig. 5).

In relation to Na\(^+\)-dependent \(^3\)H-TC uptake, the observation that LY 294002 alone was able to inhibit (or tend to inhibit) it suggests that, in control conditions, PI3K-mediated signaling is involved in promoting Na\(^+\)-dependent uptake (Fig. 5). However, the inhibitory effect of TNF-α upon Na\(^+\)-dependent uptake was not abolished by LY 294002 (Fig. 5).

Interestingly, inhibition of the JAK/STAT1 intracellular signaling pathway with fludarabine was also able to abolish the inhibitory effect of both TNF-α and IFN-γ upon total and Na\(^+\)-independent \(^3\)H-TC uptake (Fig. 6). So, the inhibitory effect of TNF-α and IFN-γ upon total and Na\(^+\)-independent uptake appears to involve the JAK/STAT1 pathway (Fig. 6). In contrast, the inhibitory effect of TNF-α upon Na\(^+\)-dependent uptake was not abolished by fludarabine (Fig. 6).

A High Concentration Of Butyrate (10 Mm) Inhibits H-tc Uptake

At lower concentrations, sodium butyrate (BT) (24h) increased \(^3\)H-TC uptake; however, a higher concentration of BT (10 mM) decreased total and Na\(^+\)-independent \(^3\)H-TC uptake (Fig. 7).

The inhibitory effect of IFN-γ upon \(^3\)H-TC total and Na\(^+\)-independent apical uptake is potentiated by a high concentration of BT (10 mM)

In order to investigate the interaction between BT and the inflammatory cytokines, the effects of TNF-α and IFN-γ (24h) upon \(^3\)H-TC apical uptake were evaluated in the presence of a high concentration of BT.
(10 mM). As shown in Fig. 8, a potentiation of the effect of IFN-γ (but not of TNF-α) upon total \(^{3}\)H-TC uptake was observed in the presence of BT.

The inhibitory effect of TNF-α and IFN-γ and of BT upon \(^{3}\)H-TC uptake is not related to a reduction in ASBT gene expression levels

Because TC uptake by intestinal epithelial cells involves ASBT, we evaluated the effect of TNF-α, IFN-γ and BT upon the expression levels of this transporter. In agreement with the lack of effect of BT upon Na\(^{+}\)-dependent \(^{3}\)H-TC uptake (Fig. 9), BT did not affect ASBT mRNA levels (Fig. 9). In contrast, both TNF-α and IFN-γ increased hASBT expression levels (Fig. 9), although they presented no significant effect on Na\(^{+}\)-dependent \(^{3}\)H-TC uptake (Fig. 4).

Interestingly, although BT did not affect Na\(^{+}\)-dependent uptake even in the presence of TNF-α and IFN-γ (Fig. 8), it was able to abolish the stimulatory effects of both cytokines upon ASBT mRNA expression levels (Fig. 9).

Discussion

In this work, we investigated the link between IBD and BA luminal absorption, by testing the effect of the pro-inflammatory cytokines TNF-α and IFN-γ and of the SCFA BT upon the apical uptake of \(^{3}\)H-TA by Caco-2 cells.

Ileal ASBT is the main responsible for the intestinal absorption of bile acids and decreased ASBT expression has been described in both pre-clinical models of IBD (murine, canine and rabbit models) and human IBD [16, 25]. Indeed, in the intestine, about 95% of luminal BA are actively reabsorbed in the distal ileum by the apical sodium-dependent bile acid transporter (ASBT, also known as SLC10A2). The other 5% BAs are transported into the colon where they are metabolized (deconjugated and dihydroxylated) by microbiota; part of them is returned to the liver while the others are excreted with feces. Although ileal ASBT constitutes the major route of absorption for most conjugated bile acids such as taurocholic acid, passive absorption present throughout the small intestine is the main route for unconjugated bile acid reabsorption [16, 26, 27]. Mechanistically, ASBT is electrogenic, requiring the cotransport of two Na\(^{+}\) ions together with a BA molecule, and the driving force is the inwardly directed Na\(^{+}\) gradient, which is maintained by both the basolateral Na\(^{+}\)/K\(^{+}\)-ATPase and the negative intracellular potential. ASBT preferentially transports conjugated BA compared with unconjugated BA [27]. Therefore, ASBT activity was assessed by the measurement of Na\(^{+}\)-dependent \(^{3}\)H-taurocholic acid (\(^{3}\)H-TC) uptake.

Uptake of \(^{3}\)H-TC uptake by Caco-2 cells was found to be time- and concentration-dependent. However, despite the fact that TC is a good substrate for ASBT [16, 26, 27] and that quantification of Na\(^{+}\)-dependent uptake of \(^{3}\)H-TC by Caco-2 cells is an established cell model for evaluation of ASBT activity, we verified that the Na\(^{+}\)-dependent component plays a minor role in \(^{3}\)H-TC uptake by Caco-2 cells. This observation stands in contrast to previous studies showing that \(^{3}\)H-TC uptake by Caco-2 cells is mainly
Na\textsuperscript{+}-dependent [22, 28]. We have no explanation for this seemingly lower Na\textsuperscript{+}-dependency of \textsuperscript{3}H-TC uptake in our experiments. However, it is worth to note that the level of ASBT expression in Caco-2 cells appears to be low and not consistent [28, 29] and that several works evaluate ASBT activity by quantifying Na\textsuperscript{+}-dependent \textsuperscript{3}H-TC uptake by Caco-2 cells, but no information as to the fraction of uptake that is Na\textsuperscript{+}-dependent is given (eg. [30, 31]). The observation that Na\textsuperscript{+}-independent \textsuperscript{3}H-TC uptake is specifically inhibited by DC and CDC argues against the possibility of Na\textsuperscript{+}-independent uptake occurring via passive absorption, although a linear correlation between \textsuperscript{3}H-TC concentration and Na\textsuperscript{+}-independent uptake was found, suggesting the involvement of a non-saturable mechanism. Further, the observation that the Km for total uptake is higher than the Km of Na\textsuperscript{+}-dependent uptake suggests a low affinity of the Na\textsuperscript{+}-independent mechanism involved in \textsuperscript{3}H-TC uptake. Although the general consensus is that ileal active transport, ABST-mediated, is the major route for conjugated BA uptake, passive or facilitative absorption is also present down the length of the small intestine. Several studies have shown that the Na\textsuperscript{+}-independent transporter OATP1A2 is expressed at the apical brush border membrane of human small intestinal epithelial cells and transports BA as well as a variety of drugs [32, 33] and a comparison between the absolute protein expression levels of 28 drug-related transporters in Caco-2 cell monolayers concluded that the expression levels of OATP1A2 and ASBT were similarly low [34]. So, it is possible that OATP1A2 is the responsible for most of \textsuperscript{3}H-TC uptake by Caco-2 cells.

The effect of IBD on \textsuperscript{3}H-TC uptake was investigated by testing the effect of TNF-\(\alpha\) and IFN-\(\gamma\) on \textsuperscript{3}H-TC uptake and ASBT mRNA expression levels. We verified that both TNF-\(\alpha\) and IFN-\(\gamma\) concentration-dependently reduced total \textsuperscript{3}H-TC uptake. We were also able to verify that both cytokines reduced Na\textsuperscript{+}-independent uptake, whereas Na\textsuperscript{+}-dependent uptake was inhibited by TNF-\(\alpha\) only. Previous studies have shown that ASBT expression is decreased by cytokines contributing to the pathophysiology of IBD, namely IL-1\(\beta\) [35–37] and TNF-\(\alpha\) [35]. These cytokines repress ASBT expression by inducing upregulation, phosphorylation, and nuclear translocation of c-fos, which then represses ASBT promoter activity via AP-1 [36, 38]. In addition to transcriptional regulation, proteosomal-mediated degradation of ASBT is stimulated by cytokines such as IL-1\(\beta\) [37]. However, the effect of proinflammatory cytokines on ASBT activity was not evaluated in these previous studies. So, the present report shows for the first time that TNF-\(\alpha\) and IFN-\(\gamma\) decrease \textsuperscript{3}H-TC uptake by a human intestinal epithelial cell line, and that TNF-\(\alpha\) inhibits Na\textsuperscript{+}-dependent (probably ASBT-mediated) transporter activity. Of note, the decrease in Na\textsuperscript{+}-dependent \textsuperscript{3}H-TC induced by TNF-\(\alpha\) is not related with a decrease in ASBT expression levels; rather, an increase in ASBT mRNA levels was observed in response to TNF-\(\alpha\) (and also IFN-\(\gamma\)). In a previous report, TNF-\(\alpha\) was found to downregulate ASBT gene expression in Caco-2 cells [35]. This difference may be related to the fact that the exposure time to TNF-\(\alpha\) was almost the double (40h) in [35].

By using specific pharmacological inhibitors of PI3K (LY294002) and JAK/STAT1 (fludorabine), we could conclude that the inhibitory effect of TNF-\(\alpha\) and IFN-\(\gamma\) upon \textsuperscript{3}H-TC apical total and Na\textsuperscript{+}-independent uptake is mediated by these intracellular signaling pathways. Interestingly, repression of ASBT expression by TNF-\(\alpha\) and IL-1\(\beta\) was previously found to be mediated by the PI3K signaling [35] and stimulation of
ubiquitin-proteasome degradation of ASBT by IL-1β was previously described to be due to JNK-regulated serine/threonine phosphorylation of ASBT protein [37]. In the present report, we thus describe that the inhibitory effect of inflammatory cytokines on total and Na+-independent (and therefore, ASBT-independent) 3H-TC uptake is similarly PI3K- and JAK (JAK2)-mediated.

The SCFA BT is a product of microbiota-mediated fermentation of dietary fibers with anti-inflammatory effects. This compound modulates various points in the inflammatory process in colonic epithelial cells and has also direct effects in intestinal immune cells (T and B cells, macrophages, neutrophils and dendritic cells), not only via long-recognized direct inhibitory effects on cell division, but also via modulation of cell signaling, epigenetic regulation and metabolism [39]. BT regulates inflammation by acting through two distinct mechanisms: (1) it interacts with cell membrane G protein-coupled receptors (GPR41, GPR43 and GPR109A) expressed in gut epithelium and immune cells, regulating downstream cell signal transduction mechanisms such as NF-κB pathway, MAPK and Ca2+, and (2) after entering colonic epithelial cells through transport proteins (such as MCT1 and SMCT1), it inhibits histone deacetylase [39–41]. Interestingly, a defect in BT epithelial uptake was described in IBD [19, 20], resulting in higher luminal concentrations of this compound. So, we decided to evaluate if BT interferes with BA uptake, as a mechanism contributing to its anti-inflammatory effect.

We tested the effect of increasing doses (in the mM range) of butyrate. High concentrations (in the mM range) of BT in the colonic lumen may be attained, for example, after digestion of a dietary fiber-containing meal (the concentrations of these fatty acids in the lumen may reach 70–130 mM, with 20–30% being BT) [42, 43]. In contrast, a low concentration (in the µM range) of BT may be attained in the intermeal period or at the bottom of colonic crypts[17].

We verified that lower levels of BT (0-5-5 mM) increased 3H-TC uptake by 10–20% and that, in contrast, higher levels of BT (10 mM) showed an inhibitory action on 3H-TC uptake. BT does not interfere with Na+-dependent uptake nor changes ASBT expression levels; rather, it negatively affects Na+-independent 3H-TC uptake. The increase in 3H-TC uptake caused by BT (0.5-5 mM) might contribute to the anti-inflammatory effect of this SCFA, because luminal BA promote intestinal inflammation [3]. In contrast, inhibition of 3H-TC by BT (10 mM) may contribute to higher deleterious luminal levels of BA. Moreover, BT (10 mM) was able to potentiate the inhibitory effect of IFN-γ upon 3H-TC uptake, which can also contribute to higher luminal BA levels. Overall, we verified that BT (in high concentrations) causes an inhibition of 3H-TC uptake and possesses an additive effect with IFN-γ in reducing 3H-TC uptake. These results thus suggest that, both in the absence and presence of intestinal inflammation, high luminal BT inhibits the intestinal epithelial cell uptake of BA, which may contribute to higher luminal levels of these compounds.

Traditionally, patients with IBS were told to increase dietary fiber intake, but several randomized controlled trials have now shown that “Fermentable Oligosaccharides, Disaccharides, Monosaccharides And Polyols” (FODMAP) restriction leads to an improvement in IBS symptoms, compared with habitual diet
Fermentation of these fibers leads to the production of BT. Our results suggest that the inhibitory effect of BT on BA colonic epithelial uptake, resulting in higher luminal levels of these compounds, may contribute to the link between BA and IBD.

In conclusion, this work shows that the proinflammatory cytokines TNF-α and IFN-γ inhibit Na⁺-independent, non-ASCT-mediated ³H-TC uptake by Caco-2 cells, which results in higher luminal levels of BA. The inhibitory effect of these cytokines on ³H-TC uptake is PI3K- and JAK/STAT1-mediated. Moreover, the SCFA BT was also found to inhibit ³H-TC uptake at high but physiologic concentrations and to possess an additive effect with IFN-γ in reducing ³H-TC uptake. So, an interaction between BT and BA appears to exist in IBD, that may participate in the link between dietary fiber intake and IBD.

Declarations

FUNDING

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Author Contribution

Conceptualization: M.C., N.A.; Research/Investigation: M.C., N.A., F.M.; Writing/original draft preparation: M.C., N.A.; Writing/review and editing: F.M., N.A., and F.M.; Visualization: N.A., F.M.; Supervision, F.M. and R.C.A.; Project administration: F.M. and F. M. All authors contributed to the article and approved the submitted version.

Ethics approval and consent to participate Not applicable

Consent for publication Not applicable

Availability of data and materials: Available upon request

Conflicts of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References


**Figures**
Figure 1

Time-course of $^3$H-TC apical uptake. Caco-2 cells were incubated for various periods of time at 37 °C with 1 μM $^3$H-TC, pH 7.4, in the presence (Total uptake; n=12) or absence of Na$^+$ (Na$^+$-independent uptake). Na$^+$-dependent uptake was calculated by subtracting Na$^+$-independent uptake from total uptake. Shown are arithmetic means ± SEM (n=6).
Figure 2

Kinetics of $^3$H-TC apical uptake. Caco-2 cells were incubated for 3 min at 37°C with increasing concentrations of $^3$H-TC (1-1000 μM), in the presence (Total uptake) or absence of Na$^+$ (Na$^+$-independent uptake). Na$^+$-dependent uptake was calculated by subtracting Na$^+$-independent uptake from total uptake. Shown are arithmetic means ± SEM (n=6-8).
Figure 3

**Effect of bile acids (24h) on \(^3\)H-TC apical uptake.** The effect of cholic acid (C), taurocholic acid (TC), deoxycholic acid (DC), chenodeoxycholic acid (CDC), taurochenodeoxycholic acid (TCDC), glycochenodeoxycholic acid (GCDC) or ursodeoxycholic acid (UDC) (300 µM) on the uptake of 1 µM \(^3\)H-TC (3 min; 37°C) by Caco-2 cells was tested. Uptake was determined in the presence (Total uptake) or absence of Na\(^+\) (Na\(^+\)-independent uptake). Na\(^+\)-dependent uptake was calculated by subtracting Na\(^+\)-independent uptake from total uptake. Total, Na\(^+\)-dependent and Na\(^+\)-independent uptake amount to 4.3±0.4, 1.7±0.5 and 2.6±0.1 pmol/mg prot, respectively. Shown are arithmetic means ± SEM (n=6). * significantly different from control (p<0.05) (Student's t test)
**Effect of increasing concentrations of TNF-α (a) and IFN-γ (b) (24h) on $^3$H-TC apical uptake.** The effect of TNF-α (a) and IFN-γ (b) (1-300 ng/ml) on the uptake of 1 µM $^3$H-TC (3 min; 37°C) by Caco-2 cells was tested. Uptake was determined in the presence (Total uptake) and absence of Na$^+$ (Na$^+$-independent uptake). Na$^+$-dependent uptake was calculated by subtracting Na$^+$-independent uptake from total uptake. Shown are arithmetic means ± SEM (n=6-9). *Significantly different from control (p<0.05) (Student’s t test)
Figure 5

Involvement of the PI3K intracellular signaling pathway in the effects of TNF-α and IFN-γ (24h) upon $^3$H-TC apical uptake. The effect of TNF-α (a) and IFN-γ (b) (100 ng/ml) on the uptake of 1 µM $^3$H-TC (3 min; 37°C) by Caco-2 cells, in the absence or presence of a PI3K inhibitor (LY 294002 1 µM), was tested. Shown are arithmetic means ± SEM (n=6-7). *P < 0.05 vs control, # P < 0.05 vs. each other; ns not significantly different (ANOVA + Student-Newman-Keuls test).
Figure 6

Involvement of the JAK/STAT1 intracellular signaling pathway in the effect of TNF-α and IFN-γ (24h) upon $^3$H-TC uptake. The effect of TNF-α (a) and IF-γ (b) (100 ng/ml) on the uptake of 1 µM $^3$H-TC (3 min; 37°C) by Caco-2 cells, in the absence or presence of a JAK/STAT1 inhibitor (fludarabine 1 µM), was
tested. Shown are arithmetic means ± SEM (n=6-7). *P < 0.05 vs control, #P < 0.05 vs. each other; ns not significantly different, ANOVA + Student-Newman-Keuls test.

Figure 7

**Effect of sodium butyrate (BT) (24h) on $^3$H-TC uptake.** The effect of BT (0.5-10 mM) on the uptake of 1 µM $^3$H-TC (3 min; 37°C) by Caco-2 cells was tested. Uptake was determined in the presence (Total uptake) and absence of Na$^+$ (Na$^+$-independent uptake). Na$^+$-dependent uptake was calculated by subtracting Na$^+$-independent uptake from total uptake. Shown are arithmetic means ± SEM (n=10-12). * Significantly different from control (p<0.05) (Student’s t test)
Figure 8

Effect of the proinflammatory cytokines in the presence of sodium butyrate (BT) (24h). The effect of TNF-α (100 ng/ml), IF-γ (100 ng/ml) and/or BT (10 mM) on the uptake of 1 µM \(^3\)H-TC (3 min; 37°C) by Caco-2 cells was tested. Uptake was determined in the presence (Total uptake) and absence of Na\(^+\) (Na\(^+\)-dependent uptake). Na\(^+\)-dependent uptake was calculated by subtracting Na\(^+\)-independent uptake from
Effects of TNF-α, IFN-γ and/or BT (24h) on ASBT mRNA levels. Caco-2 cells were exposed to TNF-α (100 ng/ml), IFN-γ (100 ng/ml) and/or BT (10 mM) for 24h and ASBT mRNA levels were then quantified by qRT-PCR. Data were normalized to the expression of β-actin. Data show arithmetic means ± SEM (n=6). *Significantly different from control (p<0.05) (ANOVA + Student-Newman-Keuls)