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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 phospho-proteins were quantified using a micro-capillary immunoelectrophoresis method on a WES instrument (ProteinSimple, San Jose, CA,

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http://www.proteinsimple.com/wes.html). Reagents were obtained from ProteinSimple and prepared according to the manufacturer's instructions, with slight modification (Klein 2014) [10,18,19]. Parallel wells were loaded with the primary antibody, the secondary HRP conjugated antibody, and a luminol substrate with hydrogen peroxide mixture. The plates were spun for 10 min at 2500 rpm. The wells containing separation fluids and running buffer were uncovered, and loaded into the WES.

2.8. Statistical analysis

Data was analyzed using WES proprietary Compass software. Quantification was determined by the area under the curve and compared to controls using paired Student's T test (2-tailed or 1-tailed). Data were analyzed using SPSS Base 9.0 (SPSS, Chicago, IL) and presented as mean averages ± standard error. Divergent trends of IκB versus pIκB against LC3A were determined by the significance of differences between correlation coefficients (r) using Fisher transformation of r values to z values.

3. Results and discussion

3.1. Colostrum suppresses the UPR marker BiP via OT receptor

We tested the impact of exogenous OT (ex vivo stimulation) on the ER-resident chaperone BiP, an important marker of cellular stress and inflammation signaling, including the unfolded protein response (UPR) to ER stress and nutrient insufficiency [2]. Our antibody recognized two families of BiP; the HSP70 family at 78 kDa (Fast BiP, GRP78), and the HSP90 family at 94 kDa (Slow BiP, GRP94). We measured BiP levels under two different protocols (Fig. S1).

Under Protocol A, we measured Slow and Fast BiP levels in unprimed and in vivo primed tissue in the presence of vehicle, LPS, OT, OT + OTA. Fast-BiP was significantly elevated in unprimed villi compared with in vivo primed villi (Fig. 1A). In both unprimed and in vivo primed villi, fast BiP was unchanged by the addition of LPS, OT, or OT + OTA. These results suggest important differences between in vivo and in previous in vitro cell culture studies, where we

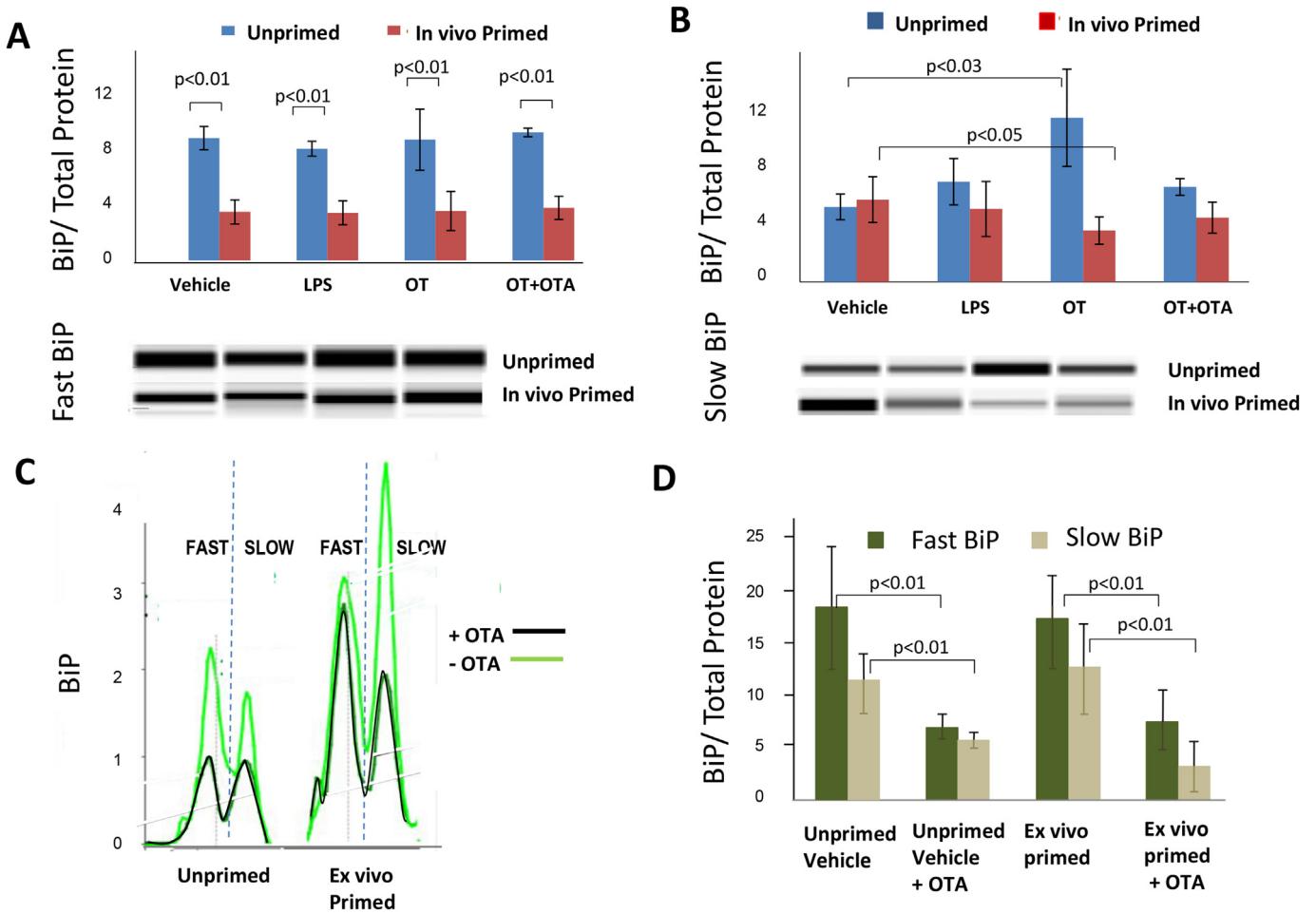

Fig. 1. The effect of in vivo and ex vivo priming on the expression of slow and fast BiP. (A–B) Protocol A: (A) Comparison of fast BiP in in vivo unprimed and in vivo primed villi exposed to vehicle, LPS, OT and OT plus OTA and representative blot. Levels of fast BiP were elevated in unprimed vs in vivo primed villi in all conditions, but were unchanged relative to one another. Note that in vivo priming reduced fast BiP. (B) Comparison of slow BiP between unprimed and in vivo primed villi exposed to vehicle, LPS, OT and OT plus OTA and representative blot. Slow BiP was significantly lowered by OT vs vehicle control ($p < 0.05$). Note also that the level of unprimed in vehicle is significantly lower than unprimed in OT ($p = 0.03$). **(C–D) Protocol B:** (C) Unprimed villi were exposed to OTA in the presence and absence of extracted colostrum ($n = 3$). Raw chemoluminescence data showing Fast BiP (GRP78) and slow BiP (GRP94) levels highlight the differential effects of OTA on Fast and Slow BiP in unprimed and ex vivo primed villi. (D) Quantification of data shown in 1C. Note that OTA significantly downregulated both fast and slow BiP. Ex vivo primed tissue + OTA decreased slow BiP compared with ex vivo primed tissue in the absence of OTA ($p = 0.01$).

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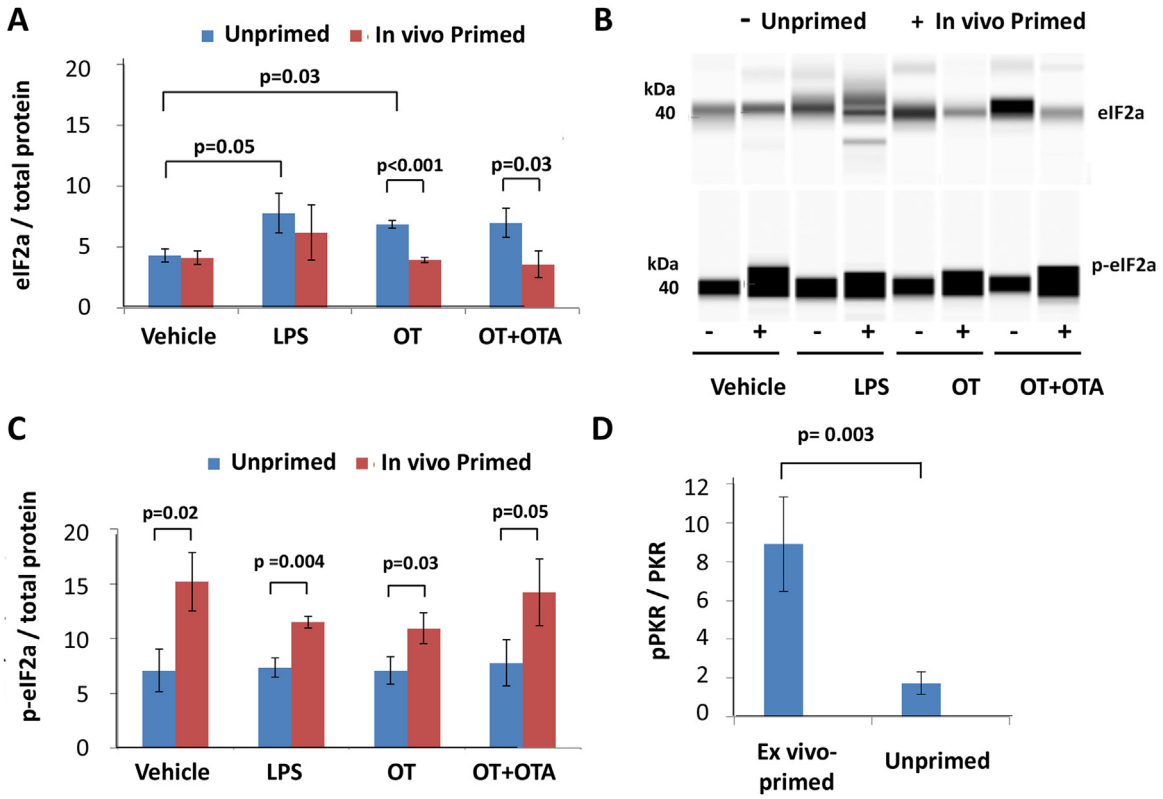

Fig. 2. Colostrum OT inhibits eIF2a and greatly increases levels of pPKR. (A–C) Protocol A: (A) eIF2a (C) p-eIF2a and (B) representative blots. eIF2a was relatively high in unprimed vs in vivo primed villi under OT and OT + OTA conditions. Under LPS eIF2a was higher in unprimed villi vs vehicle alone ($p = 0.05$) and p-eIF2a was higher in in vivo primed vs unprimed villi. **(D–C) Protocol A:** The cytoplasmic eIF2a kinase (PKR), activated by phosphorylation (pPKR), relative to the inactive form PKR is fourfold higher in colostrum primed villi than in unprimed villi, likely explaining the increase in p-eIF2a in primed villi.

reported that OT stimulation increased BiP expression [14]. In the present studies, the failure of OTA to block OT may result predetermination of fast BiP levels in vivo conditions and/or because other OT-independent mechanisms (Fig. 1A). Levels of slow BiP were significantly ($p < 0.05$) decreased by OT in primed and increased in unprimed villi ($p < 0.03$) relative to vehicle alone (Fig. 1B), suggesting that the time window of slow BiP expression occurs after fast BiP (Fig. 1A). To test whether unresponsiveness to OT had plateaud and was predetermined by in vivo conditions, we exposed unprimed villi to OTA in the presence and absence of extracted colostrum (Protocol B). OTA significantly downregulated both fast and slow BiP (Fig. 1C–D). Colostrum + OTA decreased slow BiP compared with vehicle + OTA ($p = 0.01$). OT may thus elevate BiP and is present in both, unprimed and primed villi. Furthermore, to exhibit the effect of OT or OTA on fast BiP, villus OTA exposure must be concomitant with OT. These ex vivo results confirm our previous in vitro cell culture results showing that OT exposure increased BiP associated with ER stress-sensor activation [8,10]. Thus, elevated BiP in unprimed villi likely reflect nutrient insufficiency [2] during the interval between birth and first feed, rather than ER stress. Colostrum nutrition may repress nutrient insufficiency-induced BiP by overriding OT's maintenance of high BiP levels.

Slow BiP did not increase in unprimed villi treated with vehicle, LPS, or OT + OTA relative to in vivo primed villi. OT reduced slow BiP in in vivo primed villi vs unprimed villi ($p < 0.05$). Response differences in slow versus fast BiP in unprimed villi may reflect different temporal activity windows for either ER resident chaperones [20]. Previously, we showed that OT increased BiP expression in human gut cells as an ER stress-response [8,10]. Since colostrum contains OT, we expected that colostrum would increase fast BiP in in vivo primed villi. Instead, levels of BiP were higher in unprimed vs in vivo primed villi. BiP may thus respond to nutrient insufficiency rather than ER stress under these conditions. Collectively, these data suggest that the nutrient content in colostrum may suppress BiP and override the enhancing effects of OT in colostrum on BiP levels.

3.2. Colostrum modulates newborn gut protein translation

Levels of eIF2a increased after LPS exposure consistent with an anabolic effect of LPS on inflammatory protein expression that may require uninhibited translation. Exogenous OT increased eIF2a levels significantly ($p < 0.001$) in unprimed versus primed villi, but was not blocked by OTA (Fig. 2A–B). This result is surprising, since we observed reductions in eIF2a and increased (inactive) p-eIF2a after OT stimulation in Caco2BB cells [10]. p-eIF2a was significantly increased (~1.5–2-fold) (Fig. 2B–C) in primed villi by all treatments, including vehicle alone. The effects of colostrum are thus complex and may include factors other than OT that enhance p-eIF2a. As shown, colostrum overcomes the effect of LPS by increasing the levels of p-eIF2a ($p = 0.04$). The addition of exogenous OT to OTA (OT + OTA) resulted in similar levels of p-eIF2a in colostrum-primed and vehicle. Unexpectedly, conditions that decreased BiP in in vivo primed villi increased p-eIF2a (Fig. 2C). Stress responses in unprimed villi may result from nutrient insufficiency, as opposed to ER-stress. In in vivo primed villi, OT and perhaps colostrum factors increase p-eIF2a levels.

The pPKR/PKR (dsRNA-activated kinase) ratio was more than 4-fold higher in in vivo primed vs unprimed villi (Fig. 2D). Our

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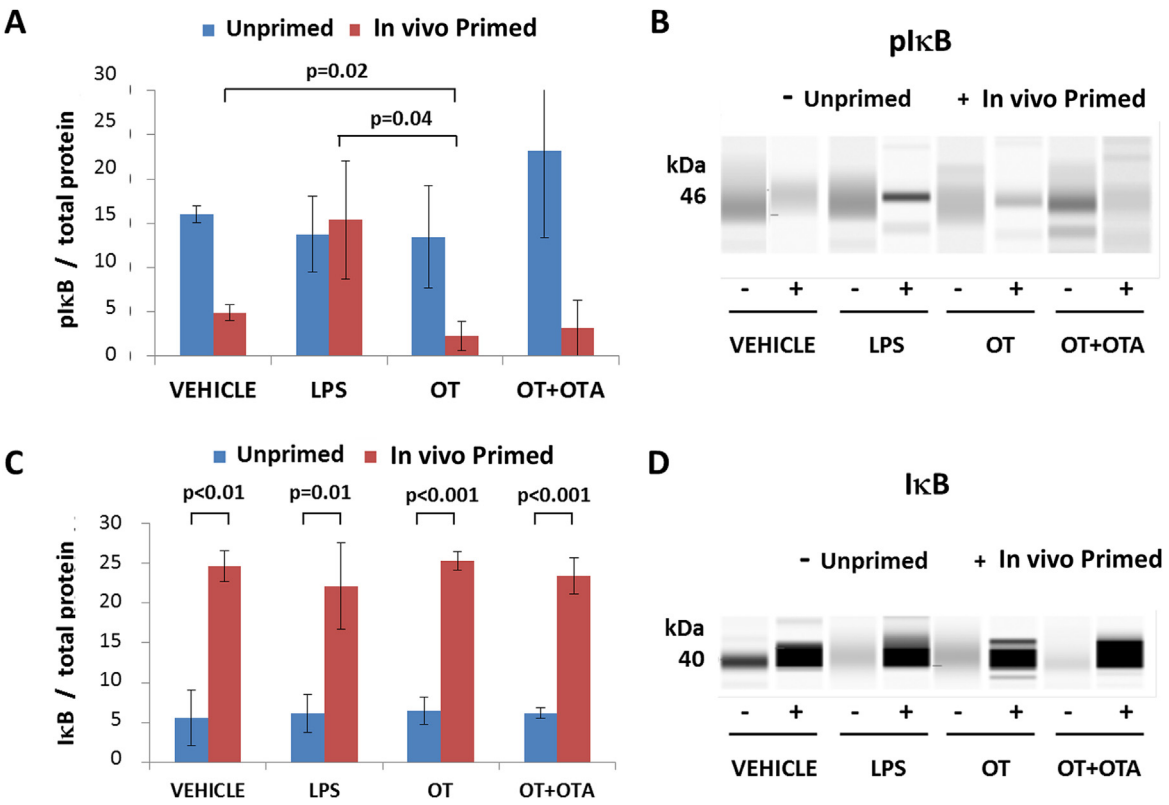

Fig. 3. pIκB and IκB expression in in vivo primed and unprimed villi. Protocol A. (**A**) Exogenous OT decreased pIκB levels in in vivo primed relative to unprimed villi. In contrast, pIκB levels under LPS were higher vs vehicle alone in in vivo primed villi, but LPS didn't increase pIkB above its effect on unprimed villi. Although not significant, the addition of OTA to OT raised the level of pIκB in unprimed villi relative to all other conditions, suggesting that OTR may be involved in inhibiting the disruption of IκB. (**B**) representative pIκB bands of Fig. 3A. (**C**) IκB was five-fold higher in primed compared with unprimed villi under all conditions. In unprimed villi, IκB was refractory to all conditions and significantly low compared to primed villi, indicating less resistance to inflammation. (**D**) Representative IκB bands from Fig. 4C.

previous studies showed that under OT, pPKR inactivated eIF2a via phosphorylation [14]. The increase in the active pPKR strongly suggests that this kinase inactivates eIF2a via phosphorylation rather than an OT-independent mechanism. Of note, colostrum had a greater impact on p-eIF2a levels than on eIF2a levels (Fig. 2). This may reflect differences in colostrum vs pPKR effects on translation compared with post-translational modification of eIF2a (Fig. 2D). Colostrum-induced increases in pPKR may result from dsRNA domains in mammary gland mRNA present in milk [14] and miRNA [21], including precursor dsRNA domains that may activate PKR [22].

3.3. Colostrum and exogenous OT inhibit inflammatory signaling

We investigated the impact of in vivo colostrum priming on IκB, the inhibitor of NF-κB (IκB), a key regulator of inflammatory signaling, and its inactive form, pIκB. pIκB was unaltered by LPS or OT in unprimed villi, but was reduced in in vivo primed villi under vehicle alone. Exogenous OT further reduced p-IκB in in vivo primed villi ($p = 0.024$), and was unaffected by OTA. IκB levels were low with all treatments in unprimed villi, but elevated 5-fold in colostrum-primed villi (Fig. 3C–D). NF-κB may be mobilized to initiate inflammatory signaling pathways prior to colostrum exposure and suppressed by colostrum OT or other colostrum factors. Compared with vehicle alone, exogenous OT did not further increase IκB in primed villi. Altogether these findings suggest that colostrum stimulates anti-inflammatory signaling to a ceiling level that is refractory to exogenous OT. This robust IκB elevation may occur in vivo to colostrum priming such that ex-vivo OTA is insufficient to show an effect. This distinguishes the present study from previous experiments where OTA-inhibition of exogenous OT occurred when both, OT and OTA were added simultaneously to cell cultures where colostrum was not previously involved [8].

3.4. Colostrum modulates autophagy

In Fig. 4, we examined the effect of OTA on the two isoforms of autophagy marker LC3A in unprimed and ex vivo primed villi (Protocol B). LC3A was elevated under all conditions relative to its precursor form. However, LC3A was attenuated by colostrum + OTA. The activation of LC3A may be partially mediated by colostrum OT.

The relative expression of LC3A in vehicle and colostrum correlated positively with IκB expression ($r = 0.91$, $n = 6$, $p = 0.01$) and negatively with pIκB ($r = -0.79$, $n = 6$, NS) (Fig. 4C). IκB increased in parallel with increased LC3A and reduced pIκB, as exhibited by the opposing regression lines (Fisher transformation of opposing correlation coefficients ($z = 3.19$, $n = 6$, $p = 0.0007$)). Unlike these results, the samples of IκB and pIκB showed a substantially lower correlation with LC3A when tested in the presence of OTA (pIκB: $r = 0.05$; IκB: $r = 0.32$) (Not shown). The difference between these r values was not significant. Thus our findings suggest that colostrum OT couples autophagy (as measured by LC3A) with the suppressor of NF-κB, the master transcription factor of inflammation. Note that this finding associates autophagy with anti-inflammation and suggests yet another mechanism by which colostrum OT may regulate inflammation. The short amino acid starvation period between birth and the first milk feeding may

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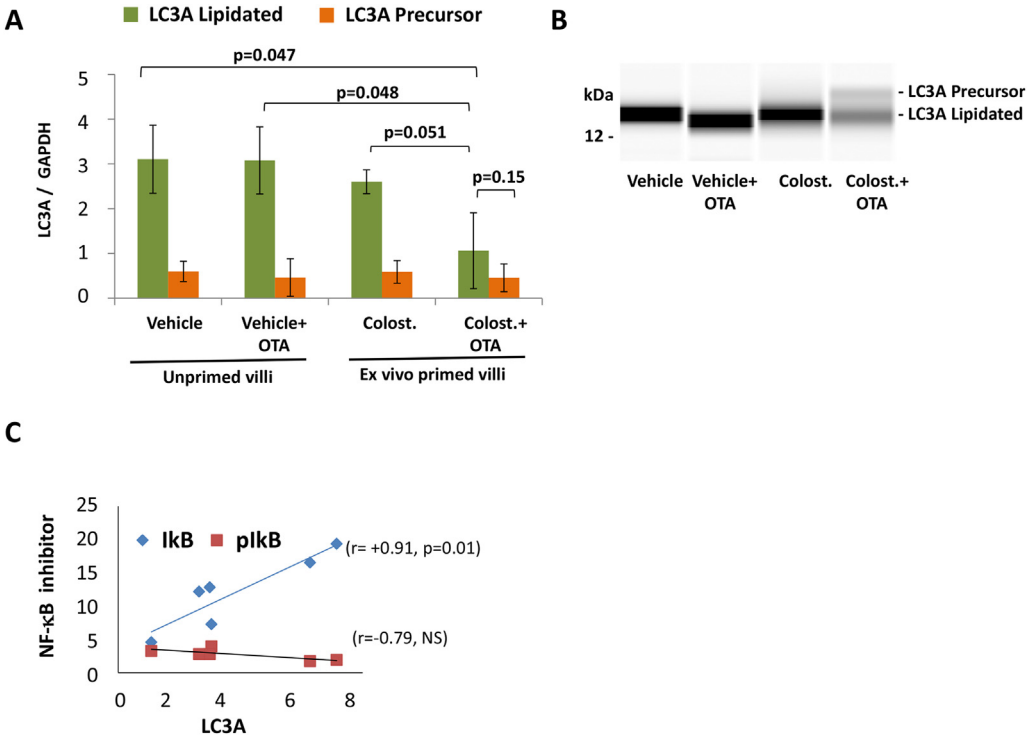

Fig. 4. Colostrum modulates autophagy coupled with upregulation NF-κB inhibition markers. Protocol B (n = 6). **(A)** Mean LC3A density was normalized to GAPDH in two LC3A isoforms, a slow running precursor (brown) and a fast running activated, lipidated form (green). LC3A expression in unprimed villi in vehicle alone or in colostrum with or without OTA shows. **(B)** Representative LC3A bands in precursor and lipidated forms under four experimental conditions. **(C)** We plot the activity of NF-κB inhibitors, IκB and pIκB, as a function of LC3A concentration. Regression lines for IκB (blue) and pIκB (red) are presented, along with their correlation coefficients. The difference between the two correlation coefficients was computed by Fisher transformation ($z = 3.91$) and indicates high significance ($p < 0.001$). This implies that OT in colostrum is responsible for coupling autophagy initiation and enhancing NF-κB inhibition markers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cause cellular stress that is possibly accommodated for by autophagy and the downregulation of mRNA translation. Intake of microbiota before and/or during suckling [3,4] may elicit an inflammatory reaction reinforced by activated immune cells from the colostrum [23]. Inflammatory NF-κB signaling involves 150 genes [11]; this is an anabolic process expected to require extensive mRNA translation, which in its turn is opposed by the downregulated translation that accompanies autophagy responses. This scenario is consistent with the increase in p-eIF2a and IκB in primed villi and the coupling of NF-κB inhibition markers with LC3A autophagy marker by ex vivo OT in unprimed villi. Colostrum is populated with maternal immune cells [24] that may infiltrate villus tissue and contribute to the two opposite processes, intracellular stress versus inflammation signaling in colostrum primed villi.

4. Summary

Our findings show that colostrum OT attenuates the impact of inflammation on postnatal gut villi during the colonization period. In addition, OT enhances autophagy to protect against amino acid insufficiency-induced stress during the interval between birth and the first feeding.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.04.011.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.04.011.

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