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Inducible nitric oxide regulates intestinal glutamine assimilation during chronic intestinal inflammation

Subha Arthur, Uma Sundaram *

Department of Clinical and Translational Sciences, Joan C Edwards School of Medicine, Marshall University, 1600 Medical Centre Drive, Huntington, WV 25701, United States

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ABSTRACT

To facilitate assimilation of glutamine, different Na-dependent glutamine absorptive pathways are present in the rabbit small intestine, specifically B0AT1 in villus and SN2 in crypt cell brush border membrane. Further, both are uniquely regulated in the chronically inflamed intestine. B0AT1 is inhibited secondary to reduced number of brush border membrane (BBM) co-transporters while SN2 is stimulated secondary to an increased affinity for glutamine. These unique changes are reversible by treatment with a broad spectrum immune modulator such as glucocorticoids. However, whether inducible nitric oxide (iNO), known to be elevated in the mucosa of the chronically inflamed intestine, may be responsible for these co-transporter alterations is not known. In the present study, treatment of chronically inflamed rabbits with L-NIL, a selective inhibitor of iNO synthase, reversed the inhibition of B0AT1 in villus and the stimulation of SN2 in crypt cells. At the level of the co-transporter in the brush border membrane, inhibition of iNO production reversed the inhibition of villus B0AT1 by restoring the co-transporter numbers while the stimulation of crypt SN2 was reversed back to normal by restoring its affinity for glutamine. Western blot analyses of BBM proteins also confirmed the kinetic studies. Thus, L-NIL treatment restores the uniquely altered Na-glutamine co-transporters in the enterocytes of chronically inflamed intestine. All these data indicate that iNO functions as an upstream immune modulator directly regulating glutamine assimilation during chronic intestinal inflammation.

1. Introduction

Inflammatory Bowel Disease (IBD) has been ascertained through years of research as a multifactor disorder influenced by several factors such as host genetic susceptibility, the intestinal microbiota, environmental factors, and the host immune system [1]. Of all these factors, inflammation of the intestinal mucosa mediated by the dysregulation of the host immune system is the major contributor to the severity and progression of the disease in the patients with IBD.

Also recently, there has been increasing recognition of the role of different immune-inflammatory mediators in the causation of the most important sequelae of IBD: malabsorption, diarrhea, malnutrition and weight loss [1]. Impairment in the absorption of NaCl resulting in diarrhea has been well established in human IBD and in animal models of chronic intestinal inflammation. Similarly, reduction in the absorption of a variety of nutrients resulting in malnutrition and weight loss has also been well documented in IBD. In a rabbit model of IBD, our laboratory has shown unique alterations in a variety of Na-dependent nutrient absorptive pathways [2–5]. We have demonstrated that assimilation of glucose, amino acids, dipeptide and bile acids are affected in the chronically inflamed intestine. At the cellular level the alteration is both secondary to the Na-extruding capacity of the enterocyte as well as a direct effect on the co-transporters in the brush border membrane (BBM). However, at the level of the protein in the BBM, the effects appear to be more unique. For example, Na-glucose (SGLT1), Na-amino acid (ASCT1) and Na-bile acid (ASBT) are all inhibited in the chronically inflamed intestine. However, at the BBM level each is inhibited by a different mechanism; SGLT1 secondary to a reduction in BBM co-transporter numbers, ASCT1 secondary to a reduction in the affinity of the co-transporter for the amino acid and ASBT secondary to both a reduction in the BBM co-transporter numbers as well as reduced affinity of the co-transporter for the bile acid [2,4]. These numerous alterations in nutrient absorptive pathways in the chronically inflamed intestine suggest that immune cell derived mediators uniquely regulate different nutrient assimilation transporters.

Glutamine is the principal respiratory fuel of the enterocytes in normal physiological conditions and is vital for restoring the health of the intestine in pathophysiological states (e.g. inflammatory bowel disease, IBD). In fact, recent research on beneficial effects

* Corresponding author. Department of Clinical and Translational Sciences, Joan C Edwards School of Medicine, Marshall University, 1600 Medical Centre Drive, Huntington, WV 25701, United States. Fax: +1 304 691 1840.
E-mail address: sundaramu@marshall.edu (U. Sundaram).

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of glutamine has upgraded glutamine from a non-essential to a 'conditionally essential' amino acid [6–12]. This important nutrient is assimilated by enterocytes through a sodium dependent co-transport process in the BBM. Through our previous publications we have demonstrated that Na-dependent glutamine absorption occurs via B0AT1 in villus cells while SN2 mediates the same in crypt cells in the rabbit small intestine [13,14]. Our laboratory has also shown in the rabbit model of chronic intestinal inflammation which resembles human IBD, these co-transporters are uniquely affected [3]. Precisely, B0AT1 was inhibited in villus cells secondary to a decrease in the BBM co-transporter numbers. In contrast, in the chronically inflamed intestine, SN2 was stimulated in crypt cells secondary to an increase in its affinity for glutamine. These unique alterations appear to be dynamically regulated in the chronically inflamed intestine, where it seems that the stimulation of SN2 might compensate for the reduction of Na-glutamine uptake by villus cells. On the contrary, there is still a net deficiency in Na-dependent glutamine uptake in the inflamed ileum. Though SN2 is stimulated it may not completely satisfy the overall demand for glutamine by the enterocytes in the inflamed intestine [3] This net decrease in mucosal glutamine uptake can be validated by another study conducted in human intestinal samples where it was demonstrated that there is a significant decrease in mucosal glutamine levels in inflamed ileum [15]. A follow-up study in the rabbit model of chronic intestinal inflammation revealed that treatment with a broad spectrum immune-modulator, methyl prednisolone, reversed the regulation of BBM co-transporters B0AT1 and SN2 to their normal function by restoring the exact mechanism which resulted in their alteration [16]. All these findings suggest that immune-inflammatory mediators known to be elevated in the chronically inflamed intestine may be responsible for the unique changes in Na-glutamine co-transport.

An important immune inflammatory mediator that is known to play a key role in the pathogenesis of inflammation is the signaling molecule nitric oxide (NO). In fact, as early as in the 1990s, it was established that inducible NO plays a detrimental role in a variety of human chronic inflammatory diseases [17,18]. Additionally, several experimental studies showed that the inhibition of iNO synthase reduced inflammation, thus providing evidence to establish its involvement as a mediator of inflammation [19–22]. With respect to chronic intestinal inflammation, many experimental studies have successfully demonstrated that selective inhibition of iNO synthase reduces the tissue damage observed following chronic up-regulation of iNO seen during inflammation [23–25]. Though a large amount of data regarding the role of iNO in a variety of human diseases has resulted over the years, we are still far from understanding the precise role of iNO in the regulation of nutrient transporters in chronically inflamed intestine. Given this background, the aim of the present study was to understand if and how BBM Na-glutamine co-transports may be regulated by iNO during chronic intestinal inflammation and to understand the molecular mechanisms involved in their regulation.

2. Methods

2.1. Induction of chronic inflammation and drug treatment

Chronic intestinal inflammation was induced in male New Zealand white rabbits (Charles River Laboratories International, Inc.) with intragastric inoculation of *Eimeria magna* oocytes as previously reported [26]. Normal rabbits and rabbits with chronic intestinal inflammation were treated intramuscularly with l-N(6)-(1-Iminoethyl)-lysine (L-NIL; 0.1 mg/day/kg body weight) or saline for 2 days (days 12 and 13 post inoculation of oocytes). Euthanasia was done on the 14th day post inoculation with an overdose of pentobarbital sodium (120 mg/kg) through the ear vein according to the Institutional Animal Care and Use Committee regulations.

2.2. Cell isolation

Villus and crypt cells were isolated from the intestine by a calcium chelation technique as previously described [26]. Previously established criteria were also utilized to validate good separation of villus and crypt cells [26]. Briefly, a 3-ft section of ileum was filled with cell isolation buffer (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; gassed with 95% O_2 and 5% CO_2; pH 7.4) at 37 °C for 3 minutes. The intestine was then gently palpitated for another 3 min to facilitate cell dispersion. The fluid with the isolated cells was then drained from the ileal loop, phenylmethylsulfonyl fluoride was added, and the suspension was centrifuged at 100 *g* for 3 min. The cell pellet was frozen immediately by plunging the tube in liquid nitrogen and stored at –80 °C until required.

2.3. Intact cell uptake studies

Uptake studies in intact villus and crypt cells and in BBMV prepared from the villus and crypt cells were done as previously described [26]. In brief, 100 mg wet weight of the cells 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). ^{3}H-glutamine (10 μCi) was added to 1 ml live cell suspension in the HEPES buffer and 100 μl aliquots were removed at 2 minute time interval. The uptake was arrested by mixing with 10 ml ice-cold choline-HEPES buffer. The ice-cold mixture was then 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 in a scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA).

2.4. Na/K ATPase measurement

Na/K ATPase was measured as P_i liberated [27] in cellular homogenates from the same amount of cells from normal or inflamed intestine as previously described [28]. Enzyme specific activity was expressed as nanomoles of P_i released per milligram protein per minute.

2.5. BBM vesicles (BBMV) preparation

BBMV from rabbit ileal villus and crypt cells were prepared by $CaCl_2$ precipitation and differential centrifugation as previously described [26]. Briefly, villus and crypt cells were suspended in 2 mM Tris–HCl buffer (pH 7.0) containing 50 mM mannitol and homogenized. $CaCl_2$ was added to the homogenate to a final concentration of 10 mM and centrifuged at 8000 *g* for 15 min, and the resulting supernatant was again centrifuged at 20,000 *g* for 30 min. The resultant pellet was then suspended in 10 mM Tris–HCl buffer, pH 7.5, containing 100 mM mannitol. $MgSO_4$ was added to the suspension to a final concentration of 10 mM and centrifuged at 2000 *g* for 15 min to remove debris. BBMV were collected as a pellet by centrifugation at 27,000 *g* for 30 min. BBMV were resuspended in a medium appropriate to each experiment.

2.6. Uptake studies in villus and crypt cell BBMV

Rapid-filtration technique was used to conduct BBMV uptake experiments [26]. Briefly, 5 μl of BBMV suspended in a vesicle medium (100 mM choline chloride, 0.1 mM $MgSO_4$, 100 mM HEPES–Tris (pH 7.4), 50 mM mannitol, and 50 mM KCl) was incubated in 95 μl of the reaction medium (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

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