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Original article

Alanyl-glutamine restores maternal deprivation-induced TLR4 levels in a rat neonatal model

Khaly Mbodji [a], Stéphanie Torre [a,b], Verena Haas [c], Pierre Déchelotte [a,d], Rachel Marion-Letellier [a,*]

[a] Appareil Digestif Environnement Nutrition (ADEN EA 4311), University of Rouen, I.F.R. 23, Institute of Biomedical Research, 22, boulevard Gambetta, 76183 Rouen cedex, France
[b] Department of Neonatal Medicine, Rouen University Hospital, 1 rue de Germont, 76000 Rouen, France
[c] Gastroenterologie, Hepatologie und Endokrinologie, Charitéplatz 1, 10117 Berlin, Germany
[d] Nutrition Unit, Rouen University Hospital, 1 rue de Germont, 76031 Rouen cedex, France

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SUMMARY

Background & aims: Increased intestinal permeability of Irritable bowel syndrome (IBS) patients has been recently associated with a decreased level of glutamine synthetase. Neonatal maternal deprivation (NMD) is considered as an IBS-like model. The aim of our study was to investigate whether early nutritional intervention with glutamine may attenuate the deleterious impact of early life stress on gut barrier function in NMD.

Methods: 124 rat pups were separated from their dam 3 h daily during postnatal days 2–14, or left undisturbed (NS). Separated rats received by gavage either with alanyl-glutamine (GLN), an isonitrogenous amino acid mix (AA, control) or an equal amount of drinking water (H_2O). Production of cytokines was measured by multiplex, expression of COX-2, PPARγ, tight junction proteins and TLR4 by western blot. Intestinal permeability was studied by Lactulose/Mannitol test.

Results: Treatment of pups with GLN or AA abolished the decrease in body weight observed in NMD. Treatment with GLN decreased in the colon (i) TLR4 expression at D20, (ii) IL-2 and -10 productions at D60 (iii) protein expression of occludin at D20 compared to AA. GLN also decreased colon expression of COX2 and PPARγ at D60 compared to NS. Colon production of IFNγ is significantly reduced by GLN compared to H_2O. No significant change in intestinal permeability was observed.

Conclusions: These results showed that an early nutritional intervention with alanyl-glutamine specifically abolished the up-regulation of TLR4 expression in NMD. Glutamine may be evaluated as a potential treatment for IBS patients.

1. Introduction

Irritable bowel syndrome (IBS) is a highly prevalent disease (4.7% in France[1]) with characteristic symptoms such as abdominal pain, bloating or illness-related anxiety. Molecular mechanisms underlying IBS remains to be elucidated. Nevertheless, stress is often associated with IBS development. Neonatal stress models such as post-infectious model[2] or neonatal maternal deprivation (NMD) have been proposed as IBS-like models.[3] Indeed, separated rats mimicked numerous hallmarks of IBS including dysregulated hypothalamic pituitary adrenal axis response.[4,5] Early stress also alters innate immunity response. Toll-like receptors (TLR) are pattern recognition receptors that are key components of innate immunity. TLR activation leads to a pro-inflammatory cascade. In fact, up-regulation of TLR has been demonstrated in IBS-like models[6] and in rat neonatal models of necrotizing enterocolitis.[7,8]

Very few drugs are currently available to treat IBS and identification of signaling pathways involved in IBS is a crucial step to develop a novel therapeutic approach. Very recently, Zhou et al demonstrated that a subset of IBS patients with an increased intestinal permeability exhibited decreased glutamine synthetase levels whereas its complementary miRNA, miR-29A increased in blood microvesicles and intestinal tissue.[9]

Nutritional intervention with glutamine showed beneficial effect on intestinal permeability,[10,11] a key event in IBS. Indeed, glutamine is a conditionally essential amino acid during injury[12] and has shown protective properties in the intestinal mucosa: Its protective effect has already been demonstrated in inflammatory bowel diseases-like models by maintenance of gut barrier function,[13,14] reduction of bacterial translocation[15] and inhibition of pro-inflammatory cytokines production.[16–18]

* Corresponding author. Tel.: +33 0 2 35 14 84 54.
E-mail address: marion_rachel@hotmail.com (R. Marion-Letellier).

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In this context, the aim of our study was to investigate whether early nutritional intervention with glutamine may contribute to maintain gut barrier function in a rat IBS-like model.

2. Materials and methods

2.1. Animals

Animal care and experimentation complied with both French regulations and European Community regulations (Official Journal of the European Community L 358, 18/12/1986) and Rachel Marion-Letellier is authorized by the French government to use this animal model (Authorization *n*°76-106). 9 Pregnant female Wistar rats (primiparous) were purchased from Janvier (Le Genest St Isle, France), on gestational days 15–16. Females were individually housed in cages containing bedding material on a 12:12-h light–dark cycle (lights on at 8:00 am).

2.2. Maternal separation protocol

Delivery was considered as D1. We performed NMD as previously described by Bueno et al.[19]. Briefly, NMD was performed daily from D2 to D14 for three consecutive hours (from 9:00 to 12:00 am). During separation, pups were removed from their home cage and kept in temperature controlled individual cages at 28 °C. Non-separated pups were not handled and were maintained in their home cages with the dams. After D15, all pups remained with their dam until weaning on D21. Pups were weighed daily until D14 and weekly from D15 to D60. The rats were killed on D20 and D60 using an overdose of anesthesia (ketamine and xylasine).

2.3. Nutritional intervention

Nutritional intervention occurred during maternal separation from D2 to D14.

In a first set of experiments (Study A): separated rats received by gavage either alanyl-glutamine (dipeptiven®) (Fresenius Kabi, Graz, Austria), 0.75 g kg^{-1} D^{-1}, or an isonitrogenous mix of 6 amino acids (GLY 0.49M, ALA 0.39M, SER 0.34M, PRO 0.31M, ASN 0.27M, HIS 0.18M).

In a second set of experiments (Study B): a supplementary group of separated rats receiving only a similar volume of sterile water by gavage instead of dipeptiven or isonitrogenous amino acids mix was added.

A litter of separated rats may contain all groups of pups (GLN, H_20 and AA).

2.4. Western blot

After sacrifice, the distal portion of the colon was removed and cut longitudinally for each rat and lightly cleaned in ice-cold PBS to remove fecal residues. Frozen colon tissues were homogenized in PBS (2 ml per sample) with 0.1% of protease inhibitor cocktail (Sigma–Aldrich, Saint-Quentin-Fallavier, France) and 1% of phosphatase inhibitor cocktail (Sigma–Aldrich). Homogenates were centrifuged (12,000 g, 15 min, 4 °C) and the supernatants were collected and stored at −80 °C. Protein concentration was determined following Bradford's colorimetric method. Aliquots of supernatants containing equal amounts of protein (25 μg) were separated on 4–12% NuPAGE gel (Invitrogen, Cergy-Pontoise, France). In the next step, the proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond, GE Healthcare, UK). After blocking in 5% nonfat dry milk, membranes were incubated with specific primary antibodies at the dilution of 1:500 (for Claudin-1 and COX-2), 1:200 (TLR4) or 1:1000 (for Occludin and PPARγ). Each filter was washed three times for 5 min and incubated with the secondary horseradish peroxidase linked anti-goat IgG (for COX-2), anti-rabbit IgG (for Claudin-1) and anti-mouse IgG (for Occludin, PPARγ and TLR4) antibodies (Santa Cruz Biotechnology, Tebu-bio, Le Perray en Yvelines, France). To check equal loading, the blots were analysed for β-actin expression using an anti-β-actin antibody (Sigma–Aldrich). Immunodetection was performed using enhanced chemiluminiscence light-detecting kit (Amersham, Arlinghton Heights, IL). Densitometric data were measured following normalization to the control (β-actin). The signals were analyzed and quantified by a Scientific Imaging Systems (ImageScanner III and ImageQuant TL software, GE Healthcare, Orsay, France).

2.5. RT-PCR

Total cellular mRNAs were extracted from colon tissues using trizol reagent (Invitrogen, Cergy-Pontoise, France) and mRNA concentration was determined by a biophotometer, Eppendorf, Hamburg, Germany). Pro-inflammatory cytokines and tights junction protein mRNAS were reverse transcripted (RT) into complementary DNA (cDNA) using a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany). Each sample (0.75 μg of mRNA) was completed with water in a final volume of 8.25 μl and then added with 9 μl of a mixture of RNAse out (1 μl), TP 5x (3 μl), dNTP 10 mM (1.5 μl), random hexamers (1 μl), and DTT (1.5 μl). RT products were amplified by polymerase chain reaction (PCR) and GAPDH cDNA (house-keeping gene) was used as a standard.

2.6. Production of cytokines

Concentrations of IL-2, -4, -10 and IFN-γ in the colon were measured by multiplex assay (Rat Fluorokine MAP kit, R&D Systems, USA). Fluorokine MAP Bead-based kit is useful tools for measuring the levels and/or activities of multiple proteins in a single sample. This assay relied on the use of polystyrene beads, each with a unique signature mix of fluorescent dyes that can be discriminated by a laser-based detection instrument (Luminex100, Luminex Corp., Austin, TX). Each bead type was coated with a specific antibody to the cytokine of interest. The cytokine antibody pairs in this multiplex assay do not cross react with other analytes in the panel. Detection limits were ranged from 0.67 to 8.72 pg ml^{-1} for IL-2, 0.18 to 1.84 pg ml^{-1} for IL-4, 1.49 to 137 pg ml^{-1} for IFNγ and 0.14 to 11.6 pg ml^{-1} for IL-10.

2.7. Intestinal permeability

Separate rats were used to measure intestinal permeability at D60 because they were subjected to possible stress when placed in individual metabolic cages. Rats (8 rats per group) received by gavage, 50 mg lactulose (L) and 100 mg mannitol (M) dissolved in 2 ml of water. 8-h urine was collected and quantitated to estimate intestinal permeability. Urinary excretion rates of lactulose (L) and mannitol (M) were determined using liquid chromatography. Results were expressed as the Lactulose/Mannitol ratio.

2.8. Statistical analysis

Statistical comparison was performed by one-way ANOVA following by Tukey's post test using GraphPad-InStat version 5.01. Statistical significance level was set at $p < 0.05$. For Table 1, statistical comparison was performed by *t*-test and a significant trend was considered for $P < 0.1$.