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Na-glutamine co-transporters B^0AT1 in villus and SN2 in crypts are differentially altered in chronically inflamed rabbit intestine

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ABSTRACT

Glutamine is a major nutrient utilized by the intestinal epithelium and is primarily assimilated via Na-glutamine co-transport (NGcT) on the brush border membrane (BBM) of enterocytes. Recently we reported that B^0AT1 (SLC6A19) mediates glutamine absorption in villus while SN2 (SLC38A5) does the same in crypt cells. However, how B^0AT1 and SN2 are affected during intestinal inflammation is unknown. In the present study it was shown that during chronic enteritis NGcT was inhibited in villus cells, however, it was stimulated in crypt cells. Our studies also demonstrated that the mechanism of inhibition of NGcT during chronic enteritis was secondary to a reduction in the number of B^0AT1 co-transporters in the villus cell BBM without a change in the affinity of the co-transporter. In contrast, stimulation of NGcT in crypt cells was secondary to an increase in the affinity of SN2 for glutamine without an alteration in the number of co-transporters. Thus, glutamine assimilation which occurs via distinct transporters in crypt and villus cells is altered in the chronically inflamed intestine.

1. Introduction

Glutamine is the primary metabolite for the intestinal epithelial cells [1] and is involved in modulation of several cell signaling pathways, such as induction of heat shock protein (HSP) expression [2], regulation of redox status [3], activation of immune cells [4], influence on epithelial cell apoptosis and modulation of proteome under apoptotic conditions [5,6]. Glutamine is efficacious in inhibiting drug related toxicity and reduction of severe mucositis [7].

In the intestinal epithelial cells, glutamine can be absorbed by several transport systems [8], among them Na-dependent co-transporters are most important which include system A, system N and system B^0 [9]. In system A, three distinct isoforms have been cloned to date — SNAT1 (SLC38A1), SNAT2 (SLC38A2) and SNAT4 (SLC38A4) [10–14]. System N includes two isoforms, SNAT3 (SLC38A3) and SNAT5 (SLC38A5), also known as SN1 and SN2 respectively [15–18]. System B^0 includes two distinct isoforms B^0AT1 and B^0AT2 [19,20]. Recently, we have demonstrated that villus cells transport glutamine primarily via B^0AT1 [21]. Further, for the first time; we reported the presence of nutrient absorptive transporter on the brush border membrane (BBM) of secretory crypt cells, specifically SN2 [22].

Chronic intestinal inflammation is the hallmark of Crohn's disease (CD) and ulcerative colitis (UC) collectively called inflammatory bowel disease (IBD) [23]. Patients with IBD have an increased risk of developing colorectal cancer (CRC) and the risk appears to be related to the cumulative effect of chronic inflammation [24].

There is increasing evidence supporting the protective role of glutamine supplementation to restore the health of the intestine as nutritional therapy for IBD [25,26], but the mechanism and regulation of NGcT during chronic intestinal inflammation are completely unknown. Therefore, in this study, for the first time, we report the regulation of NGcT mediated by B^0AT1 in villus and SN2 in crypt cells during chronic intestinal inflammation.

2. Materials and methods

2.1. Induction of chronic inflammation

Chronic intestinal inflammation was produced in rabbits as previously reported [27]. Pathogen-free New Zealand White male rabbits weighing 2–2.2 kg were intragastrically inoculated with *Eimeria magna* oocytes or sham inoculated with 0.9% NaCl (control animals). All animal handling, treatments and euthanization were carried out according to a protocol approved by the West Virginia University IACUC.

Abbreviations: NGcT, Na-glutamine co-transport; BBM, Brush Border Membrane

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2.2. Cell isolation

Animals were euthanized with an overdose of pentobarbital sodium through the ear vein. Villus and crypt cells were isolated from normal and chronically inflamed rabbit small intestine by a Ca^{++} chelation technique as previously described [27]. Briefly, a 3-ft section of ileum was filled with buffer containing 0.15 mM EDTA, 112 mM NaCl, 25 mM $NaHCO_3$, 2.4 mM K_2HPO_4, 0.4 mM KH_2PO_4, 2.5 mM L-glutamine, 0.5 mM β-hydroxybutyrate, and 0.5 mM dithiothreitol, and gassed with 95% O_2, and 5% CO_2, pH 7.4, at 37 °C. The intestine was incubated in this solution for 3 min and gently palpitated for another 3 min to facilitate cell dispersion. The fluid was then drained from the ileal loop, phenylmethylsulfonyl fluoride was added, and the suspension was centrifuged at 100 *g* for 3 min. Cells used for BBM vesicle (BBMV) preparation were frozen immediately in liquid nitrogen and stored at −80 °C until required.

2.3. Uptake studies in villus and crypt cells

Intact cell uptakes were done as previously described [27]. In brief, villus or crypt cells (100 mg wet wt) were washed and resuspended in HEPES buffer containing 0.2 mM glutamine, 4.5 mM KCl, 1.2 mM KH_2PO_4, 1.0 mM $MgSO_4$, 1.25 mM $CaCl_2$, 20 mM HEPES, and either 130 mM sodium chloride or choline chloride and was gassed with 100% O_2 (pH 7.4 at 37 °C). Ten μCi of ^{3}H-glutamine was added to 1 ml cell suspension in the HEPES buffer and 100 μl aliquots were removed at desired time interval. The uptake was arrested at 2 min by mixing with 10 ml ice-cold stop solution (choline-HEPES buffer). The mixture was filtered on 0.65 μm Millipore (HAWP) filters and washed twice with ice cold-stop solution. The filter was dissolved in 5 ml Ecoscint and the radioactivity was determined.

2.4. Na-K-ATPase measurement

Na-K-ATPase was measured as Pi liberated by the method of Forbush [28] in cellular homogenates from the same amount of cells from normal or chronically inflamed ileum as previously described [29]. Enzyme specific activity was expressed as nmoles of Pi released per milligram of protein per minute.

2.5. BBMV preparation

BBM vesicles (BBMV) from rabbit intestinal villus and crypt cells were prepared by $CaCl_2$ precipitation and differential centrifugation as previously described [29]. Briefly, frozen villus and crypt cells were thawed and suspended in 2 mM Tris–HCl buffer (pH 7.0) containing 50 mM mannitol. The suspension was homogenized and $CaCl_2$ was added to 10 mM final concentration. The suspension was then centrifuged at 8000 *g* for 15 min, and the resulting supernatant was centrifuged at 20,000 *g* for 30 min. The pellet was then suspended in 10 mM Tris–HCl buffer, pH 7.5, containing 100 mM mannitol, and homogenized. $MgSO_4$ was added to the suspension to a final concentration of 10 mM. The suspension was centrifuged at 2000 *g* for 15 min to remove debris, and the BBMV were collected by centrifugation at 27,000 *g* for 30 min. BBMV were resuspended in a medium appropriate to each experiment. BBMV purity was assured with marker enzyme enrichment (e.g., alkaline phosphatase).

2.6. Uptake studies in villus and crypt cell BBMV

BBMV uptake studies were performed by the rapid-filtration technique as previously described [21,22]. In brief, 5 μl of BBMV, resuspended in the buffer containing 100 mM choline chloride, 0.1 mM $MgSO_4$, 100 mM HEPES–Tris (pH 7.4), 50 mM mannitol, and 50 mM KCl, were incubated in 95 μl of the reaction medium containing 100 mM HEPES–Tris buffer (pH 7.4), 0.2 mM glutamine, 10 μCi [^{3}H] glutamine, 0.1 mM $MgSO_4$, 50 mM KCl, 50 mM mannitol, and 100 mM of either NaCl or choline chloride. Whenever pH 6.0 uptake buffer was needed, 100 mM HEPES–Tris was replaced with 100 mM MES–Tris. The vesicles were voltage clamped with 5.6 μM valinomycin and 15 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone. At desired times, uptake was arrested by mixing with ice-cold stop solution (50 mM HEPES–Tris buffer, pH 7.4, 0.1 mM $MgSO_4$, 50 mM KCl, and 100 mM choline chloride). The mixture was filtered on 0.45-μm Millipore (HAWP) filters and washed twice with 5 ml of ice-cold stop solution. Filters with BBMV were dissolved in Ecoscint scintillation fluid, and radioactivity retained on the filters was counted in a Beckman Coulter LS 6500 liquid scintillation counter.

2.7. RTQ-PCR studies

Total RNA was isolated from normal and chronically inflamed rabbit intestinal villus and crypt cells by use of TRIzol reagent from Invitrogen Life Technologies. Real-time quantitative PCR (RTQ-PCR) was performed using total RNA isolated by a two-step method. First-strand cDNA synthesis from total RNA was performed by using SuperScript III from Invitrogen Life Technologies using an equal mixture of oligo(dT) primer and random hexamers. The cDNA generated was used as template for real-time PCR using TaqMan Universal PCR master mix from Applied Biosystems (Foster City, CA) according to the manufacturer's protocol. 5′-GGATCCTGCTGTGCCTCA-3′, and 5′-CGAGGTAGGGTAGCG-TAGAAGT-3′ were the forward and reverse primer and 5′-FAM-CAC-CATCCGCGGAATCGAGACAAC-TAMRA-3′ was the TaqMan probe for RTQ-PCR of rabbit B^0AT1. 5′-CTGGGACAGAGGGCATTC-3′, and 5′-CGGATTTGATGATGAACAGGT-3′ were the forward and reverse primer and 5′-FAM-CCACCGTCATCTGTCTGCACAATGTTG-TAMRA-3′ was the TaqMan probe for RTQ-PCR of rabbit SN2. Rabbit β-actin was run along with the B^0AT1 and SN2 RTQ-PCR as an internal control by using rabbit β-actin-specific primers and probes. The expression of β-actin was used to normalize the expression levels of B^0AT1 and SN2 between the individual samples. The sequences of the rabbit β-actin primers and probes were 5′-GCTATTTGGCGCTGGACTT-3′, 5′-GCGGCTCGTAGCTCTTCTC-3′ and 5′-FAM-AAGAGATGGCCACGGCCGCGAAC-TAMRA-3′. All experiments were performed in triplicate and repeated (n = 5) with RNA obtained from separate animals. Serial dilution experiments of cDNA were performed to establish that the efficiency of PCR was the same between β-actin, B^0AT1 and SN2 transporters (data not shown).

2.8. Western blot studies

Polyclonal antibody against B^0AT1 was raised in goat by using the custom antibody services provided by Invitrogen. Antigenic peptide with the sequence-CDRFNKDIEFMIGHKPN-coupled to Keyhole limpet hemocyanin was used as the immunogen. SN2 polyclonal antibody was raised in chicken against antigenic peptide with the sequence CRIVPSD TEPLFSWPK by use of the same services provided by Invitrogen. Western blotting for B^0AT1 and SN2 was performed according to the standard protocols. Briefly, plasma membranes from villus cells were solubilized in RIPA buffer (50 mM Tris–HCl pH 7.4, 1% Igepal, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Equal volume of 2x SDS/sample buffer (100 mM Tris, 25% glycerol, 2% SDS, 0.01% bromphenol blue, 10% 2-ME, pH 6.8) was added and the proteins were separated on a 4–20% Ready Gel (Bio-Rad Laboratories, Hercules, CA). The separated proteins were transferred on to polyvinylidene difluoride membrane and probed with the primary antibody against rabbit B^0AT1 and SN2 overnight at 4 °C. Secondary antibodies coupled to horseradish peroxidase were used to monitor the binding of the primary antibody. ECL Western blotting detection reagent (GE Healthcare Bio-Sciences, Piscataway, NJ) was used to detect the B^0AT1 and SN2 specific signal. The resultant chemiluminescent signals were quantitated by using a Molecular

Dynamics (Sunnyvale, CA) densitometric scanner. All experiments were performed in triplicate.

2.9. Immuno-localization of B^0AT1 and SN2 in the rabbit small intestine and enterocytes

Immunolocalization of B^0AT1 and SN2 protein in the normal and chronically inflamed rabbit small intestine was determined by immunohistochemistry (IHC) technique. Normal and chronically inflamed rabbit intestinal tissue was fixed in 10% (vol/vol) neutral-buffered formalin (Richard-Allan Scientific, Kalamazoo, MI) and embedded in paraffin. Sections measuring 4 μm thick were made using a microtome and mounted on glass slides. Paraffin was removed from the sections by incubating the slides with xylene, and sections were hydrated gradually by incubating in graded ethanol. Antigen retrieval was performed by incubating the sections with 10 mM sodium citrate buffer, pH 6, at 95 °C for 5 min. Nonspecific binding sites in the tissue sections were blocked by incubation with bovine and goat serum for B^0AT1 and SN2 respectively for 1 h at room temperature. The tissue sections were then incubated overnight with B^0AT1 and SN2 primary antibody. Excess antibody was removed by washing with PBS. Sections were then incubated with bovine anti-goat IgG secondary antibody coupled to tetramethyl rhodamine isothiocyanate (TRITC; Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-chicken IgG secondary antibody coupled to FITC (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 h. Excess secondary antibody was removed by washing with PBS and the tissue sections were mounted with ProLong Gold Antifade Reagent (Invitrogen Life Technologies). Finally, the signal generated by TRITC and FITC was observed under Zeiss LSM510 confocal microscope and photographed. Localization of B^0AT1 and SN2 in the isolated villus and crypt cells from the normal and inflamed intestine was performed using the same method except that the cells were fixed in 4% paraformaldehyde and permeabilized in chilled methanol for 10 s.

2.10. Data presentation

When data were averaged means ± SE were shown, except when error bars are inclusive within the symbol. All uptakes and RTQ-PCR were done in triplicates. The number (n) for any set of experiments refers to numbers of rabbits. Student's *t*-test was used for statistical analysis.

3. Results

3.1. Whole cell glutamine uptake

In villus cells from the chronically inflamed intestine, NGcT was significantly reduced in comparison to villus cells from normal rabbit intestine (Fig. 1A). In contrast, in crypt cells during chronic enteritis NGcT was significantly increased (Fig. 1B). The alteration of NGcT during chronic intestinal inflammation may be either secondary to altered cellular Na gradient or at the level of the Na-glutamine co-transporter in the BBM or both. Since Na-K-ATPase provides the favorable Na gradient for NGcT, we measured Na-K-ATPase activity.

3.2. Na-K-ATPase activity

In villus cells from the chronically inflamed intestine, Na-K-ATPase activity was significantly inhibited (Fig. 1C) while it was significantly increased in crypt cells compared to normal (Fig. 1D). Thus, inhibition of NGcT in villus cells and stimulation in crypt cells during chronic

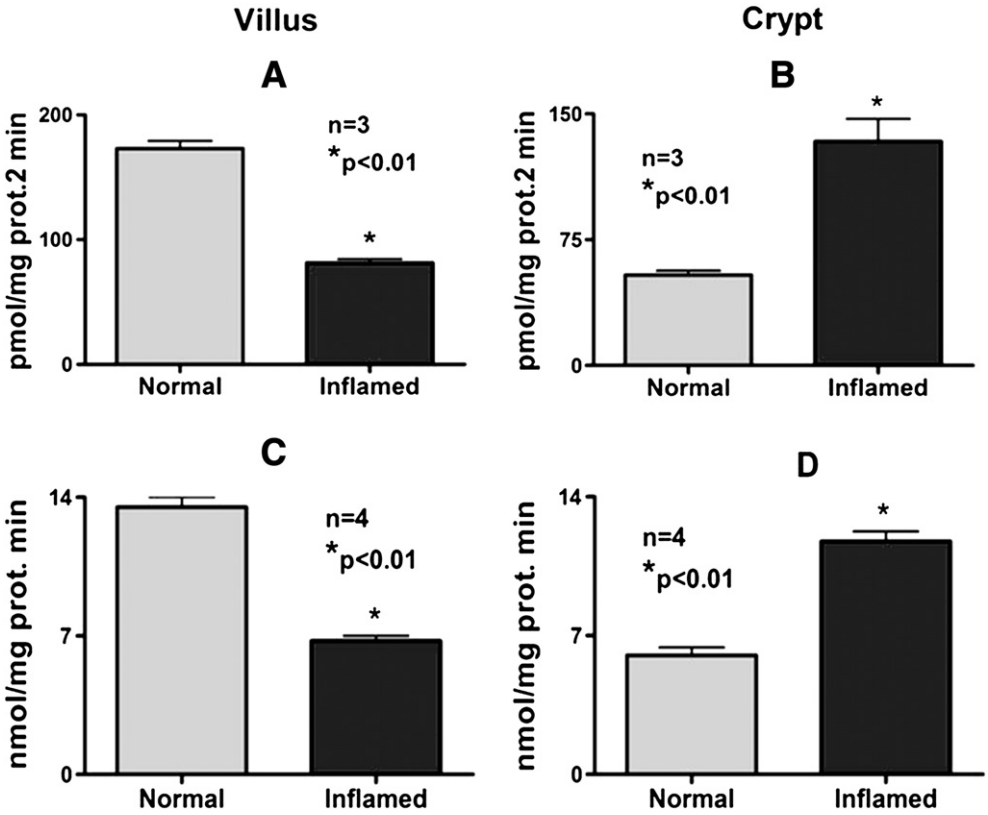

Fig. 1. Effect of chronic intestinal inflammation on Na-glutamine co-transport. A. Na-dependent ^{3}H-glutamine uptake (pmol/mg protein) is significantly diminished in villus cells from the chronically inflamed intestine (173 ± 6.3 in normal; 81.4 ± 1.8 pmol/mg protein.2 min in villus cells from inflamed intestine, n = 3, *p<0.01). B. Na-dependent ^{3}H-glutamine uptake is significantly increased in crypt cells during chronic enteritis (53.9 ± 2.7 pmol/mg protein.2 min in normal; 133.5 ± 13.5 in crypt cells from inflamed intestine, n = 3, *p<0.01). C. Na-K-ATPase which provides the favorable Na gradient for Na-glutamine co-transport is decreased in villus cells from the chronically inflamed intestine (13.5 ± 0.5 nmol/mg protein.min in normal villus cells; 6.7 ± 0.3 in inflamed, n = 4, *p<0.01). D. Na-K-ATPase is significantly increased in crypt cells from the chronically inflamed intestine (6 ± 0.4 nmol/mg protein.min in normal; 11.8 ± 0.5 during chronic enteritis, n = 4, *p<0.01).

enteritis are, at least partially, secondary to altered Na extrusion capacity of cells. We then determined if NGcT was directly affected at the co-transporter level in the BBM of villus and crypt cells.

3.3. BBM vesicle (BBMV) glutamine uptake in villus and crypt cells

NGcT was significantly decreased in villus cell BBMV (Fig. 2A) but increased in crypt cell BBMV during chronic intestinal inflammation (Fig. 2B). These results indicate that the alteration of the NGcT during chronic intestinal inflammation is secondary to an alteration at the level of co-transporter in the villus and crypt cell BBM.

3.4. BBM vesicle (BBMV) glutamine uptake in all enterocytes

To determine whether during intestinal inflammation there is an overall net Na-dependent glutamine co-transport inhibition, stimulation or no change, we looked at uptake in all enterocytes from normal and chronically inflamed intestine. BBMV uptake of enterocytes in normal was 200 ± 16.8 pmol/mg protein·30 s and in chronically inflamed it was 77 ± 8.4 pmol/mg protein·30 s, ($n = 3$, $p < 0.003$). These results indicate that despite an apparent attempt at compensation by the crypt cell, there is net overall Na-dependent glutamine uptake inhibition in the chronically inflamed intestine as compared to the normal. To determine the mechanism of these alterations we performed kinetic studies.

3.5. Kinetic studies

Kinetic studies demonstrated that the mechanism of inhibition of NGcT in villus cells was secondary to a reduction in the maximal rate of uptake (V_{max}) without a change in the affinity (Fig. 2C). However, in crypt cells the mechanism of stimulation of NGcT was secondary to increased affinity ($1/K_m$) for glutamine without a change in the V_{max} (Fig. 2D). These studies indicate that the mechanism of inhibition of NGcT in villus cells is likely secondary to reduced co-transporter numbers, while the mechanism of stimulation of NGcT in crypt cells during chronic enteritis is likely secondary to enhanced affinity of the co-transporters for glutamine.

Our laboratory has shown in previous studies that B^0AT1 and SN2 are responsible for Na-glutamine co-transport in villus and crypt cells respectively [21,22]. Thus, to explore the mechanism of alteration at the level of the specific co-transporter we performed molecular studies.

3.6. Relative abundance of B^0AT1 and SN2 mRNA

To determine the molecular mechanisms of alterations of B^0AT1 and SN2 we quantified mRNA abundance by RTQ-PCR. The abundance of B^0AT1 mRNA in villus cells and SN2 mRNA in crypt cells was found to be reduced during chronic enteritis (Fig. 3A and B).

3.7. Quantitation of B^0AT1 and SN2 protein in BBM

Since mRNA levels may not necessarily correlate with protein in the BBM, Western blot analyses of B^0AT1 and SN2 protein were performed on extracts of BBM. B^0AT1 protein levels were markedly diminished in villus cell BBM during chronic enteritis (Fig. 3C) and this was confirmed by densitometric quantitation (Fig. 3E). In contrast, in crypt cell BBM, SN2 was unaffected during chronic intestinal inflammation (Fig. 3D and F). For loading control we analyzed the expression of Ezrin using mouse anti Ezrin monoclonal antibody.

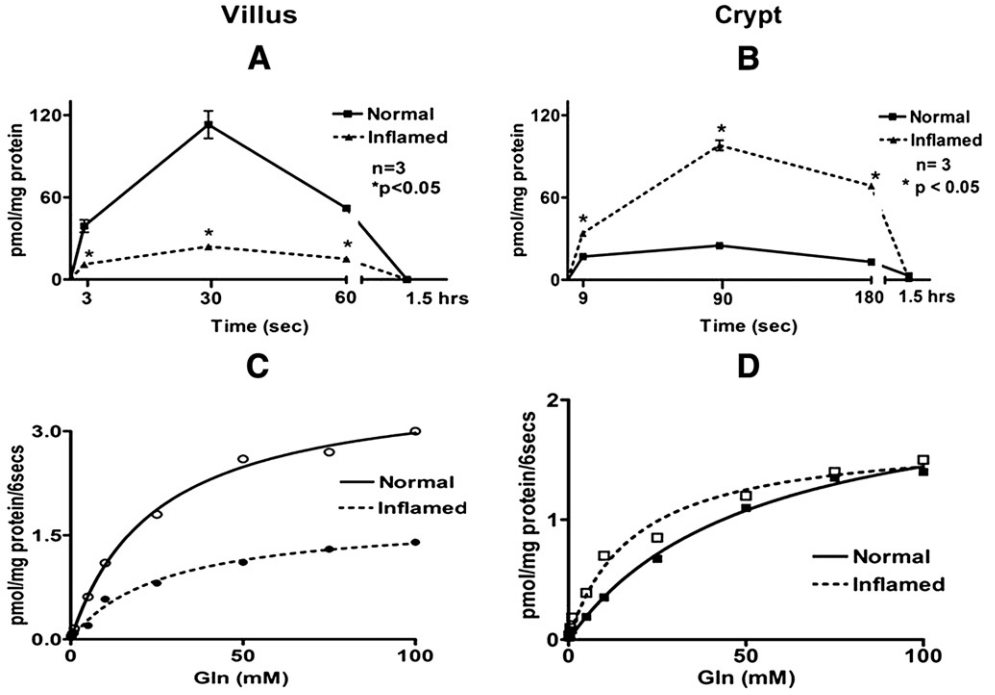

Fig. 2. BBMV studies of NGcT during chronic intestinal inflammation. The Na-dependent glutamine uptake was determined as a function of ^{3}H-glutamine uptake in the presence of extracellular Na minus uptake in the absence of extracellular Na. A. Na-dependent glutamine uptake was significantly decreased in villus cell BBMV from the chronically inflamed intestine at all time points measured ($n = 3$, $^*p < 0.05$). B. In contrast, Na-dependent glutamine uptake was significantly increased in crypt cell BBMV during chronic intestinal inflammation ($n = 3$, $^*p < 0.05$). C. Kinetic studies of glutamine uptake of villus BBMV from the normal and inflamed intestine. The figure is representative of 4 BBMV experiments from 4 different animals. The figure shows Na-dependent glutamine uptake as a function of varying concentrations of extracellular glutamine. As the concentration of extracellular glutamine was increased, the uptake of glutamine was stimulated and subsequently became saturated in the villus cell BBMV from the normal and chronically inflamed intestine. Analysis of the data by Michaelis–Menton equation yielded the kinetic parameters. In villus cells the maximal rate of uptake (Vmax) was significantly diminished (3.7 ± 0.3 pmol/mg protein.6 s in normal; 1.75 ± 0.1 pmol/mg protein.6 s in inflamed, $n = 3$, $^*p < 0.05$). However, the affinity was unchanged (24.6 ± 2.8 mM in normal; 26.7 ± 4.3 mM in inflamed). D. Kinetics of glutamine uptake in crypt cell BBMV from the normal and inflamed intestine. In crypt cells the maximal rate of uptake (Vmax) was not altered (2.2 ± 0.2 pmol/mg protein.6 s in normal, 1.9 ± 0.1 pmol/mg protein.6 s in inflamed). However, the affinity (Km) was increased significantly during chronic intestinal inflammation (51.8 ± 3.2 mM in normal; 18.07 ± 1.6 mM in inflamed, ($n = 3$, $^*p < 0.05$).

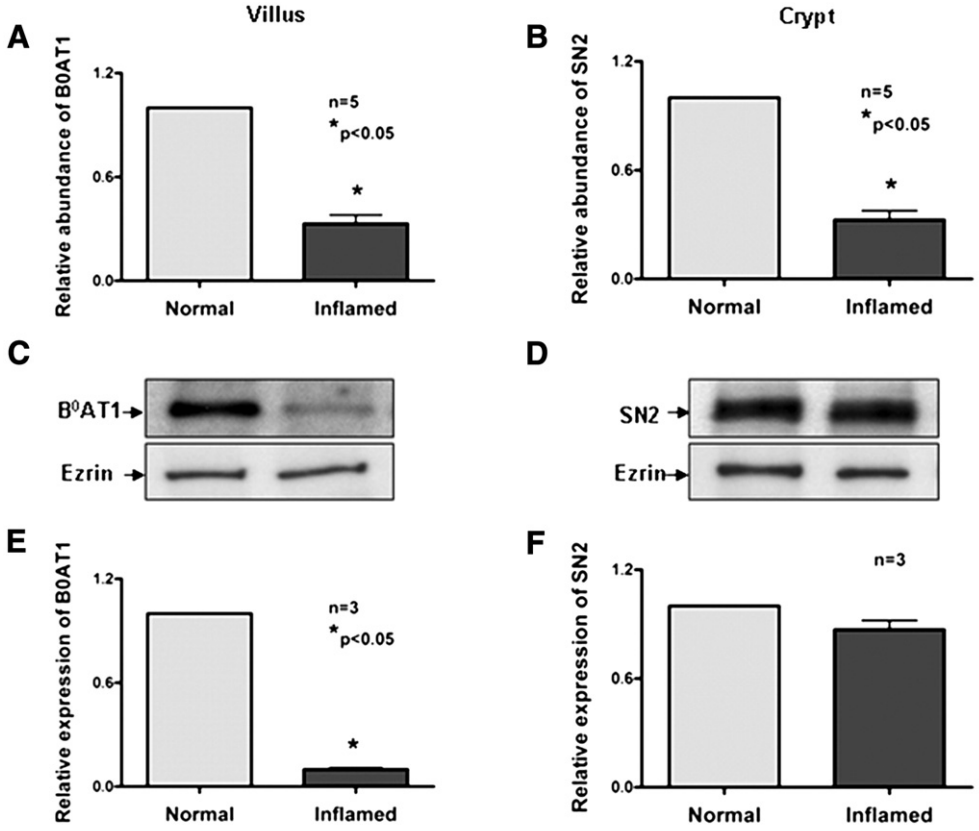

Fig. 3. Effect of chronic intestinal inflammation on B^0AT1 and SN2 mRNA and protein. A. In villus cells, B^0AT1 mRNA expression quantitated by RTQ-PCR was significantly reduced during chronic intestinal inflammation ($n=5$, $^*p<0.05$). B. SN2 mRNA abundance was also decreased in crypt cells during chronic enteritis. The expression of mRNA was normalized using β-actin in all conditions. Since, mRNA levels may not correlate with functional protein levels, immunoreactive protein levels of B^0AT1 and SN2 were measured in villus and crypt cell BBM C. Representative figure of B^0AT1 Western blot from 3 independent experiments each with different animals. B^0AT1 protein is markedly diminished in villus cell BBM during chronic enteritis. D. In contrast, in crypt cells SN2 protein levels were unaffected in the BBM during chronic intestinal inflammation. Ezrin was used as loading control for all conditions. E and F. Densitometric quantitation of Western blots of villus and crypt cells respectively confirmed the observations shown in panels C and D.

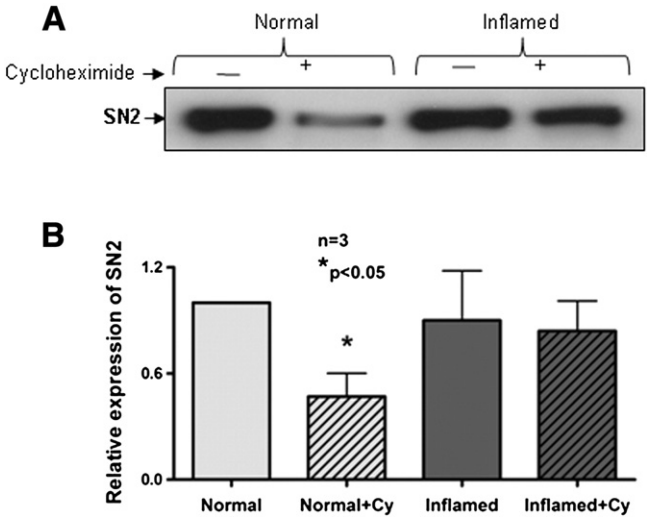

Fig. 4. SN2 protein stability. Down regulation of SN2 mRNA expression and unchanged SN2 protein level at BBM may indicate increased stability of existing protein on BBM. A. Representative Western blot of SN2 in normal and inflamed crypt cells which were treated with protein synthesis inhibitor cycloheximide (15 μg/ml) in-vitro for 24 h. As expected cycloheximide decreased SN2 protein expression in crypt cells from the normal but not from the chronically inflamed intestine. B. Densitometric quantitation confirmed these findings. Thus, these data indicate that the stability of SN2 protein is increased in crypt cells BBM during chronic inflammation.

In villus cells, message and protein levels of B^0AT1 were inhibited in tandem during chronic enteritis. In contrast, SN2 mRNA expression was decreased in the crypt cell whereas the protein level in BBM remained unchanged during inflammation. This may either be due to increased de novo protein synthesis or increased stability of the existing protein in the BBM during chronic inflammation. To examine the mechanism, we treated the crypt cells with protein synthesis inhibitor cycloheximide (15 μg/ml) in-vitro for 24 h. We measured lactate dehydrogenase activity to assess the extent of cytotoxicity of cycloheximide treatment and observed that the cell viability was not significantly affected in the treated cells (data not shown). Cycloheximide treatment significantly inhibited the expression of SN2 (~50%) in the crypt cells isolated from normal rabbit intestine, but there was no significant change in SN2 expression between inflamed and inflamed cycloheximide-treated cells (Fig. 4A and B). These observations indicate that the stability of SN2 protein is increased during chronic inflammation in the BBM of the crypt cells which helps to maintain its level in the BBM despite decreased mRNA levels.

3.8. Localization of B^0AT1 and SN2 along the villus-crypt axis

Previous immunohistochemical studies demonstrated the presence of B^0AT1 along the villus, whereas SN2 was present in the crypts in the normal rabbit small intestine [21,22]. In the chronically inflamed intestine B^0AT1 expression is significantly diminished in the villus region (Fig. 5A and B). In contrast, SN2 expression is increased in multiple rows

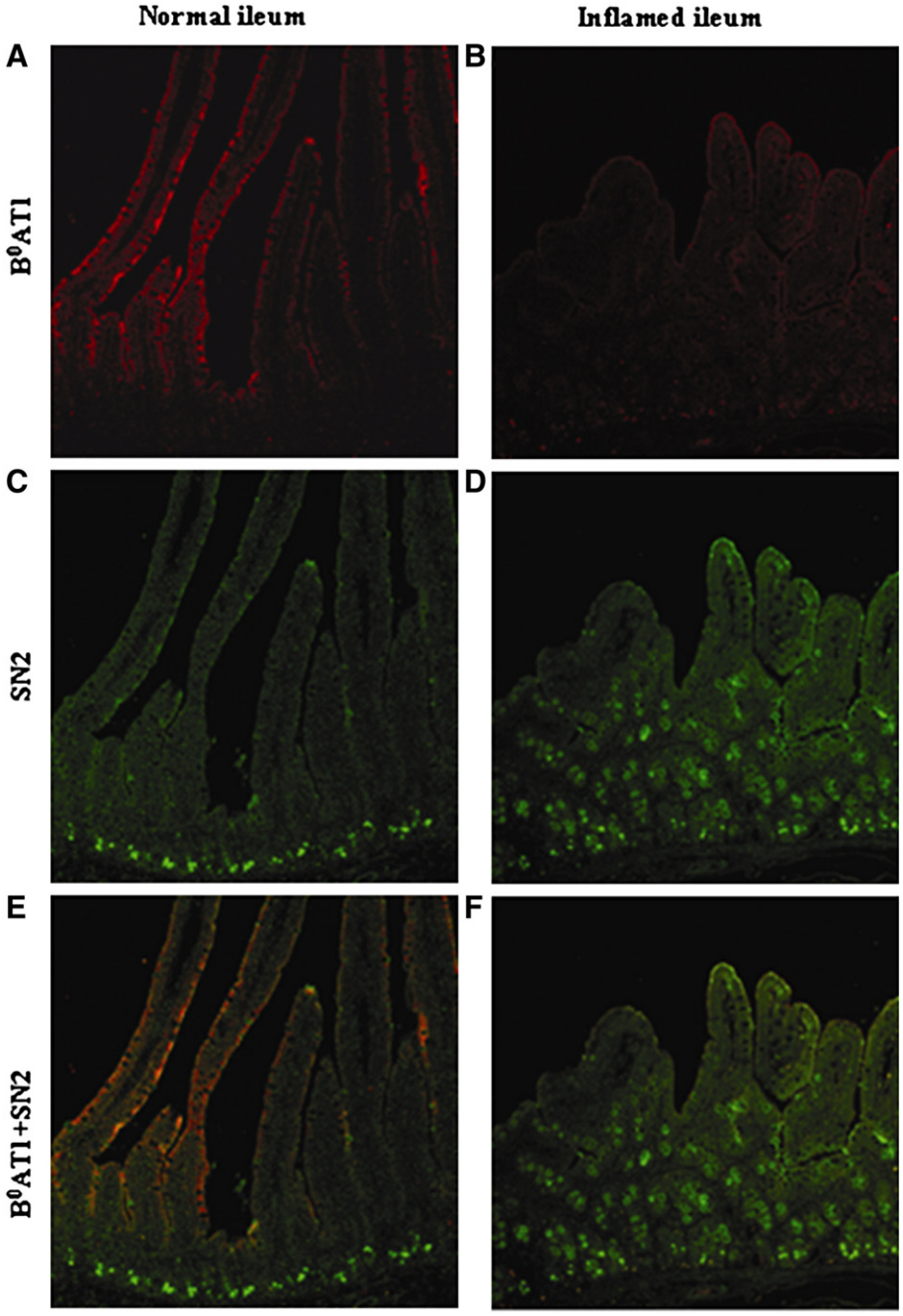

Fig. 5. Distribution of B^0AT1 and SN2 along the intestinal villus-crypt axis. A. B^0AT1 expression is robustly demonstrated along the villus axis in the normal intestine. B. B^0AT1 expression is diminished in the chronically inflamed intestine. C. In contrast, SN2 is only expressed in the crypt region in normal intestine. D. SN2 expression is increased in multiple rows of crypts cells in chronically inflamed intestine. E. Co-localization of both B^0AT1 and SN2 demonstrates the unique distribution along the villus-crypt axis in the normal intestine. F. Co-localization studies confirmed the alterations in B^0AT1 and SN2 along villus-crypt axis in the chronically inflamed intestine.

of crypts cells (Fig. 5D) consistent with crypt hyperplasia known to occur during chronic enteritis [30] as compared to the normal intestine (Fig. 5C). Co-localization of both B^0AT1 and SN2 with our custom antibodies demonstrated their unique distribution in the normal intestine in Fig. 5E and their alterations during chronic enteritis in Fig. 5F. These results indicate that B^0AT1 protein level is markedly decreased along the villus axis during chronic enteritis. However, SN2 is increased in crypt region due to the increased number of crypt cells during chronic enteritis. To analyze the amount of B^0AT1 and SN2 in individual villus and crypt cells we performed immunocytochemistry.

3.9. Expression of B^0AT1 in villus and SN2 in crypt cells

Immunocytochemical studies were performed using isolated villus and crypt cells from normal and chronically inflamed rabbit intestine. B^0AT1 is distinctly expressed in the BBM of normal villus cells (Fig. 6A) and it is significantly diminished in these cells from the chronically inflamed intestine (Fig. 6B and C). SN2 is expressed in the BBM of crypt cells isolated from the normal intestine (Fig. 6D); however, it remains unaffected in these cells from the chronically inflamed intestine (Fig. 6E and F). These studies indicate that B^0AT1 is markedly

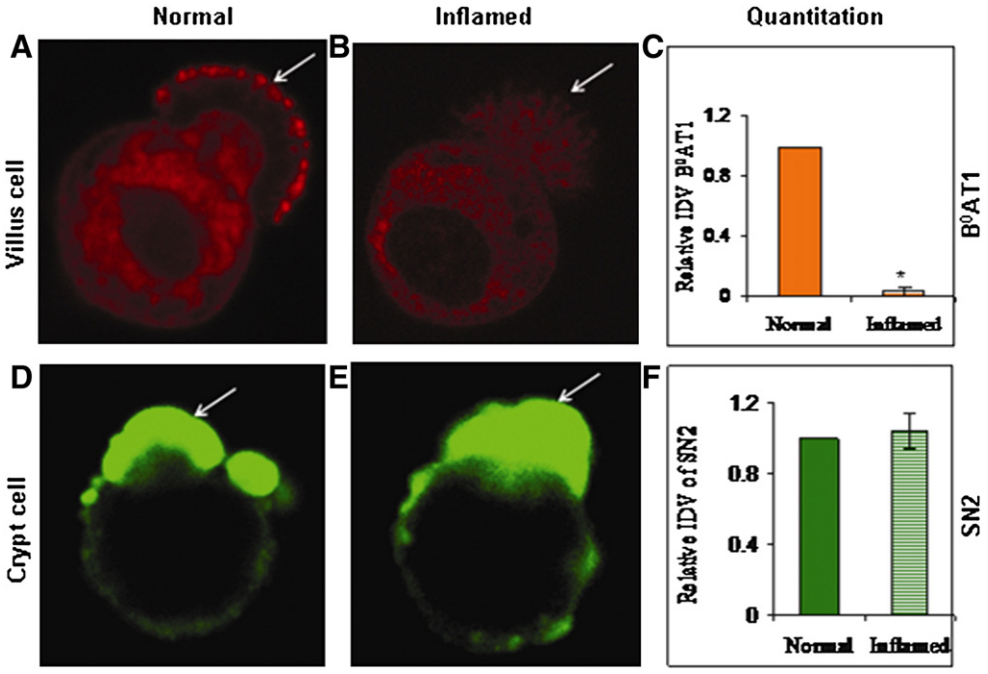

Fig. 6. Immunocytochemisty of B^0AT1 in villus cell and SN2 in crypt cells. To quantitate B^0AT1 and SN2 protein at the BBM, immunocytochemistry was performed in isolated villus and crypt cells. A. Representative villus cell demonstrating that B^0AT1 is distinctly expressed in the BBM of normal intestine. B. B^0AT1 expression is diminished in villus cells from the chronically inflamed intestine. C. Densitometric analysis showed that the alteration is significant ($n=6$, $^*p<0.05$). D. Representative crypt cell demonstrating that SN2 is expressed only in the BBM in the normal intestine. E. SN2 expression is unchanged in the BBM of crypt cells from the chronically inflamed intestine. F. Densitometric quantitation confirmed these findings. These data indicate that B^0AT1 is present in the BBM of villus cells and that it is markedly diminished during chronic intestinal inflammation. In contrast, SN2 is present in the BBM of crypt cells and its levels are unaffected in the chronically inflamed intestine.

diminished in villus cell BBM while SN2 is unaffected in crypt cell BBM from the chronically inflamed intestine.

4. Discussion

The significance of the amino acid glutamine for the normal growth and differentiation of intestinal enterocytes, as well as for numerous other critical functions has been well documented. Our laboratory has previously demonstrated that Na dependent co-transport pathways facilitate glutamine absorption but through different co-transporters in the villus and the crypt cells [21,22]. In the rabbit small intestine we reported that Na-glutamine co-transport is facilitated by B^0AT1 in the BBM of villus cells, while in the crypt cells it is facilitated by SN2 [22]. While the assimilation of glutamine may be important to maintain the normal health of the small intestinal mucosa it may play a more vital role in chronic pathophysiological conditions to help restore the mucosal integrity and function. Thus, we studied how glutamine assimilation may be regulated in a mammalian model of chronic small intestinal inflammation.

NGcT mediated by B^0AT1 in villus cells was inhibited during chronic intestinal inflammation. The mechanism of inhibition was secondary to both an altered Na extruding capacity of the villus cells as well as a direct effect at the level of the co-transporter on the BBM. At the level of the co-transporter, B^0AT1 was inhibited secondary to a reduction in the co-transporter numbers on the BBM. This is most likely transcription mediated as reflected by a decrease in the steady state mRNA of B^0AT1 in villus cells from the chronically inflamed intestine. In contrast, in crypt cells from the chronically inflamed intestine NGcT mediated by SN2 was significantly increased. This increase was also at least in part secondary to the altered Na-K-ATPase activity in the basolateral membrane of these cells. Nevertheless at the level of the co-transporter, SN2 was also directly affected in the crypt cells during chronic intestinal inflammation. The mechanism of stimulation of SN2 was found to be secondary to an increase in the affinity of co-transporter for glutamine without a change in the number of co-transporters. Chronic intestinal inflammation leads to villus atrophy, crypt hyper proliferation and a variety of biochemical and functional abnormalities especially electrolyte and nutrient malabsorption.

Previous reports from our lab and others have shown significant inhibition of electrolyte [31,32], glucose [29], alanine [33], bile acid [34] and peptide [35] transport in villus cells during chronic intestinal inflammation. Several novel observations were made in the course of this study. NGcT villus cells were inhibited by a mechanism that appears to be unique to glutamine among other amino acids. For example, we have previously demonstrated that sodium neutral amino acids co-transport, specifically sodium alanine co-transport, mediated by ATB^0 in villus cells in the rabbit small intestine was inhibited secondary to a decrease in the affinity of the co-transporter for alanine without change in the co-transporter numbers [33]. Similarly, in rat intestinal epithelial cells, we demonstrated that the sodium alanine co-transport mediated by ASCT1 was inhibited by a chronic immune inflammatory mediator LTD_4, also by decreasing the affinity of the co-transporter for alanine without a change in the number of co-transporters [36]. Further, we had also demonstrated that H^+-dipeptide co-transport in rabbit intestinal villus cells was also inhibited during chronic intestinal inflammation secondary to a decrease in the affinity of the co-transporter for the dipeptide without a change in the number of co-transporters on the BBM [35]. In contrast, Na glutamine co-transport in villus cells is inhibited secondary to a decrease in co-transporter number, but not affinity of the transporter to its substrate during chronic enteritis. Thus, B^0AT1 appears to be uniquely regulated during chronic intestinal inflammation.

Though, folate and thiamine transports have shown to be present in mouse crypt cells [37,38], NGcT mediated by SN2 is the only absorptive transport pathway to be demonstrated in otherwise secretory crypt cells of the rabbit small intestine. While all the other sodium solute co-transport pathways present only on the BBM of villus cells have been

demonstrated to be inhibited by different mechanisms during chronic intestinal inflammation, the Na-glutamine co-transporter SN2 found in the BBM of crypt cells, in contrast is stimulated during chronic enteritis. Further, while NGcT mediated by B^0AT1 in villus cells is inhibited secondary to decrease in co-transporter numbers, the same transport process mediated by SN2 in the crypt cells is stimulated secondary to an alteration in the affinity and increase in protein stability of the co-transporter without a change in the number of the BBM co-transporters. The increased stability of the SN2 protein seen during inflammation, may be a result of changes in the phosphorylation or nitrosylation levels of the protein itself which could be mediated by a multiple of immune inflammatory mediator or cell signaling pathways. Therefore, the exact mechanism of alteration of the SN2 protein and immune-inflammatory mediators that may be responsible for these alterations are to be elucidated.

It is conceivable that crypt hypertrophy and stimulation of SN2 in crypt cells are compensatory mechanisms to offset the loss of glutamine absorption due to villus atrophy and inhibition of B^0AT1 during chronic intestinal inflammation [39–43]. But, the stimulation of glutamine absorption by the crypt cells does not satisfy the overall demand for glutamine by the enterocytes. This is likely because the villus absorptive area (BBM) is much, much greater than that of the crypt, on the order of 1000–10,000×, so the relative number of transporters in toto will still be strongly biased in the direction of the villus thus, accounting for the observed inhibition of overall glutamine uptake.

As glutamine is critical for intestinal epithelial growth and differentiation, better understanding of the immune regulation of glutamine absorption during chronic intestinal inflammation may lead to more efficacious and novel nutritional treatments for IBD. Further, there is increasing evidence in supporting a protective role of glutamine supplementation during DMBA induced carcinogenesis [44,45], cancer progression and chemotherapy efficacy [46], in postoperative complication in CRC surgery [47]. It has also been reported that SN2 expression is upregulated in cancerous tissues from eye, kidney, head and neck, in contrast, neoplastic tissues did not exhibit increased expression of SN2 [48]. Thus it could be speculated that NGcT SN2 may be a potent therapeutic target for regulation of the process of carcinogenesis.

In conclusion, multiple novel observations have resulted from this study. NGcT mediated by B^0AT1 in villus and SN2 in crypt cells is altered in chronically inflamed intestine. While B^0AT1 was reduced in villus, SN2 was increased in crypt cells during chronic enteritis. The mechanism of these alterations was also distinct — B^0AT1 was inhibited secondary to a reduction in mRNA expression and the number of co-transporters while SN2 was increased secondary to an apparent increase in the affinity of the co-transporters. Thus, glutamine assimilation is uniquely regulated in the chronically inflamed intestine.

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