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Enteral leptin administration affects intestinal autophagy in suckling piglets

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ABSTRACT

Leptin has been shown to play an integral role in the endocrine regulation of metabolism. Moreover, a substantial amount of this peptide has been found in colostrum and milk. The aim of the study was to investigate the effects of exogenous leptin, administered intragastrically, on the process of autophagy and the changes in cell hyperplasia and hypertrophy in the small intestine mucosa. Three groups (n = 6) of neonatal piglets were used in the study. The pigs were fed either by their sows (sow-reared piglets) or with only milk formula, or with milk formula together with leptin administered via a stomach tube (10 μg/kg BW) every 8 h for 6 d. We have shown that pure milk formula feeding significantly elevates ($P < 0.05$) autophagy compared with that observed in sow-reared piglets. Compared with the control group, feeding milk formula supplemented with leptin resulted in a significant decrease ($P < 0.05$) in immunodetection of microtubule-associated protein 1 light chain 3, as well as significantly accelerated epithelial cell renewal ($P < 0.05$). We demonstrated that autophagy is involved in the remodeling of the small intestine mucosa and that leptin, when administered enterally, may be an important factor for its regulation.

1. Introduction

Autophagy is a highly conserved process by which cells degrade and recycle their own components (cytosol and organelles) within lysosomes [1]. When autophagy proceeds at a regular, basal rate, it maintains normal cellular homeostasis by removing cellular debris and defective proteins, therefore playing a crucial role in cell renewal, especially in the case of long-lived and highly active cells. Autophagy is also triggered in response to environmental cues, such as starvation, radiation [2], and an increase in reactive oxygen species [3]. As a starvation response, autophagy is activated after depletion of a range of nutrients, including nitrogen, carbon, and amino acids, as well as certain hormones such as insulin and glucagon in mammals [4]. Autophagy is reported to be associated with various pathophysiological conditions, including cancer, cardiomyopathy, muscular diseases, and neurodegenerative disorders [1]. Recently, autophagy has also been linked to intestinal pathophysiology such as bowel inflammation [5] or necrotizing enterocolitis [6,7]. In addition, autophagy has been shown to play a crucial role in maintaining amino acid and energy homeostasis in neonates during the early neonatal period, thus ensuring survival during the transitional hours immediately after birth, when neonates must adapt to changes from placental to maternal milk feeding [8,9]. Previous studies have shown that in neonatal piglets the first week of life is a crucial period, because of intensive development of the gastrointestinal tract, a complex process that involves both growth and changes in function which provide irreversible gut maturation. In the early postnatal

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period the development of the gastrointestinal tract in mammals is highly controlled and regulated by bioactive peptides in the mother's colostrum and milk. This unique mechanism of delivering bioactive peptides from the mother to the offspring through the act of nursing is called lactocrine signaling [10,11]. Replacement of mother's milk with the milk formula (deficient in bioactive peptides) inhibits the structural and functional maturation of the small intestine mucosa [12,13]. We have previously found that enteral administration of leptin (in a dose that is naturally present in colostrum and milk) to neonatal piglets fed with milk formula reversed the maturation of the small intestine mucosa to that observed in sow-reared (SR) piglets [12]. It has also been previously reported that leptin, a satiety hormone, injected in pharmacologic doses, influences autophagy in several peripheral tissues [14]. However, to our knowledge the effect of leptin on the autophagy of the small intestine epithelium has not yet been studied. Our previous studies have shown that ghrelin, the most prominent functional opponent for leptin actions, when administrated enterally to the piglets fed with the milk formula and maintained in the same experimental model as in the present study, increased autophagy in the small intestine epithelium [15]. Therefore, the aim of the present study was to elucidate whether enterally administrated leptin affects autophagy in the small intestine mucosa of neonatal piglets. We examined the level and cellular localization of light chain 3 (LC3; the main marker of autophagy) with the use of immunodetection in piglets fed mother's milk, milk formula, or milk formula supplemented with leptin. To better understand the mechanism of leptin action in the small intestine of neonates, we also determined the intensity of proliferation and apoptosis in the intestine epithelium.

2. Materials and methods

The experiments and treatments were conducted in compliance with the European Union regulations, concerning the protection of experimental animals (EC Directive 86/609/EEC with amendments). The study protocol was approved by the Third Local Ethics Committee in Warsaw, according to the Polish Law for the Care and Use of Animals (Resolution no. 26/2003).

2.1. Animals

A total number of 18 male neonatal piglets (Polish Landrace × Pietrain), from 4 different litters, were purchased from a commercial pig farm. For the first 24 h, all piglets were kept with their sows. After this period, 12 of them were transported to the laboratory (6 piglets for each trial) and randomly divided into 2 groups (n = 6). Piglets (n = 12) were housed in individual cages equipped with single-armed artificial sows microprocessor-operated system (Research Center Foulum-model, Pig's oline, Boss' Produkter a/s, Ulstrup, Denmark), which provides equal amounts of milk replacement formula with a frequency appropriated for piglets of a particular age. Milk replacer formula for piglets (protein, 19.8%; fat, 19.7%; Lakti R, Polfarma, Poland) was supplied to each piglet every 75 min (20 times per 24 h) by means of an artificial sow. Body weight was recorded every morning. The control group (n = 6) received the milk formula via the artificial sow and 5 mL 0.9% NaCl via a stomach tube every 8 h. The leptin group (n = 6) received the milk formula via the artificial sow together with leptin (leptin mouse recombinant; Sigma, St. Louis, MO, USA) via a stomach tube (10 μg/kg BW) every 8 h. The leptin dose was chosen, based on the results of a previous study by Woliński et al [12] who showed that this dose reflects the natural concentration of leptin in sow's milk during the first week of lactation, and moreover when administered enterally plays an important biological role within the small intestine of neonatal pigs. The remaining 6 piglets were kept and reared by their sows (SR) under commercial farm conditions.

After 6 d of vehicle or hormone treatment, as well as sow-rearing, the pigs were sacrificed by means of a pentobarbiturate (Vetbutal; Biowet, Gorzów, Poland) overdose. The jejunum was dissected out, and the middle part was collected and immediately processed for further analysis. Identification of the intestinal segments was performed according to the gut morphology of each segment, as previously described [16].

2.2. Sample preparation

For immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, samples of middle jejunum were immediately fixed in 10% neutral formalin solution. After 24-h fixation period, the intestinal samples were routinely embedded in paraffin. The paraffin-embedded samples were cut into 4.5-μm sections and applied to silane-treated glass slides. For all further staining, the sections were dewaxed in xylene and rehydrated in decreasing grades of ethanol and then washed in PBS buffer. The antigen retrieval was performed by 20-min microwave heating (500 W) in citrate buffer.

For the Western blot procedures and biochemical analysis, the intestine was flushed with the ice-cold isotonic solution, and samples of the mucosa from the middle jejunum were scraped off from the intestine and then weighed. For measurements of protein and DNA/RNA concentrations, the mucosal scrapings were collected in sterile tubes, frozen, and stored in −80°C until analysis. For Western blot analysis, the scrapings were immediately immersed in cell lysis buffer (20 m*M* HEPES, pH 7.4; 250 m*M* NaCl; 1% Nonidet P-40; 5% glycerol; 3 m*M* EDTA; 3 m*M* ethylene glycol tetraacetic acid; 1 m*M* phenylmethylsulfonyl fluoride; 1 m*M* Na_3VO_4; 50 m*M* NaF; 20 m*M* β-glycerophosphate; 30 m*M* Na-pyrophosphate, inhibitors of proteases) and then homogenized and centrifuged (20 min; 20,000 × *g*). The supernatant was frozen and stored in –80°C until further analysis.

2.3. TUNEL assay

The number of apoptotic epithelial cells was determined with a TUNEL assay, in paraffin-embedded