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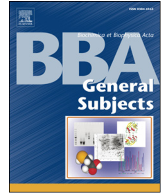

Targeting immunoproteasome and glutamine supplementation prevent intestinal hyperpermeability

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ABSTRACT

Background: Intestinal hyperpermeability has been reported in several intestinal and non-intestinal disorders. We aimed to investigate the role of the ubiquitin proteasome system in gut barrier regulation in two mice models: the water avoidance stress model (WAS) and a post-inflammatory model (post-TNBS).
Methods: Both models were applied in C57BL/6 male mice ($n = 7$–8/group); Proteasome was targeted by injection of a selective proteasome inhibitor or by using knock-out mice for β2i proteasome subunit. Finally, glutamine supplementation was evaluated.
Results: In both models (WAS at day 10, post-TNBS at day 28), we observed an increase in proteasome trypsin-like activity and in inducible β2/constitutive β2 subunit protein expression ratio, associated with an increase in intestinal permeability. Moreover, intestinal hyperpermeability was blunted by intraperitoneal injection of selective proteasome inhibitor in WAS and post-TNBS mice. Of note, knock-out mice for the β2i subunit exhibited a significant decrease in intestinal permeability and fecal pellet output during WAS. Glutamine supplementation also improved colonic permeability in both models.
Conclusions: In conclusion, the proteasome system is altered in the colonic mucosa of WAS and post-TNBS mice with increased trypsin-like activity. Associated intestinal hyperpermeability was blunted by immunoproteasome inhibition.

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1. Introduction

Intestinal permeability is altered not only in intestinal diseases such as irritable bowel syndrome (IBS) or inflammatory bowel diseases (IBD), but also in extra-intestinal disorders such as obesity and is involved in the pathophysiology of these diseases [1–3]. The paracellular permeability of the intestinal barrier is regulated by a complex protein system that constitutes tight junctions (TJ). Mainly three of the TJ proteins have been studied: the transmembrane proteins, occludin and claudin-1, and the cytosolic protein, zonula occludens-1 (ZO-1). Occludin and claudin-1 interact with ZO-1 which binds to the actin cytoskeleton to control paracellular permeability [4,5]. TJ proteins have been reported to be altered in different conditions such as IBD [6,7], IBS [8] or obesity [9]. Interestingly, occludin has been reported as a target of proteasome degradation through interaction with Itch ubiquitin ligase [10] and we have previously shown that down-regulation of occludin protein expression may be related to its increased degradation by the proteasome system in IBS patients [11].

The ubiquitin proteasome system is involved in the regulation of several cellular pathways by selective degradation of proteins i.e. cell proliferation, apoptosis, inflammatory response and antigen presentation [12]. Recently, by using a proteomic approach, several proteins involved in stress, inflammatory responses, and metabolism, have been described as targets of proteasome degradation [13,14]. Targeted proteins are polyubiquitinated and then processed by the constitutive 20S proteasome, a large ATP-dependent multicatalytic complex which degrades the proteins to short peptides and releases ubiquitin [12]. In mammalian cells, the three catalytic β subunits of the constitutive 20S proteasome, β1, β2 and β5 can be replaced by inducible β-type subunits (βi), β1i, β2i and β5i. This then becomes an alternative proteasome complex, called immunoproteasome [15]. We [16] and others [17] have reported that IFNγ up-regulates proteasome activity in intestinal epithelial cell lines by increasing βi subunit protein expression. The proteasome system is altered during IBD [17,18] and also in the colonic mucosa of IBS patients [19]. However, modifications of proteasome seem to

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have different patterns [11,17]. In addition, experimental data suggest that proteasome inhibition prevented colitis in mice [20–22] but to our knowledge, the role of proteasome on intestinal permeability remains poorly documented.

Previous data reported the beneficial effects of glutamine, a conditionally essential amino acid, on intestinal permeability in vitro [23,24], in both animal models [25] and in humans [26,27]. Interestingly, Zhou et al. showed that glutamine synthetase protein expression was reduced in the colonic mucosa of IBS patients with intestinal hyperpermeability [28] and recently proposed that a decrease in claudin-1 protein expression in IBS patients may be linked to glutamine synthetase protein expression and to the proteasome system [3]. In IBD, Sido et al. also reported alteration of glutamine metabolism in colitic rats [29]. Finally, glutamine supplementation was able to regulate the proteasome system by regulating ubiquitination processes [14,30,31] and was able to restore colonic tight junction protein expression in different conditions [23–25,32,33].

Therefore, we aimed to investigate the regulation of the proteasome system in animal models associated with increased intestinal permeability and the effects of its inhibition.

2. Material and methods

2.1. Animal experimentation

Male C57Bl/6 mice were obtained from Janvier Labs (Le Genest St Isle, France) and were acclimatized at 23 °C with a 12-h light-dark cycle before study (dark phase: 9:30 PM–9:30 AM). Knock-out mice for *MECL1* gene (or β2i proteasome subunit) were a kind gift from Professors Marcus Groettrup and Mickael Basler of Konstanz University (Germany) with the permission of Professor John Monaco from Cincinnati University (Ohio). The final author (MC) is authorized by the French government to use animal models (authorization no. 76–107) and the protocol was approved by CENOMEXA, the Ethics Committee of Normandy for Animal EXperimentation (acceptance number: N/04-11-12/27/11-15). In addition, the authors confirm that all experiments were performed in accordance with relevant guidelines and regulations (Official Journal of the European Community L 358, 18/12/1986) and followed ARRIVE guidelines [34].

2.1.1. Water avoidance stress

The water avoidance stress (WAS) model was induced by placing mice on a small platform in a tank (25 cm in height and 40 cm in length) of water (25 °C) during 1 h per day (from 9 h00 to 10 h00 AM) during 10 consecutive days. Control mice were placed on the same platform without water during the same time period. Fecal pellet output was monitored during each session. Mice were euthanized at day 10 immediately after the 1 h WAS period.

2.1.2. Post-inflammatory model

Under anesthesia, TNBS (0.5 mg, trinitrobenzen sulphonic acid) in 50% ethanol was instilled into the colon to produce colitis and was followed up for 28 days. Control mice received intracolonic instillation of 50% ethanol solution. Mice were euthanized at different times (0, 7, 14, 21 and 28 days after TNBS instillation) to establish post-inflammatory state.

2.1.3. Proteasome inhibitor injections

Daily intraperitoneal injection of a selective Inhibitor of immunoProteasome (IP), PR-957, was performed from day 21 to day 28 in TNBS mice and from day 1 to day 10 in WAS mice at 12 mg/kg. IP was diluted in phosphate buffered saline (PBS) solution (KH PO4 0.2 g, KCl 0.2%, Na2HPO4 1.15 g, NaCl 8 g, H2O qsp 1000 mL).

2.1.4. Glutamine supplementation

Glutamine (Gln) was diluted in drinking water to provide 2 $g \cdot kg^{-1}$ of body weight per day^{-1}. In the post-TNBS model, Gln was provided from day 21 to day 28. In the WAS model, Gln was administered from day 1 to day 10. In both models, glutamine solution was prepared and replaced every day.

2.2. Euthanasia and sampling

Mice were anesthetized with an intraperitoneal injection of ketamine/Largactil (40 and 1 mg·kg-1, respectively). Blood samples were collected, centrifuged (4 °C, 3000 rpm, 15 min) and plasma was frozen at −80 °C. Colonic samples were collected, washed with ice-cold PBS, immediately frozen in liquid nitrogen and stored at −80 °C for RT-qPCR, western blot analysis and evaluation of proteasome activities. Samples of fresh colon were collected to evaluate paracellular permeability.

2.3. Intestinal permeability

Colonic permeability was assessed by measuring FITC-dextran (4 kDa) fluxes in Ussing chambers with an exchange surface of 0.07 cm^2 (Harvard Apparatus, Holliston, United States). FITC-dextran (5 mg/mL) was placed in the mucosal side. After 3 h at 37 °C, medium from the serosal side was removed and stored at −80 °C.

We also evaluated intestinal permeability in vivo in some WAS model series. At day 10, after a 6-h fasting period, FITC-dextran (4 kDa, 40 mg/mL) was administered by gavage at 10 μL/g of body weight. Three hours later, the last WAS procedure was performed and mice were then immediately euthanized for blood collection.

The fluorescence level of FITC-dextran either in serosal medium or in plasma (excitation at 485 nm, emission at 535 nm) was measured in 96-well black plate with spectrometer Chameleon V (Hidex, Turku, Finland). Values were converted to concentration using a standard curve.

2.4. qRT-PCR

Mucosal total RNA was extracted from samples as previously described [35]. After reverse transcription of 1.5 μg total RNA into cDNA by using 200 units of SuperScript™ II Reverse Transcriptase (LifeTechnologies, Cergy-Pontoise, France), qPCR was performed by SYBR™ Green technology on BioRad CFX96 real time PCR system (BioRad Laboratories, Marnes la Coquette, France) in duplicate for each sample. GAPDH was used as the endogenous reference gene. Specific primers are displayed in Table 1.

2.5. Western blot analysis

Colonic mucosa was homogenized in ice-cold lysis buffer containing 0.1% protease inhibitor cocktail (Sigma Aldrich) as previously described

Table 1
Sequences of used primers for qPCR.

Gene		Sequence (5′ > 3′)	Product size (bp)
TNFα	Forward	TGTCTACTCCTCAGAGCCCC	166
	Reverse	TGAGTCCTTGATGGTGGTGC	
IL-1β	Forward	CCCAAAAGATGAAGGGCTGC	169
	Reverse	AAGGTCCACGGGAAAGACAC	
CXCL-1	Forward	ACTCAAGAATGGTCGCGAGG	84
	Reverse	GGGACACCTTTTAGCATCTTTTGG	
GAPDH	Forward	CATCACTGCCACTCAGAAGA	316
	Reverse	AAGTCACAGGAGACAACCT	

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