Effects of Bile Duct Ligation and Ghrelin Treatment on the Colonic Barrier and Microbiome of Mice

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Abstract

Introduction: Cholestatic liver disease (CLD) is associated with intestinal barrier dysfunction. The peptide hormone ghrelin may exert both hepatoprotective and barrier-strengthening effects. Here, we have evaluated these effects under the conditions of experimental cholestasis. Methods: C57BL/6J mice with bile duct ligation (BDL) or sham surgery were treated with ghrelin or solvent for 9 days. Liver injury was assessed by histological and laboratory analyses. Paracellular macromolecule permeability and transmural electrical resistance (TMER) of colonic tissues were measured using a Ussing chamber. Expression of tight junction (TJ) genes was quantified by real-time PCR. Amplicon metagenomic sequencing was employed to analyze bacterial 16S rRNA from colonic stool samples. Results: Mice with BDL exhibited weight loss and signs of severe liver injury. These changes were unaffected by ghrelin treatment. FITC-4-kDa-dextran flux was increased and TMER decreased after BDL. Treatment with ghrelin tended to reduce these effects. Furthermore, application of ghrelin was associated with higher mRNA levels of claudin-4, occludin, and ZO-1 in colonic tissues of mice with BDL. Reduced alpha-diversity of the microbiome was observed in solvent-treated mice with BDL but not in ghrelin-treated animals. Conclusion: Ghrelin treatment did not improve weight loss and liver damage but increased gene expression of colonic TJ proteins and restored the alpha-diversity of the microbiome. Since protective effects of ghrelin might be masked by the severity of the model, we suggest follow-up studies in models of milder CLD.

Keywords

Cholestasis · Ghrelin · Experimental liver injury · Intestinal barrier · Mouse

Introduction

Cholestasis frequently results from biliary tract obstructions, which may be caused by choledocholithiasis, inflammatory (e.g., autoimmune mediated) diseases, or tumors [1, 2]. In turn, cholestasis exacerbates preexisting liver injury, triggers fibrosis and eventually cirrhosis, and increases the risk of bacterial infections with the potential for sepsis [2]. Cholestatic liver disease (CLD) is associated with further complications, such as malnutrition and sarcopenia, cardiac dysfunction [3], and kidney injury [4].
Moreover, a dysfunction of the intestinal barrier develops that promotes bacterial translocation and systemic inflammation [5–9]. Mechanistic links between cholestasis and the “leaky gut” are not fully understood yet but include an altered architecture of tight junctions (TJs) in the presence of pro-inflammatory cytokines [7, 8]. Taken together, increased intestinal permeability aggravates the causative liver/biliary tract disease, and there is a clinical need for complementary therapies that specifically target this aspect.

The gastric peptide hormone ghrelin exerts appetite- and food intake-increasing effects that are mediated centrally via increased hypothalamic neuropeptide Y secretion [10]. In addition, local effects of ghrelin on gastrointestinal organs have been described, including a hepatoprotective action in experimental CLD [11, 12]. Of note, several studies suggested that ghrelin can ameliorate experimental colonic inflammation and promote wound healing [13–16]. In contrast, De Smet et al. [17] observed an enhancement of dextran sulfate sodium (DSS)-induced colitis by ghrelin, and Tian et al. [18] reported that the knockdown of ghrelin-O-acyltransferase, the enzyme that acylates inactive ghrelin to produce active ghrelin, attenuated experimental colitis in mice. The reasons for these discordant findings are currently unclear. Moreover, the pathomechanisms of primary colitis are fundamentally different from those of intestinal damage secondary to CLD. These mixed data prompted us to investigate the effects of ghrelin on colonic barrier function in an experimental model of biliary obstruction. For the sake of clinical importance, we decided for a model of severe CLD, bile duct ligation (BDL) in mice [8, 11, 19].

Materials and Methods

Animal Studies

C57BL/6J mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and bred in the local animal facility of the Rostock University Medical Center. Animal experiments were executed in accordance with the EU directive 2010/63/EU and approved by the Landesamt für Landwirtschaft, Lebensmittel sicherheit und Fischerei Mecklenburg-Vorpommern (LALLF/MV). The mice had access to water and rodent chow diet ad libitum. All animals received care according to the guidelines and the Ethical Committee for Animal Care (LALLF/MV).

Male mice at an age of approximately 3 months were randomly assigned to the experimental groups. Prior to surgery, the mice were anesthetized with isoflurane (5% for induction, 1.5% for maintenance), and carprofen (Pfizer GmbH, Berlin, Germany) at 5 mg/kg was injected subcutaneously for pain prevention. For BDL, the common bile duct was exposed after midline laparotomy, ligated three times using 5-0 polyester suture (Ethicon, Norderstedt, Germany), and transected between the two most distal ligations. In mice with sham surgery, the common bile duct was also exposed but neither ligated nor cut. The abdominal wall was closed in two layers using 6-0 prolene (Catgut, Markneukirchen, Germany).

Treatment was initiated on the day of surgery and continued until d9. Ghrelin (acyl form, Ser [n-octanoyl]) was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA), dissolved in 0.9% NaCl, stored in aliquots at ~20°C until usage, and administered by intraperitoneal injection at 200-μg/kg body weight. The dose was chosen based on published data [11, 12, 14, 20, 21]. Control mice received injections of solvent only. Experiments were finished on d10 after surgery. Differing from this, some pilot studies with completely untreated mice were continued until postoperative day 14 (see figure legends).

The animals were sacrificed by an overdose of ketamine/xylazine hydrochloride followed by cervical dislocation. Blood samples, further tissues, and stool from the colon were harvested and stored under appropriate conditions (as detailed below) until they were assayed.

Hematoxylin and Eosin Staining

Routine hematoxylin and eosin (H&E) staining was performed on 4-μm sections of formalin-fixed paraffin-embedded material using standard procedures. Samples of colonic tissue were always derived from the same region of the colon (directly adjacent to the cecum). In case of liver tissue, at least two independent samples from different lobules were evaluated.

Ussing Chamber Analyses

Fresh colonic tissue was immediately placed on ice in recording buffer (2.4 mM NaH2PO4, 0.4 mM Na2HPO4, 5 mM KCl, 25 mM NaHCO3, 1.2 mM CaCl2, 1.2 mM MgCl2, 115 mM NaCl, and 5 mM glucose [2, 3], pH 7.4). Subsequently, gut segments were cut longitudinally, carefully cleaned from stool residues, and analyzed in a Ussing chamber with 5 mL half-cell volume and 0.24 cm2 surface area with AgCl electrodes (Karl Mussler, Scientific Instruments, Germany) as described previously [22, 23]. The chambers were continuously gassed with 5% CO2 and 95% O2 at 37°C. Transmural electrical resistance (TMER) was measured in the open circuit mode using a Voltage/Current Clamp VCC6 (Karl Mussler, Scientific Instruments), and a correction for background resistance was performed. Paracellular permeability was assessed by measuring the mucosal-to-serosal FITC-4-kDa-dextran (FITC-Dx) flux. Therefore, FITC-Dx was added at 40 ng/mL into the mucosal compartment. Fluorescence intensity in the serosal chamber was measured every 30 min for 3 h using a microplate reader.

Gene Expression Studies

RNA was isolated using the RNEasy Mini kit (Qiagen, Hilden, Germany). Approximately 30 mg of frozen tissue (stored at ~150°C) was minced in liquid nitrogen, suspended in buffer provided with the kit, and homogenized using a tissue lyser at 50 Hz for 5 min. Seventy percent ethanol was added, and the mixture was processed according to the manufacturer’s instructions. On-column DNase digestion was performed to remove genomic DNA. Unless indicated otherwise, all subsequent procedures were performed with reagents/instruments from Thermo Fisher Scientific (Karlsruhe, Germany). 250 ng of mRNA per sample was reverse transcribed into cDNA using TaqMan™ Reverse Transcription.
Reagents and random priming. Levels of target cDNA were quantified by real-time PCR with a ViiA 7 sequence detection system. Therefore, qPCR MasterMix (Eurogentec, Seraing, Liège, Belgium) and the following mouse-specific TaqMan™ gene expression assays with fluorescently labeled MGB probes were employed: Mm00516703_s1 (claudin-2), Mm00515514_s1 (claudin-4), Mm00516817_m1 (claudin-7), Mm00517635_m1 (claudin-15), Mm00500912_m1 (occludin), Mm00493699_m1 (ZO-1; tjd1), and Mm04209403_g1 (keratin-8, house-keeping gene control). PCR conditions were 95°C for 5 min, followed by 45 cycles of 15 s at 95°C/1 min at 60°C. The relative amount of target mRNA was expressed as $2^{-\Delta\Delta C_T}$, where $\Delta C_T = C_{\text{target gene}} - C_{\text{keratin-8}}$.

**Clinical Chemistry**

Activities of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), and alkaline phosphatase (AP) as well as plasma levels of glucose, bilirubin, and albumin were determined using the chemistry analyzer DxC 700 AU (Beckman Coulter, Krefeld, Germany) and systems reagents from Beckman Coulter for this analyzer [7]. Calibration of glucose and total bilirubin procedures for serum specimens was accomplished by use of the Chemistry Calibrators Cat #DR0070 (glucose) and Cat #DR0070 (total bilirubin), which are traceable to the National Institutes of Standards and Technology Standard Reference Material 965a and 916a. The methods of ASAT, ALAT, AP, and albumin are in accordance with the reference methods of the International Federation of Clinical Chemistry.

**Microbiome Studies**

Stool samples were obtained from the colon upon tissue harvest and stored at −80°C. DNA was extracted with the ZymoBIOMICS DNA Miniprep Kit (ZymoResearch, Irvine, CA, USA) and sent to Novogene (Cambridge, UK) for further analysis. Amplicon metagenomic sequencing was used to target bacterial 16S rRNA (V3–V4) using NovaSeq 6000 PE250 (Illumina, Cambridge, UK).

**Statistical Analyses**

All statistical analyses, except for the microbiome studies, were performed using SPSS, version 27 (IBM, Ehningen, Germany). Pairwise comparisons of normally distributed data were performed using a two-tailed Student’s t test. For data that were not normally distributed, the Kruskal-Wallis test (multigroup comparisons) was applied, followed by the Mann-Whitney U test (pairwise comparisons) if applicable. Bonferroni-adjusted values of $p < 0.05$ were considered as statistically significant. Analysis of the microbiome data was conducted by Novogene.

**Results**

**Ghrelin Has No Effect on Weight Loss and Liver Injury after BDL**

Sham-operated mice maintained their initial weight of approximately 26 g throughout the trials. Mice with a ligation of the bile duct (BDL mice) exhibited a significant weight loss (Fig. 1). We noticed that relative body weights (normalized to day of surgery) of ghrelin-treated mice were numerically higher at most of the time points than those of untreated controls. However, these differences were not statistically significant.

BDL mice developed a severe CLD with extended necroses, inflammation, bile duct proliferation, and a beginning fibrosis. There were no gross morphological differences between ghrelin-treated animals and controls (Fig. 2). As expected, BDL also caused strong increases in bilirubin levels as well as ASAT, ALAT, and AP activities in blood plasma (Table 1). Furthermore, lower plasma levels of glucose were observed. There were no significant effects of ghrelin treatment. Albumin levels did not differ between groups, indicating that liver synthesis performance was not affected despite BDL over the 10-day experimental period.
Electrophysiological and Barrier Properties of Colon Tissue after BDL

H&E staining of colonic tissues showed no apparent pathological changes for any of the experimental groups (intact surface epithelium, mucosa, and submucosa; Fig. 3a). However, TMER measurements indicated a reduced resistance of colonic tissues in untreated BDL mice compared to sham-operated animals (Fig. 3b, left panel). In addition, FITC-Dx assays revealed an increased paracellular macromolecule permeability of colonic tissues after BDL (Fig. 3c, left panel). Administration of ghrelin tended to reduce both the effects of BDL on TMER and on paracellular permeability (Fig. 3b, c, right panels).

Limitations that may explain the lack of statistical significance are the small sample size and the high standard deviations of the FITC-Dx assay data. BDL mice with treatment (Fig. 3c, right panel) were subjected to significantly more stress than untreated BDL mice (Fig. 3c, left panel) because of the multiple injections, and effects on the measured values and their variation cannot be excluded.

Enhanced Expression of Claudin-4, Occludin, and ZO-1 in the Colon of Ghrelin-Treated BDL Mice

Barrier properties are influenced by intestinal TJ proteins [24]. BDL tended to decrease mRNA levels of the
barrier-strengthening proteins claudin-4, occludin, and ZO-1 (Fig. 4). However, these effects were not statistically significant. Upon treatment with ghrelin, BDL mice but not animals with sham surgery expressed significantly higher colonic mRNA levels of claudin-4, occludin, and ZO-1 than solvent-treated controls. The mRNA levels of

Fig. 3. Effects of BDL and ghrelin on colon morphology and electrophysiological and barrier properties of colonic tissues. Mice with BDL or sham surgery remained untreated (b, c – left panels) or were treated by daily injections of ghrelin (200 μg/kg) or solvent as indicated (all other panels). The animals were sacrificed between days 10–14 (b, c – left panels) and on day 10 (all other panels). Afterward, colonic tissues were subjected to follow-up investigations. a FFPE sections were stained with H&E. Exemplary pictures for the different experimental groups are shown (original magnification, ×100). b, c TMER and FITC-Dx were measured in an UC as described in the "Methods" section. 100% FITC-Dx permeability corresponds to time point 0 h. Data are presented as mean ± SEM. *p < 0.05 versus sham (b) T-test; (c) Mann-Whitney U test). UC, Ussing chamber; FFPE, formalin-fixed paraffin-embedded.
Ghrelin Treatment in Mice with Bile Duct Ligation

Fig. 4. Effects of BDL and ghrelin on gene expression in colonic tissues. Mice with BDL or sham surgery were treated by daily injections of ghrelin (200 μg/kg) or solvent only. On d10, animals were sacrificed, and RNA was isolated from colonic tissues followed by reverse transcription into cDNA. The mRNA levels of the indicated genes were quantified by TaqMan PCR and normalized to the housekeeping gene keratin-8 by expression as $2^{-ΔCt}$ values. Data are presented as mean ± SEM. *p < 0.05 versus BDL + solvent (Mann-Whitney U test, Bonferroni adjusted).

Claudin-2 (Fig. 4), claudin-7, and claudin-15 (data not shown) were neither influenced by the surgical procedure nor by the kind of treatment.

Effects of BDL and Ghrelin on the Colonic Microbiome

An initial principal component analysis of the colonic microbiome data revealed that samples of BDL mice clustered separately from samples after sham surgery, while treatment with ghrelin displayed no effect (Fig. 5a). BDL surgery followed by solvent treatment significantly decreased alpha-diversity of the colonic microbiome (Fig. 5b). Noteworthy, upon ghrelin treatment, alpha-diversity was as high as in mice with sham surgery, and the Shannon index was significantly higher than in the group of BDL mice with solvent treatment (Fig. 5b). At the phylum level (Fig. 5c), Verrucomicrobiota showed a significantly higher relative abundance in BDL mice than in animals with sham surgery (both groups with solvent treatment). Significant effects of ghrelin were observed only for phyla with very low relative abundance (<0.01; not included in Fig. 5c for reasons of clarity). Specifically, the relative abundance of Campilobacterota and Gemmatimonadota was higher when BDL mice were treated with ghrelin instead of solvent ($p = 0.048$ and 0.03, respectively).

Discussion

In this study, we used a model of severe CLD, BDL in C57BL/6J mice to evaluate effects of ghrelin on the colonic barrier, which is affected by cholestasis under both experimental and clinical conditions [5–9]. Our investigation was stimulated by previous studies that had indicated protective effects of ghrelin on the colonic mucosa in the context of experimentally induced inflammation [13–16]: in rats with experimental colitis (induced by DSS or acetic acid), ghrelin exerted anti-inflammatory effects [13, 15] and accelerated wound healing [16]. In mice with DSS colitis, ghrelin treatment improved the intestinal barrier function [14]. However, contradictory findings have been reported as well [17, 18], and the effects of ghrelin on intestinal damage secondary to CLD have not been studied before. Ghrelin was administered in its active acylated form and at a dose of 200 μg/kg body weight per day, which is in the upper range of doses commonly used in rodents [11, 12, 14, 20, 21]. Treatment with ghrelin significantly increased mRNA levels of claudin-4, occludin, and ZO-1 in colonic tissues of BDL mice. However, this potentially protective effect of ghrelin in the colon of BDL mice did not translate into statistically significant increases in TMER values and decreases in FITC-Dx permeabilities. Together, these data suggest a limited efficiency of ghrelin in intestinal barrier dysfunction secondary to experimental CLD.

Effects of ghrelin on the liver have been addressed in previous studies [11, 12] and were not the main focus of this investigation. Evaluation of liver histology and laboratory findings indicated no improvement of tissue damage by ghrelin treatment. It should be noted that the severity of the model prevented extension of treatment beyond day 10 after BDL. This time might have been too
short for liver fibrosis to develop (only mild changes were observed), and antifibrotic effects of ghrelin, as previously reported [11, 12, 25], can therefore not be assessed.

Weight loss of untreated C57BL6/J mice with BDL progressed steadily and reached almost 25%, at postoperative day 10. As an orexigenic hormone, ghrelin stimulates appetite [10]. This effect, however, was either lost or insufficient to attenuate weight loss in mice with severe CLD. A limitation of our investigation is that the consumption of diet by the mice could not be quantitated.

**Fig. 5.** Effects of BDL and ghrelin on the colonic microbiome. Mice with BDL or sham surgery were treated by daily injections of ghrelin (200 μg/kg) or solvent only. On d10, animals were sacrificed, and stool was collected directly from the colon. a PCA to identify clustering among the samples. b Analysis of alpha-diversity (shown as Shannon diversity index). Box plots indicate the median, the 25th and 75th percentiles in the form of a box, and the 5th and 95th percentiles as whiskers. *p < 0.05 versus BDL + ghrelin, *p < 0.05 versus sham + solvent (Wilcoxon test). c Analysis of relative abundance of operational taxonomic units at the phylum level. *p < 0.05 versus sham + solvent (integration of nonparametric multiple testing, p value correction included). Data analyses were performed by Novogene. PCA, principal component analysis.
To our knowledge, this is the first study on the effects of ghrelin on the microbiome of mice with CLD. In accordance with previous investigations [26, 27], changes of the colonic microbiome after BDL were observed. Analysis of microbiome data after ghrelin treatment showed mixed results: on the one hand, clustering depended on the type of surgery (BDL vs. sham) but not on the type of treatment (ghrelin vs. solvent). On the other hand, ghrelin treatment prevented the reduction in alpha-diversity observed in solvent-treated BDL mice. As previously reported by Cabrera-Rubio et al. [26], BDL induced a significant increase of Verrucomicrobiota. The sole member, Akkermansia muciniphila, is a mucosal sugar metabolizer that colonizes the outer mucus layer, promotes mucin production, and has barrier-enhancing properties [28]. In vitro studies demonstrated that Akkermansia-derived extracellular vesicles ameliorate lipopolysaccharide-induced barrier damage by increasing tight junction function [29, 30]. Moreover, an in vivo study by Plovier et al. [31] demonstrated that even administration of pasteurized A. muciniphila and a stable protein isolated from the outer membrane of A. muciniphila (Amuc_1100) improved barrier function, lowered endotoxin levels, and restored tight junction protein and antimicrobial peptide expression in mice with metabolic disease. However, the metabolic properties of commensal microbiota depend on the host luminal microenvironment and can be influenced by immune cell activation and host nutritional status [32, 33]. Malnutrition, as occurs in cholestasis [34], favors the growth of Akkermansia and other taxa that can degrade mucus [26]. We hypothesize that excessive growth of Akkermansia may have negative effects on barrier properties and microbial homeostasis. One mechanism, recently demonstrated for A. muciniphila [35], may be that expansion of A. muciniphila under nutrient restriction leads to erosion of colonic mucus, thereby allowing pathobionts to colonize the nearby epithelial layer. By providing an epithelial niche and nutrients from the host, pathobionts can proliferate excessively and disrupt microbial homeostasis. Furthermore, disruption of the mucus may lead to a breakdown of the physical barrier and facilitate luminal antigens to penetrate the epithelial layer, resulting in pro-inflammatory responses. Thus, the normalization of Verrucomicrobiota abundance by ghrelin therapy suggests an improvement of physiological status in the colon of BDL mice.

**Conclusion**

Ghrelin influenced BDL-induced colon dysbiosis and increased the colonic expression of claudin-4, occludin, and ZO-1. However, there was only a numerical but not statistically significant improvement in macromolecular permeability and electrical resistance. In addition, ghrelin treatment did not increase body weight or reduce liver injury. The irreversible anatomical changes of BDL lead to severe and progressive liver injury without the possibility of adaptation. Thus, C57BL/6J mice reach humane endpoints after few weeks. This time may be too short and the primary damage too severe for efficient treatment with ghrelin. Studies on effects of ghrelin on the intestinal barrier should therefore be extended to models with milder CLD in which prolonged ghrelin treatment is possible.

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**Statement of Ethics**

All animal experiments were approved by the local Animal Use and Care Committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern; file numbers 7221.3-1-046/18 and 7221.3-1.1-015/20).

**Conflict of Interest Statement**

The authors have no conflicts of interest to declare.

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Author Contributions

Luise Ehlers and Robert Jaster designed the study and wrote the manuscript; Luise Ehlers, Leonard Andreas Wolfgang Netz, Johannes Reiner, and Robert Jaster performed the experiments; Luise Ehlers, Leonard Andreas Wolfgang Netz, Johannes Reiner, Peggy Berlin, Karen Bannert, Manuela Bastian, Dietmar Zechner, Georg Lamprecht, and Robert Jaster analyzed the data and reviewed and edited the draft.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Amplicon sequencing data were deposited on the European Nucleotide Archive (ENA) under the study accession PRJEB55321. Further inquiries can be directed to the corresponding author (Robert Jaster).

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