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Bovine lactoferrin regulates cell survival, apoptosis and inflammation in intestinal epithelial cells and preterm pig intestine

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ABSTRACT

Bovine lactoferrin (bLF) may modulate neonatal intestinal inflammation. Previous studies in intestinal epithelial cells (IECs) indicated that moderate bLF doses enhance proliferation whereas high doses trigger inflammation. To further elucidate cellular mechanisms, we profiled the porcine IEC proteome after stimulation with bLF at 0, 0.1, 1 and 10 g/L by LC-MS-based proteomics. Key pathways were analyzed in the intestine of formula-fed preterm pigs with and without supplementation of 10 g/L bLF. Levels of 123 IEC proteins were altered by bLF. Low bLF doses (0.1–1 g/L) up-regulated 11 proteins associated with glycolysis, energy metabolism and protein synthesis, indicating support of cell survival. In contrast, a high bLF dose (10 g/L) up-regulated three apoptosis-inducing proteins, down-regulated five anti-apoptotic and proliferation-inducing proteins and 15 proteins related to energy and amino acid metabolism, and altered three proteins enhancing the hypoxia inducible factor-1 (HIF-1) pathway. In the preterm pig intestine, bLF at 10 g/L decreased villus height/crypt depth ratio and up-regulated the Bax/Bcl-2 ratio and HIF-1α, indicating elevated intestinal apoptosis and inflammation. In conclusion, bLF dose-dependently affects IECs via metabolic, apoptotic and inflammatory pathways. It is important to select an appropriate dose when feeding neonates with bLF to avoid detrimental effects exerted by excessive doses.
Biological significance: The present work elucidates dose-dependent effects of bLF on the proteomic changes of IECs in vitro supplemented with data from a preterm pig study confirming detrimental effects of enteral feeding with the highest dose of bLF (10 g/L). The study contributes to further understanding on mechanisms that bLF, as an important milk protein, can regulate the homeostasis of the immature intestine. Results from this study urge neonatologists to carefully consider the dose of bLF to supplement into infant formula used for preterm neonates.

1. Introduction

Lactoferrin (LF) is a multifunctional 80-kDa protein present in both human (1–7 g/L) and bovine (0.2–1.5 g/L) milk and colostrum [1,2]. Its bioactivity has been well documented, including its iron-binding, anti-microbial, immune-stimulating and anti-inflammatory effects [2–4]. LF has been suggested to protect preterm infants against the development of necrotizing enterocolitis (NEC), a devastating disease with high morbidity and mortality rates [3]. The high homology in protein sequence (77%) between human (hLF) and bovine lactoferrin (bLF) suggests that supplementation of bLF to infant formula may exert beneficial effects in a similar manner to that of hLF in human milk [5].

In preterm infants, bLF at a dose of 0.1 g/day protects against late-onset sepsis and acts in synergy with probiotics to decrease NEC sensitivity [6]. In rodents, oral administration of 0.24–0.4 g/kg bLF decreased dextran sulfate sodium-induced colitis and inflammatory cytokine secretion [7]. A high dose of oral bLF (20 g/L) protected germ-free newborn pigs against endotoxin-induced lethal shock [8], possibly due to the binding of bLF to lipopolysaccharide (LPS), which prevents LPS-induced inflammation [9,10]. However, we have previously shown in preterm pigs that bLF-enriched formula at a relatively high dose (10 g/L, 1.2 g/kg/day) did not protect against NEC, but tended to exacerbate the disease severity and increase intestinal permeability [11]. In porcine intestinal epithelial cells (IECs), low levels of bLF (0.1–1 g/L) stimulated cell proliferation whereas a high level of 10 g/L inhibited cell proliferation and induced inflammatory cytokine production via NF-κB signaling [11]. bLF may activate NF-κB following its binding to TNF receptors, as shown for monocytic cells [12], or to TLR-4, as

Abbreviations: iTRAQ, isobaric tag for relative and absolute quantitation; LF, lactoferrin; NEC, necrotizing enterocolitis.

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shown for macrophages [13]. Most IECs express the LF receptor (LfR) [14], and upon binding of bLF, the LfR undergoes internalization, which enables the intracellular uptake of bLF and causes the activation of ERK and cell proliferation [4]. Consequently, the bLF concentration and expression of IEC receptors, such as LfR and TLR-4, may be crucial in determining which intracellular signaling pathways and regulatory effects are initiated by bLF stimulation.

In this study, the porcine IEC cell line PsIc1, derived from not fully differentiated crypt cells of a 6-month-old pig, was used as a model of immature IECs. We hypothesized that bLF dose-dependently affects the fate and functions of IECs, as reflected by the differentiated cellular proteome following stimulation with bLF at 0, 0.1, 1 and 10 g/L. The chosen doses of 0.1 and 1 g/L were close to LF levels in bovine and human mature milk [1,2], whereas 10 g/L is a relatively high dose, slightly higher than the average level of LF in human colostrum. The proteins involved in key cellular pathways identified by proteomics were further analyzed in the small intestine of formula-fed preterm pigs with a supplementation of 10 g/L bLF. Our study aimed to help understand the mechanisms by which different bLF doses may exert different, or even contrasting, effects on the immature intestine.

2. Materials and methods

2.1. Cell culture

Porcine IECs (PsIc1, Bionutritech, Lunel, France) at passages 5–25 were cultured at 37 °C and 5% CO_2 using an advanced DMEM medium supplemented with 2% heat-inactivated fetal bovine serum, 40 U/mL penicillin, 40 μg/mL streptomycin and 2 mM glutamax (Life Technologies, Nærum, Denmark).

2.2. Proteomics of IECs treated by bLF

A high-throughput approach using LC-MS-based proteomics with iTRAQ labeling was applied [15]. PsIc1 cells were cultured until 90–95% confluency and maintained in serum-free medium for 24 h, when cells reached 100% of confluency, prior to stimulation with bLF (Morinaga Milk Industry, Japan) at different doses of 0, 0.1, 1 and 10 g/L (LF0, LF0.1, LF1 and LF10, respectively) in triplicates (4 treatments × 3 replicates = 12 samples) for another 24 h. bLF was 15% iron-saturated with a low endotoxin content (1.6 EU/mg protein) detected by Limulus Amebocyte Lysate Chromogenic Endotoxin Quantitation Kit (Fisher Scientific, Slangerup, Denmark). Thereafter, the IECs were collected and lyzed by sonication in TES buffer (10 mM Tris pH 7.6, 1 mM EDTA and 0.25 M sucrose), and the protein concentration was determined using the BCA protein assay (Thermo Scientific, Denmark). The extracted proteins were precipitated in cold acetone and reduced with dithiothreitol, and free cysteines were blocked with methyl methanethiosulfonate before trypsin digestion overnight. The obtained peptides were labeled with 4-plex iTRAQ tags (Applied Biosystems, Forster, CA, USA), and a common reference sample was made by pooling equal amounts of protein from all 12 samples. This reference was then used in each of the 4-plexed samples, as specified in Supplementary Table S1 online. The procedure from lysis to labeling has previously been described in detail [16]. After labeling, the 4-plexed samples were dried at room temperature and stored at −80 °C until LC-MS/MS analysis.

The LC-MS/MS analysis and raw-data file processing were performed as previously described [17]. In brief, the iTRAQ-labeled tryptic peptide samples were analyzed on a Dionex RSLC UPLC system with a nanopump module coupled with a Thermo-Electron Q-exactive Plus mass spectrometer (MS, Thermo Scientific, Waltham, USA). Duplicate runs of each 4-plexed sample were loaded onto a C18 reversed-phase column (Dionex; Acclaim PepMap100 C18, with a 5 μm pre-column and a 50 cm Acclaim Pepmap RSLC, 75 μm ID main column, Thermo Scientific) and eluted with a linear gradient from 96% formic acid (1%) and 4% acetonitrile to 65% formic acid and 35% acetonitrile. The 12 precursor-ions with the highest intensity were selected for higher energy collisional dissociation (HCD) fragmentation using *m/z* 100 as fixed lower set point. The resulting raw files were analyzed using Thermo Proteome Discoverer 1.4 (Thermo Scientific) and MaxQuant LFQ, as previously described [18]. The protein abundance in treatment groups was reported relative to that in the common reference sample. Data were searched against Uniprot *Sus scrofa* (UPID000008227) and *Bos taurus* (UPID000005136) protein reference proteome databases and in-house compiled database optimized for a novel PeptideAtlas database for *Sus scrofa*. The MS proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002755.

The intracellular uptake of bLF in the IECs was determined by SDS-PAGE with 15 μg of total protein loaded into the gel, as previously described [19].

2.3. Preterm pig study and gut morphology

Twenty-eight preterm pigs from four sows (Large White × Danish Landrace × Duroc) were delivered by caesarean section at 90–92% of gestational age (105–106 days). The pigs were housed, and administered parenteral and enteral nutrition for five days prior to euthanasia as previously described [11]. The enteral nutrition was either infant formula (CON, n = 15) or infant formula enriched with 10 g/L bLF (LF, n = 11) [11]. The diets were optimized for piglet's needs with energy and macronutrient composition according to Table 1, as previously reported [11]. Commercial ingredients used were: protein (Lacprodan DI-9224, Arla Foods Ingredients (AFI), Denmark), lactose (Variolac 960, AFI), maltodextrin (Ross Polycose, Abbott Nutrition, USA), lipids (Liquigen and Calogen, Nutricia), vitamins and minerals (SHS Seravit, Nutricia). The bLF used was from the same source as in the cell study. At euthanasia, the clinical sign of NEC was recorded, based on our established macroscopic NEC scoring system from 1 to 6 [11]. NEC was regarded as positive when a score of 3 or higher was observed in any gastrointestinal regions (stomach, proximal, middle, distal intestine and colon). Proximal small intestinal sections were removed, fixed by formaldehyde and embedded in paraffin prior to staining with hematoxylin and eosin to measure villus height and crypt depth, as previously described [11]. For each individual section, the length of 10 random villi and crypts was measured and the average values for each pig were used for statistics. The protocol of the animal study was approved by the Danish National Committee on Animal Experimentation.

2.4. Western blot for intestinal tissues

Middle small intestinal tissues of the healthy CON and LF pigs (n = 5/group), that did not develop NEC, were homogenized in an extraction buffer containing 1% Triton X-100 and 1% of protease inhibitor cocktail [15]. Thereafter, 25 μg protein of all tissue samples was resolved on a 16% SDS-PAGE gel and transferred onto PVDF membranes (Life Technologies) [15]. Membranes were then probed with primary and secondary

Table 1
Macronutrient compositions of formula diets for CON and LF pigs.[a]

	CON	LF
Energy, MJ/L	4.51	4.51
Protein, g/L	79	79
Whey protein, g/L	79	69
Supplemented bLF, g/L	0	10
Carbohydrates, g/L	62.5	62.5
Maltodextrin, g/L	47.5	47.5
Lactose, g/L	15	15
Fat, g/L	57	57

[a] Energy and macronutrient composition was calculated according to the ingredient specifications provided by the suppliers (Eurofins Steins Laboratorium, and Morinaga Industry).