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Colostrum oxytocin modulates cellular stress response, inflammation, and autophagy markers in newborn rat gut villi

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ABSTRACT

Little is known about the role of oxytocin (OT) in colostrum during early gut colonization. We previously showed that transient OT receptor (OTR) expression on newborn rat enterocytes coincides with the milk-suckling period, and that OT activates endoplasmic reticulum stress sensors in cultured enterocytes. Here, we explored whether colostrum-OT attenuates stress in newborn villi primed and unprimed by colostrum by measuring levels of stress markers including BiP (an ER chaperone), eIF2a (translation initiation factor), and pPKR (eIF2a kinase). We also measured two inflammation-signaling proteins NF-κB and its inhibitor IκB. To test the impact of colostrum on autophagy, we measured a marker of autophagy initiation, LC3A. Colostrum increased inactive p-eIF2a, p-PKR and IκB and reduced p-IκB, BiP and LC3A. LPS increased and OT decreased p-IkB. BiP (GRP78) was higher in unprimed than primed villi. Together, these data suggest that colostrum OT attenuates the impact of inflammation on postnatal gut villi and that OT enhances autophagy to protect against amino acid insufficiency-induced stress during the interval between birth and the first feeding.

1. Introduction

The presence of the neuropeptide oxytocin (OT) in milk and its role in milk letdown are well known. Less known is the role of OT delivered in mothers' milk on newborn gut development. Recent in vivo studies in rodent gut have demonstrated multiple important effects of OT/OT receptor (OTR) signaling on enteric neurons (1). Other experiments have shown that OT in combination with secretin is anti-inflammatory in animal models of colitis (2) and OTR deficient mice have altered gastrointestinal structure, motility, macromolecular permeability, mucosal maintenance and inflammatory responses [1]. OT appears to play an important role in early gut function and development. For instance, OTR expression is developmentally regulated during the milk-suckling period, and toward the end of the suckling period, OTR expression appears to migrate toward stem cells in the crypts [4].

There are several stressors to the newborn gut. These include temporary starvation, particularly of essential amino acids prior to first feed [2]. The first feeding of colostrum exposes the gut to high concentrations of foreign microbiota [3,4], as well as the anti-inflammatory OT [5–7]. Another stressor includes early colonization by microbiota. In vitro experiments utilizing lipopolysaccharide (LPS) to mimic exposure of the newborn gut to bacterial endotoxin have shown that inflammatory signaling in enterocytes may be attenuated by OT to reduce cellular stress [[8]. Other studies in gut cells support this hypothesis. OT downregulates the PI3K/Akt/mTORC protein synthesis pathway [9], a pathway dysregulated in autism and linked to mRNA translation and [12]. OT also modulates central sensors of the unfolded protein response (UPR) [10], a transcriptional program that strictly limits mRNA translation and clears unfolded proteins to resolve endoplasmic reticulum (ER) stress [14]. LPS activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB), which is a transcription factor that

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regulates the expression of ~150 genes, including pro-inflammatory proteins that are normally downregulated by NF-KB inhibitor, IKB [11]. Phosphorylated IKB (pIKB) releases NF-KB, which translocates to the nucleus to serve as an active transcription factor [12,13]. Collectively, these studies show that exposure to OT suppresses activation of the inflammatory pathway and stimulates the UPR.

In the present study, we tested the impact of colostrum OT on markers of cellular stress and inflammation signaling in early postnatal villus tissue. We also measured LC3A, a marker of autophagy, to test whether amino acid insufficiency stress stimulates or inhibits inflammation during the interval between birth and first feeding before villus tissue is exposed to colostrum [14]. We examined protein expression in harvested villi that were unprimed (prior to first feed) and in vivo primed (subsequent to first feed). We exposed in vivo unprimed and colostrum primed tissue at a subsequent ex vivo stage to vehicle alone, LPS, exogenous OT or OTR antagonist (OTA). In a second protocol, we compared ex vivo the effects of vehicle versus colostrum in the presence and absence of OTA on villi obtained from unprimed rats using extracted colostrum. Our results suggest that colostrum OT plays a pivotal anti-inflammatory role that includes autophagy in the newborn rat gut villi.

2. Materials and methods

2.1. Reagents

Oxytocin (OT; Phoenix Pharmaceuticals Inc., Burlingame, CA) and the oxytocin receptor antagonist (OTA; desGly-NH2-d(CH2)5 [D-Tyr2,Thr4]OVT (ST-11-61)) were donated by Dr. Maurice Manning, University of Toledo, OH; OTA is 102-fold more efficient than OT in its interaction with OTR [15]. LPS-EB standard, derived from E coli 0IIIB4 and TLR4 ligand were obtained from InvivoGen (San Diego, CA).

2.2. Antibodies

The following antibodies were used: anti-rabbit IgG horseradish peroxidase (HRP) conjugate and anti-mouse IgG HRP conjugate (WES Automated Western Blot kits; ProteinSimple, Santa Clara, CA). Rabbit anti-phospho-eIF2a (Ser51, 9721; Cell Signaling Technology (CST)), Inc., Danvers, MA, mouse mAb anti-eIF2a (2103; CST), rabbit anti-Phospho-PKR (Thr451, 07-886; Millipore), rabbit mAb anti-PKR (12297; CST), rabbit anti-LC3A (4108; CST), rabbit mAb anti-GAPDH (2118; CST). Mouse mAb anti-phospho-IκB (9246; CST), mouse mAb anti-IκB (4814; CST). Rabbit anti-BiP (3183; CST) was used to detect BiP/GRP78 and BiP/GRP94, which are two separate chaperones coded by different genes. However, they share an important structural motif, KDEL, at their C-terminus, which serves as an ER retention signal. Due to this shared motif, the antibody cross-reacts with both chaperones [16,17].

2.3. Animals

Pregnant Sprague-Dawley rats (Hilltop Lab Animals, Scottsdale, PA) were housed in AAALAC-approved facilities of the New York State Psychiatric Institute and Columbia University. Individually-caged pregnant dams were provided food and water ad libitum with light/darkness cycles of 12h each. The Institutional Animal Care and Utilization Committees of Columbia University and the New York State Psychiatric Institute approved all experimental procedures.

2.4. Villi harvest and colostrum extraction

Timed-pregnant rats were inspected every 2 h on their anticipated delivery date. After birth, all females from 3 litters were euthanized by intraperitoneal injection of ketamine and xylazine. The stomach and duodenum were dissected, and the duodenal section was spread open under the microscope and gently washed with phosphate buffered saline (PBS) at room temperature. Using a laboratory microscope, the duodenal villi were gently scraped and aspirated into PBS, washed four times and spun at 200 rpm for 5 min at room temp to remove milk traces. Villi were harvested within 6 h after birth at two different time points, prior to first feed (unprimed) and after first feed (primed). All villi were then isolated and washed with PBS. Gastric colostrum was extracted from some pups post feeding for use in unprimed villi experiments under Protocol B (ex vivo; Supplemental Fig. 1).

2.5. Villi stimulation and protein extraction

Villi were incubated (5% CO2 and 37 °C in a humid atmosphere) for 30 min in a vehicle containing Dulbecco modified essential medium (DMEM) purchased from American Type Culture Collection, Manassas, VA, glucose 4.5 g/L, bovine transferrin 10 ng/ml, standard penicillin and streptomycin, 2 mM glutamine, and 10% fetal calf serum purchased from GIBCO, Grand Island, NY. At this point, villi tissue was examined according to two different protocols. Under Protocol A (Supplemental Fig. 1), we examined the response of villi exposed to colostrum in vivo (in vivo primed) to OT, OT + OTA, LPS or vehicle alone and compared them to unprimed villi by assaying markers of stress and inflammation. Under Protocol B we examined the levels of stress and inflammation in villi not yet exposed to colostrum (unprimed). Therefore, we exposed only unprimed villi to vehicle alone or with colostrum, both in the presence or absence of OTA. In this protocol the colostrum was extracted from the stomach of a animals immediately after suckling. The colostrum was diluted (1:10) to adjust its viscosity to the vehicle. After 30min incubation we assayed villus protein extracts for markers of stress and inflammation.

2.6. Preparation of protein extracts

Villi under Protocol A and B were quickly washed twice with ice cold PBS. The villi were incubated for 30min in ice cold 0.1 ml of a protein extraction cocktail [Bicine/Chaps Cell Lysis Kit (p/n CBS403)] containing protease and phosphatase inhibitors following the manufacturer's instructions (www.proteinsimple.com). All samples were equalized to have the same protein concentration according to a Bradford based assay from Bio-Rad against a bovine serum albumin (BSA) standard curve. Samples were stored at −70 °C in 10 μl aliquots. Samples for WES analysis were prepared using 4 parts of protein extract mixed with 1 part of a master mix containing SDS, dithiothreitol, and sample buffer (5X). This mixture was heated to 95 °C for 5 min and loaded onto plates provided by ProteinSimple and loaded into the WES instrument.

2.7. Assaying markers of nutrient insufficiency stress and inflammation

The effects of stimulation in both protocols were measured by assaying markers of stress and inflammation; eIF2a, PKR, BiP, NFKb, IKb and LC3A as indicated in the results section. Total- or phosphoproteins were quantified using a micro-capillary immunoelectrophoresis method on a WES instrument (ProteinSimple, San Jose, CA,