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ORIGINAL PAPER

Oxytocin modulates markers of the unfolded protein response in Caco2BB gut cells

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Abstract We have shown that oxytocin receptor (OTR) expression in neonatal rat enterocytes is robust from birth to weaning, but OTR function during this period is unknown. We previously reported that oxytocin (OT) stimulation of Caco2BB cells (enterocytes in vitro) inhibits the mammalian target of rapamycin complex 1 (mTORC1) signaling. The unfolded protein response (UPR) is known to protectively reduce translation during endoplasmic reticulum (ER) stress. Because the mTORC1 pathway is linked to cellular stress, we investigated markers of UPR in OT-stimulated Caco2BB cells. We report that OT modulates several factors involved in sensing and translation of ER stress. High OT (62.5 nM) reduced translation initiation factor 4E-BP1 phosphorylation (Ser65), which is known to inhibit cap-dependent translation via its rate-limiting eukaryotic translation initiation factor 4E (eIF4E). Importantly, high OT increased phosphorylation of eukaryotic translation initiation factor 2a (eIF2a) phospho-Ser51, which inhibits eIF2a. High OT also increased protein kinase RNA-like endoplasmic reticulum kinase phosphorylation, a sensor of ER stress and a kinase of eIF2a. Both high and low OT activated inositol requiring enzyme1 (IRE1), which generates the transcription factor X-box binding protein 1 (XBP1) and induces the UPR. We also show that OT modulates XBP1 splicing and induces tribbles 3 (TRIB3; a negative regulator of Akt and protein involved in autophagy) and immunoglobulin binding protein (BiP; ER-chaperone). Taken together, these results indicate that OT modulates sensors of ER stress and autophagy. These findings support our hypothesis that transiently elevated OTR expression in neonatal gut may serve a protective function during a critical postnatal developmental period.

Keywords Autism spectrum disorder · Inflammatory bowel disease (IBD) · Crohn's disease · Ulcerative colitis · Signaling · Translation factors · X-box protein 1 · Simon

Abbreviations

4E-BP1	Translation initiation factor 4E-binding protein 1
ASD	Autism spectrum disorder
ATF4	Activated transcription factor 4
ATF6	Activation transcription factor 6
BiP	Immunoglobulin binding protein
CHOP	C/EBP homology protein
eIF2a	Eukaryotic translation initiation factor 2a
eIF4E	Eukaryotic translation initiation factor 4E
FGM	Fresh growth medium
IBD	Irritable bowel disease
IRE1	Inositol requiring enzyme 1
ER	Endoplasmic reticulum
GADPH	Glyceraldehyde-3-phosphate dehydrogenase

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mTORC1	Mammalian target of rapamycin complex 1
OT	Oxytocin
OTR	Oxytocin receptor
PDK1	Phosphoinositide-dependent kinase-1
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PI3K	Phosphoinositol-3 kinase
PTEN	Phosphatase and tensin homolog
TRIB3	Tribbles 3
TSC2	Tuberosclerosis protein
UPR	Unfolded protein response
XBP1	X-box binding protein 1

Introduction

The affiliative and nurture hormone, oxytocin (OT) is perceived as a stress-attenuating hormone. This role of OT has been demonstrated across psychological, neurological and physiological levels (Amico et al. 2004; Brunton and Russell 2008). OT is also involved in modulating inflammation that accompanies disorders such as IBD (Cetinel et al. 2010; Welch et al. 2010). OT is also likely relevant to gut development and function, particularly in a subset of individuals with autism spectrum disorder (ASD). The role of OT and its receptor (OTR) in gut function is supported by three of our findings. First, we have demonstrated that OTR expression is developmentally regulated in rat enterocytes (Welch et al. 2009). This finding is significant in light of high concentrations of OT in milk (Takeda et al. 1986). Second, when combined with secretin, OT reduces colonic inflammation in adult rats (Welch et al. 2010). And third, mice lacking the OTR have altered gastrointestinal motility, inflammation, macromolecular permeability, and mucosal maintenance (Margolis et al. 2013). Other findings point to a connection between OT and ASD, as well as between gut function and ASD: intranasal OT treatment improves ASD symptoms in adults (Bartz and Hollander 2008); ASD patients show hypertrophy of epithelial secretory Paneth cells (Horvath et al. 1999); a subset of individuals with ASD showed a deficiency of digestive enzymes (Horvath and Perman 2002b). All of these findings suggest that understanding the role of OT in gut development and function could shed new light on the physiology of ASD phenotypes, and lead to novel therapeutic interventions.

To gain insight into the signaling pathway(s) modulated by OT stimulation of the OTR in gut epithelium, we previously explored the response of the phosphoinositol-3 kinase (PI3K)/Akt pathway to OT in human gut epithelial cell lines (Klein et al. 2011). We found that OT modulated the abundance of $pAkt^{T308}$ via PI3K in a dose-dependent manner; notably, 62.5 nM OT maximally stimulated the Akt/PI3K pathways in comparison with higher (up to 1,000 nM OT) or lower OT concentrations. Subsequently, we found that OT attenuated the stimulation of the PI3K/Akt pathway induced by fresh growth medium (FGM) (Klein et al. 2013). OT also modulated the phosphatase and tensin homolog (PTEN) (Klein et al. 2011) and the mammalian target of rapamycin (mTOR) pathway (Klein et al. 2013). Several of these OTR signal transducers are associated with both ASD and protein translation; mutations in PI3K, PTEN, and tuberosclerosis protein (TSC2), members of the Akt/mammalian target of rapamycin complex 1 (mTORC1) pathway that control ribosomal protein and translation factor activity have been linked with ASD (Kelleher and Bear 2008). Further support for aberrant protein translation in ASD comes from the finding of mutations in a negative regulator of mRNA translation for fragile X mental retardation protein 1 (FMRP1), as well as mutations in the eukaryotic translation factor 4E (eIF4E), both of which are linked to autism (Kelleher and Bear 2008; Neves-Pereira et al. 2009; Gkogkas et al. 2013; Wang and Doering 2013). It is therefore highly probable that OT may regulate protein translation in neuronal circuits important to normal social behaviors and, in enterocytes essential for normal gut development and/or function.

Processes that regulate mRNA translation serve several essential cellular functions. These include synaptic plasticity in neurons that underlies memory formation (Costa-Mattioli et al. 2009), nutrient sensing and autophagy orchestrated by mTOR (Foster and Fingar 2010), and protective mechanisms during endoplasmic reticulum (ER) stress (Koumenis et al. 2002; Ma and Hendershot 2003), which are especially important in secretory cells (Martinon and Glimcher 2011). ER stress occurs when the rate of translation of proteins exceeds their proper rate of folding (Calfon et al. 2002). This stressful situation induces a transcriptional program called the unfolded protein response (UPR). The UPR causes attenuation of global translation and selectively upregulates chaperones that act to clear unfolded proteins from the stressed ER (Song et al. 2009). During the UPR, signaling branches are activated by three parallel sensors of stress in the ER membrane: (1) protein kinase RNA-like endoplasmic reticulum kinase (PERK), (2) activation transcription factor 6 (ATF6) and (3) inositol requiring enzyme 1 (IRE1) (Bernales et al. 2006). The kinase PERK directly inhibits mRNA translation by phosphorylating translation initiation factor 2a (eIF2a) (Bertolotti et al. 2000). ATF6 transduces ER stress after its cleavage, which yields a fragment that translocates to the nucleus to regulate gene expression (Sato et al. 2011), including the gene for X-box binding protein 1 (XBP1) (Yoshida et al. 2001). The kinase and endoribonuclease domains of IRE1 are crucial for differentiation of plasma cells (Todd et al. 2009) and for secretory cell function (Hetz and Glimcher 2009). A substrate of IRE1, XBP1, mRNA, is then spliced and becomes itself a transcription factor (Lee et al. 2003; Yoshida et al. 2006).

Here, we demonstrate that OT stimulation of Caco2BB gut cells modulates several UPR pathway markers, including eIF2a, PERK, IRE1, XBP1, and the ER-resident chaperone immunoglobulin binding protein (BiP). We show also that OT can induce tribbles 3 (TRIB3), which is elicited by cellular stress or nutrient deficiency. Taken together these results indicate that OT modulates sensors of ER stress and autophagy.

Materials and methods

Cells and culture reagents

The Caco2BB cells (C2BBe1 clone; American Type Culture Collection, Manassas, VA) were grown (5 % CO2 and 37 °C in a humid atmosphere) in Dulbecco-modified essential medium (DMEM, glucose 4.5 g/L) fortified with bovine transferrin 10 ng/ml that was supplemented with standard penicillin and streptomycin, 2 mM glutamine, and 10 % fetal calf serum (GIBCO, Grand Island, NY). We stimulated Caco2BB cells at different concentrations and time-points with FGM plus OT and with FGM alone. The concentrations of OT used activated both the high affinity state of the OT receptor (OTR; kD from 1 to single-digit nanomolar with 7.5 nM OT (designated as "low OT")) or approximately mid-range between high and low affinity states of the OTR (kD 100 nM with 62.5 nM OT ("high OT")). These OT concentrations were determined in previous studies that examined effect of serial dilutions of 1000 nM OT on the PI3K/Akt signaling pathway in Caco2BB cells. Higher concentrations of OT (>125 nM) were not used to avoid non-specific stimulation of various vasopressin receptors.

Reagents

Experiments used OT (Phoenix Pharmaceuticals Inc., Burlingame, CA) and the oxytocin receptor antagonist (OTA; desGly-NH2-d(CH2)5[D-Tyr2,Thr4]OVT (ST-11-61) 5 nM); donated by Dr. Maurice Manning, University of Toledo, OH, and is 10^2-fold more efficient than OT in its interaction with OTR (Manning et al. 1995).

Antibodies

The following antibodies were used: anti-rabbit IgG horseradish peroxidase (HRP) conjugate and anti-mouse IgG HRP conjugate (Simon Automated Western Blot kits; ProteinSimple, Santa Clara, CA). Rabbit anti-Phospho-4E-BP1 (Thr37/46, 9459; Cell Signaling Technology (CST) Inc., Danvers, MA), rabbit anti-Phospho-4E-BP1 (Ser65, 9451; CST), rabbit anti-4E-BP1 (9452; CST), rabbit anti-phospho-eIF2a (Ser51, 9721; CST), mouse mAb anti-eIF2a (2103; CST), rabbit anti-Phospho-PERK (Thr980, 3179; CST), mouse mAb anti-spliced XBP1 (MAB4257; R&D Systems), rabbit mAb anti-phospho-IRE1 (Ser724, 3881-1; Epitomics, Burlingame CA), rabbit mAb anti-IRE1 (3294; CST), mouse mAb anti-α-tubulin (T6074; Sigma, St. Louis, MO); rabbit anti-BiP (3179; CST), rabbit mAb anti-GAPDH (2118; CST), rabbit mAb anti-XBP1u+s (ab109221; abcam), rabbit mAb anti-TRIB3 (TA303408; ORIGENE).

OTR stimulation and protein extraction

Stimulation experiments with OT were performed in cell cultures 24 h after seeding of 25×10^4 cells/cm^2. OTR antagonist (5 nM final concentration) was added with OT. Continuous stimulation for 30 min was terminated by placing the cultures on ice. The cultures were quickly washed twice with ice cold phosphate-buffered saline (PBS) and 0.1 ml of protein extraction cocktail was added for 5 min. We extracted the protein samples using the Bicine/Chaps NanoPro Cell Lysis Kit (p/n CBS403) following the manufacturer's instructions (www.proteinsimple.com). Samples were adjusted to equalize protein concentrations and were stored at −70 °C in 10-μl aliquots until use. The extraction cocktail contained protease inhibitors and phosphatase inhibitors. The protein extracts were scraped and spun at 10,000×*g* for 30 min at 4 °C. A sample of each extract was processed for protein determination and the remainder was stored at −70 °C. Protein concentrations where measured by a paper spot protein assay against a bovine serum albumin (BSA) standard curve. Protein samples (4 μl) were applied to 3 mm filter paper, stained with Coomassie blue in 40 % methanol and 10 % acetic acid, washed with the same solution without dye and dried. Proteins were eluted with 3 ml of 2 % SDS and concentrations were quantified on an ELISA reader at 650 nm.

Simon™ automated western blotting and analysis

All reagents for running the simple western on Simon™ (http://www.proteinsimple.com/simon.html) were obtained from ProteinSimple (San Jose, CA) and prepared according to manufacturer's recommendations. These reagents include: biotinylated molecular weight ladder, streptavidin-HRP, fluorescent standards, luminol-S, hydrogen peroxide, sample buffer, DTT, stacking matrix, separation matrix, running buffer, wash buffer, matrix removal buffer. For antibodies, ProteinSimple antibody diluent, goat anti-rabbit secondary antibody, and goat anti-mouse secondary antibody were also purchased from ProteinSimple. The capillaries, containing a proprietary UV-activated chemical linked reagent, were also obtained from ProteinSimple.

All samples and reagents were prepared according to the recommended ProteinSimple manual. Samples were diluted to adjust protein concentration to 3 or 4 μg in 2.5 μl with sample buffer (ProteinSimple) and further diluted 1:2 by

adding 2.5 μl of the 2X master mix (containing 80 mM DTT, 2X Sample buffer and 2X fluorescent standards). The final samples of 5 μl each were boiled 5 min, placed on ice for 5 min, and after a short centrifugation they were applied to proper wells. Both a stock of 1 M DTT and 1:1 mixture of luminol-S and peroxide (150 μL) were prepared fresh daily and kept on ice until use. Fluorescent standards and biotinylated molecular weight ladder were used according to the manufacturer's instructions. Aliquots (5 μL of biotinylated ladder, and 14 μL for fluorescent standards) were stored at −20 °C and removed for each run on Simon™. All primary antibodies were diluted with antibody diluent provided by ProteinSimple.

The Simon™ instrument was prepared by adding 2 ml of matrix removal buffer (ProteinSimple) to Trough 1, 2 mL of wash buffer (ProteinSimple) to Trough 2, and 0.8 ml of running buffer (ProteinSimple) to Trough 3. A clip of 12 capillaries and the 384-well plate containing samples, antibodies, and matrices were then placed inside the instrument. The Simple Western was run using the following settings. The capillaries were filled with separation matrix for 100 s, stacking matrix for 16 s and protein samples for 12 s. The samples were then separated by applying a voltage of 250 V for 40 min. Once the separation was complete, the samples were immobilized to the wall of the capillary using the default immobilization conditions and then washed with matrix removal buffer for 140 s to remove the separation matrix inside the capillaries. Capillaries were then washed with wash buffer for 150 s before being blocked with antibody diluent for 15 min to prevent non-specific binding by the primary antibody. Next, the capillaries were incubated with primary antibody for 3 h, washed, and incubated with HRP conjugated secondary antibodies (ProteinSimple) for 1 h. After removal of unbound secondary antibody, the capillaries were incubated with the luminol-S/peroxide substrate and chemiluminescent signal was collected using the charge-coupled device (CCD) camera of Simon™ with six different exposure times (30, 60, 120, 240, 480, and 960 s). Data analysis was performed using the Compass Software (ProteinSimple) on Simon™.

Statistical analysis

Band density differences at each time point or each reagent concentration were computed against controls using a paired Student's t test (two-tailed or one-tailed in the absence or presence of expectation, respectively; $\alpha = 0.05$). Data were collected from three to four replicates per most conditions and 2 replicates for the inhibition with OTA and were analyzed using SPSS Base 9.0 (SPSS, Chicago, IL). All plots present mean±standard error. Where baseline antibodies did not produce a clean and interpretable figure, we substituted a household reference protein.

Results

OT modulates phosphorylation of 4E-BP1

We recently found that OT modulates the activity of mTORC1 by Raptor^{S792} phosphorylation (Klein et al. 2013). We analyzed the response of the mTORC1 substrate 4E-BP1, a negative regulator of eIF4E, to test whether OT influences translation factors downstream of mTORC1. For all experiments, we have defined high (62.5 nM) and low (7.8 nM) concentrations of OT based upon convention from our prior work (Klein et al. 2011) where high 62.5 nM OT maximally stimulated the Akt/PI3K pathways. Protein extracts from Caco2BB cells treated with high or low concentrations of OT for 30 min were fractionated by capillary electrophoresis and analyzed for phosphorylation. Phosphorylation of 4E-BP1^{S65} inhibits eIF4E binding and thereby enables cap-dependent translation, whereby the ribosome scan is initiated from the 5′ end of the mRNA cap until the start codon, as guided by initiation factors that include eIF4E. It has been suggested that p4E-BP1$^{T37/46}$ does not directly disrupt the inhibition of eIF4E. Instead, it primes 4E-BP1 for further S65 phosphorylation, which in turn disrupts the eIF4E inhibition and enables cap-dependent translation (Gingras et al. 1999). Low OT increased both total 4E-BP1 and p4E-BP1^{S65}, but minimally decreased p4E-BP1$^{T37/46}$ compared with controls (0 nM OT). High OT increased levels of total 4E-BP1, as well as p4E-BP1$^{T37/46}$ in both 27- and 11-kDa bands. In contrast, high OT decreased levels of p4E-BP1^{S65}, as compared with 27 kDa control. The elevated level of unphosphorylated 4E-BP1 with high OT relative to low OT is consistent with inhibition of cap-dependent translation activity with high OT treatment (Fig. 1a).

Only high OT reduced the ratio of p4E-BP1^{S65} relative to total 4E-BP1 (Fig. 1b). The unexpected inverse correlation between p4E-BP1^{S65} and p4E-BP1$^{T37/46}$ indicates that OT has an opposite effect on phosphorylation of these two sites (Fig. 1c). High OT treatment resulted in a gradual decrease in p4E-BP1^{S65} over a time course from 20 to 60 min. Total 4E-BP1 did not change with OT dose or time (Fig. 1d). The time-dependent effects of high OT observed here for 4E-BP1 are similar to those previously observed for the PI3K/Akt pathway (Klein et al. 2013; Klein et al. 2011). In summary, high OT increases 4E-BP1 levels while maintaining low levels of p4E-BP1^{S65}. This 4E-BP1 increase is consistent the inhibition of cap-dependent translation by eIF4E mentioned above.

High OT stimulates eIF2a and its kinase PERK

Phosphorylation of eIF2a is known to inhibit its translation activity (Kimball et al. 1998). eIF2a is in the pathway downstream of Akt and mTORC1, which we found to be modulated

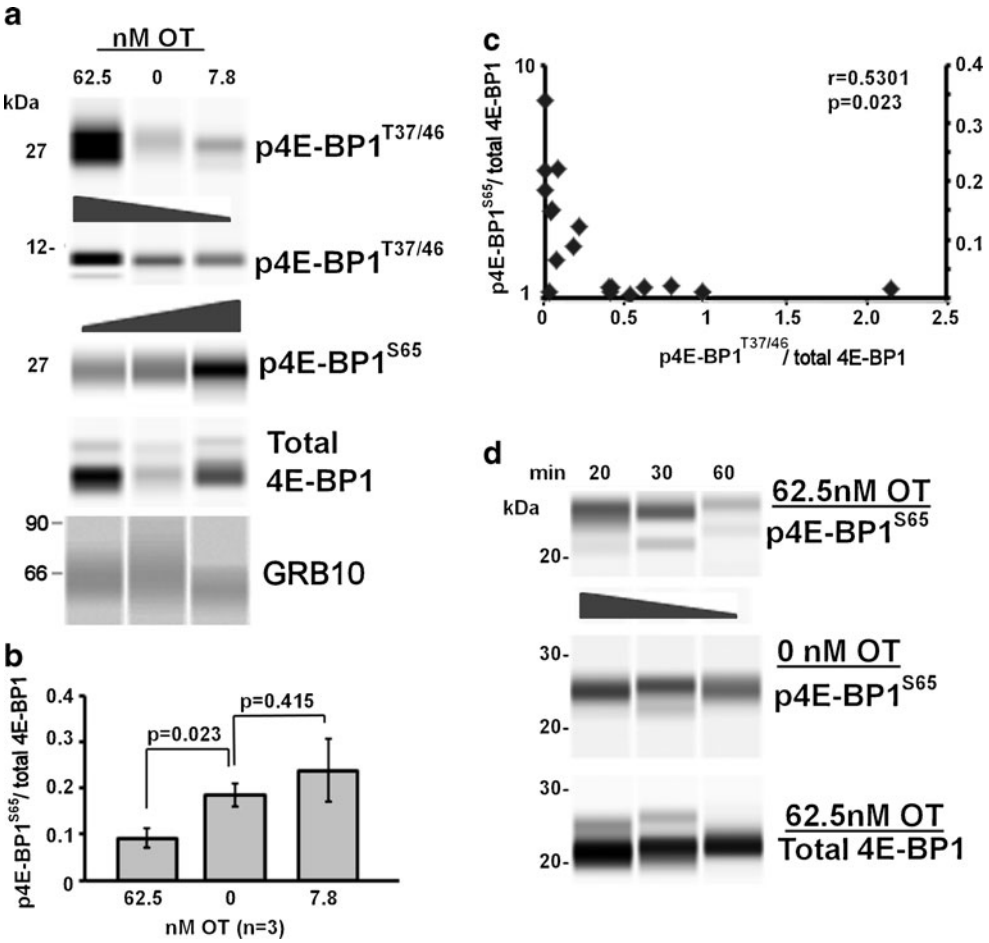

Fig. 1 OT regulation of 4E-BP1 phosphorylation. Caco2BB cells were stimulated with high OT (62.5 nM), control low OT (7.8 nM) or control medium (0 nM). Extracted proteins were analyzed using the Simon device for automated Western blot analysis. **a** At 30 min, two bands of p4E-BP1$^{T37/46}$ at 27 and 11 kDa, and only one band of p4E-BP1^{S65} at 27 kDa were detected. GRB10 is included as a loading control. **b** To correct for unequal antibody efficiency, the results were indexed relative to each control and presented as a fraction of total 4E-BP1 ($n=3$). A lower ratio indicates greater levels of 4E-BP1relative to p4E-BP1^{S65}. **c** Plot shows regression of p4E-BP1$^{T37/46}$ vs p4E-BP1^{S65} indices, each relative to total 4E-BP1; $n=18$. **d** Time course of p4E-BP1^{S65} levels after high OT (*upper panel*) are compared with plain growth medium alone (*middle panel*) and total 4E-BP1 levels (*bottom panel*) at 20, 30 and 60 min of stimulation. Note the decrease in p4E-BP1^{S65} as a function of time at high OT levels

by OT. Thus, we measured the level of eIF2a and its phosphorylated form (p-eIF2a^{S51}) in response to OT stimulation. Low OT increased total eIF2a levels, but did not alter p-eIF2a^{S51}, as compared with control. Conversely, high OT greatly increased p-eIF2a^{S51} and resulted in a more marginal increase in total eIF2a (Fig. 2a). The ratios between phosphorylated and total eIF2a were significantly higher with high OT compared with controls ($p=0.039$), and this effect was sensitive to OTR antagonist (OTA, $p=0.026$) (Fig. 2b). These results indicate the OT can modulate eIF2a activity. OTA has significant antagonistic effects on the levels of p-eIF2a and total eIF2a.

eIF2a is a substrate of the ER stress sensor PERK, the protein kinase RNA-like endoplasmic reticulum kinase (Harding et al. 2001), both are important elements of the UPR (Bertolotti et al. 2000). Since phosphorylation of ER stress sensor PERK increases its catalytic activity, we assayed the levels of PERK phosphorylation in response to OT. High OT increased pPERK^{T980}, which parallels increased p-eIF2a with this dose. Low OT marginally increased pPERK. The phosphorylation of PERK stimulates its kinase activity. Thus, increases in p-eIF2a may reflect translation inhibition by OT as early as the translation initiation stage. Interestingly, with high OT treatment, both p-eIF2a and pPERK were partially insensitive to OTA. This finding raises the question as to whether OT exerts its effect on this UPR sensor and/or eIF2a via additional kinases (Fig. 2c–d).

OT activates XBP1

Activation of transcription factors XBP1 and ATF6 (which is also responsible for XBP1 transcription) is also characteristic of the UPR. Upon ER stress, XBP1 mRNA is unconventionally spliced in the cytosol (Hetz et al. 2011) to form XBP1s, which is catalyzed by IRE1 after its endonuclease domain is induced by ER stress-elicited dimerization and autophosphorylation. We assessed OT effects on levels of spliced XBP1 (XBP1s), as an indicator of UPR activation. High and low OT (for 30 min, after 30 min preincubation in FGM) increased levels of XBP1s, suggesting that OT is also involved in activation of this branch of the UPR. In addition, the increase was OTA sensitive, implying that this response acts via the OTR (Fig. 3a–b). The IRE1 response to ER stress may act differentially from other ER stress sensors during the inflammatory response (Martinon et al. 2010), in which XBP1s acts to sustain inflammation. To understand the possible role of OT as

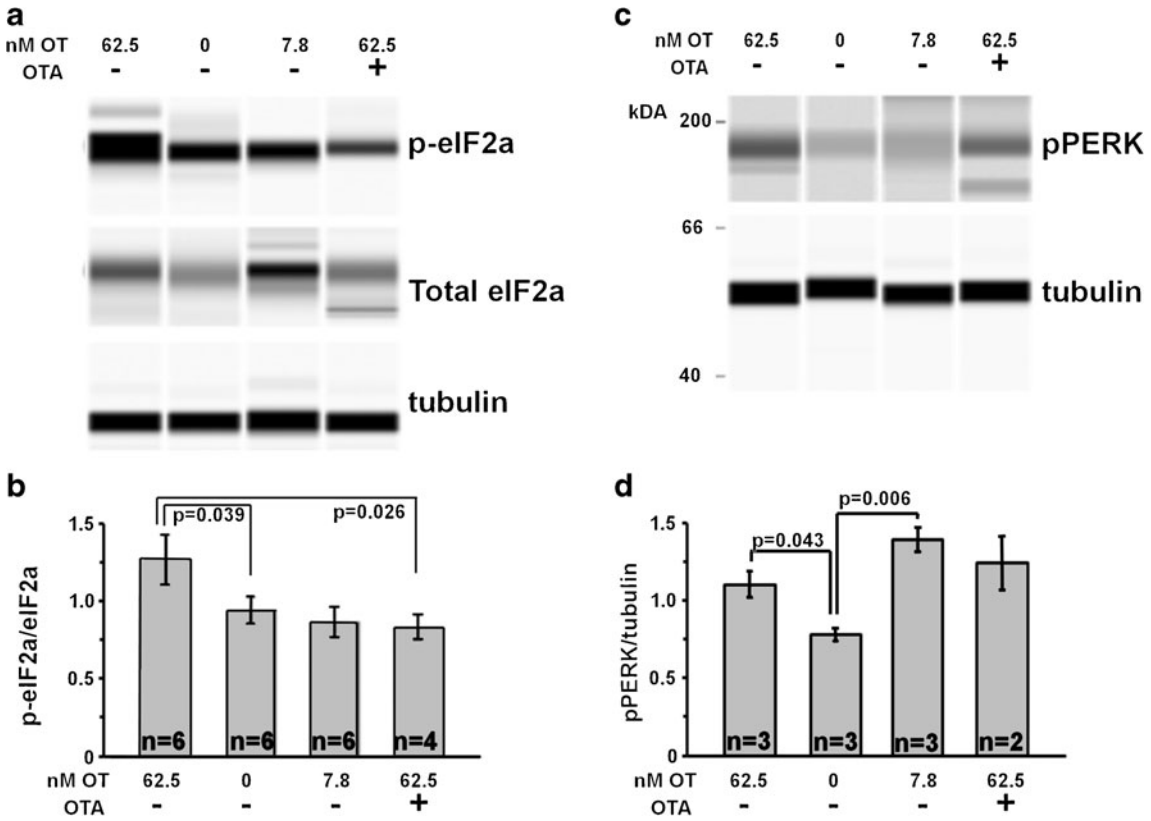

Fig. 2 High OT stimulates the phosphorylation of eIF2a and its kinase PERK. Caco2BB cells were stimulated for 30 min with either high OT, plain medium control, or low OT ($n=6$), or with high OT+5 nM OTA (OT receptor antagonist; $n=4$). Extracted proteins were analyzed using the Simon device for automated Western blot analysis. Using anti-p-eIF2a^{S51}, anti-total eIF2a and anti-tubulin, we show **a** immunolabeling and **b** quantitation of the blots. **a** Representative blot shows p-eIF2a^{S51} and total eIF2a (relative to tubulin) in response to OT with and without OTA. **b** Ratios of p-eIF2a/total eIF2a are shown for all assays. **c**–**d** Immunolabeling and quantitation of the blots. **c** Representative blot shows p-PERK^{T980} (relative to tubulin) in response to OT with and without OTA. **d** Ratios pPERK/tubulin are shown for all assays in (**c**). Note that OTA blocked OT in both **a** and **c**, strongly suggesting that OT acts via its specific receptor

a regulator of the cellular stress response, we exposed Caco2BB cells to FGM for 30 min (Fig. 3a,b) or 90 min (Fig. 3c–f), followed by exposure to OT for 30 min. It was noted that the ratios of inducted proteins were similar in the two time periods (Fig. 3). With low OT, IRE1 still exhibited phosphorylation at Ser724, thereby maintaining its endoribonuclease activity for XBP1 mRNA splicing. Under high OT, however, the levels of pIRE1^{S724} were already low to undetectable, although the total IRE1 was much higher (Fig. 3c–d). A parallel effect of OT upon XBP1 was observed in cells preincubated in this assay for a longer period (90 min), which also increased the level of pIRE1^{S724} (Fig. 3e–f). The synchronized alteration in levels of the two proteins is consistent with the fact that XBP1s is a substrate of the active enzyme (pIRE1a^{S724}).

To examine the extent to which OT modulates the splicing of XBP1u (unspliced) in response to FGM during the first 30 min of Caco2BB stimulation, we analyzed the abundance of XBP1u and XBP1s (spliced) between two treatment groups: FGM alone and FGM+62.5 nM OT (Fig. 4). FGM progressively increased the relative abundance of XBP1u at 10, 20, and 30 min. XBP1s showed even larger abundance at 10 and 20 min, but declined relative to XBP1u after 30 min. The addition of OT to FGM increased XBP1u but decreased XBP1s at 10 min (Fig. 4b). This effect is diametrically opposed to the effect of FGM alone (Fig. 4a). However, after 20 and 30 min (Fig. 4c, d) the ratio of XBP1s to XBP1u in the presence and absence of OT changed substantially. XBP1s abundance significantly increased with 30 min of OT treatment relative to XBP1u as compared to the effect of FGM alone (Fig. 4e). Thus, Figs. 3 and 4 show that OT modulates the kinetics of XBP1 splicing, as elicited by FGM alone. Collectively, these results strongly suggest that OT is involved in modulating the sensors of the UPR signaling.

Induction of TRIB3 by oxytocin

We previously observed that OT downregulates pAkt^{T308} (PDK1 substrate) but not pAkt^{S473} isoforms (Klein et al. 2013). Selective inhibition of PDK1 activity may depend upon induction of UPR via activated transcription factor 4

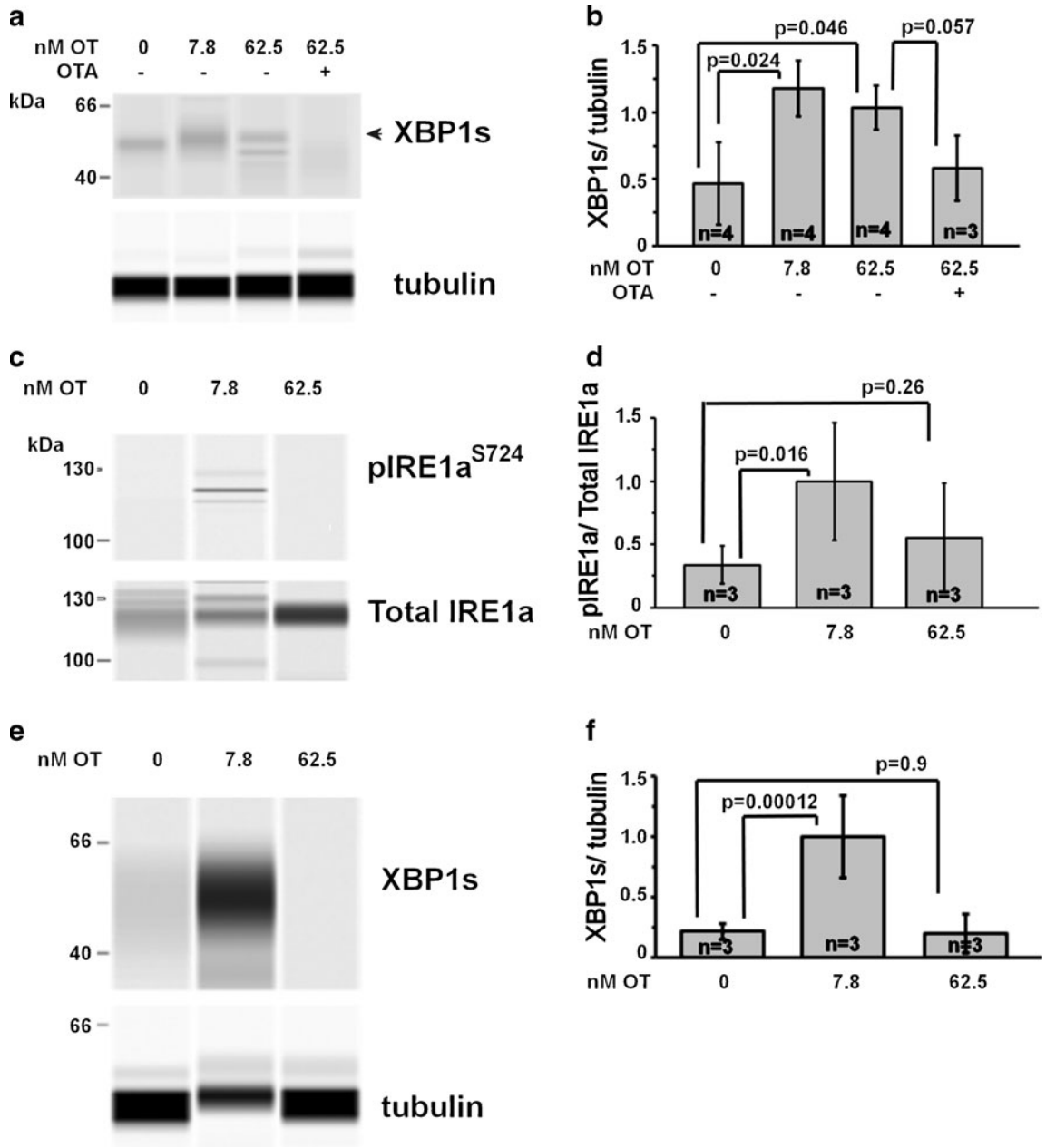

Fig. 3 OT increases the spliced form of XBP1 and differentially phosphorylates IRE1. **a**–**b** Caco2BB cells were preincubated in FGM for 30 min and then stimulated with OT for 30 min. **a** Blot shows increased XBP1s levels in response to OT and dependence on OTR activation. **b** Ratio of XBP1s/tubulin shows differential response to high and low OT. **c**–**f** Caco2BB cells were stimulated for preincubated in FGM for a longer period (90 min) and then stimulated with OT for 30 min. **c** Blot shows pIRE1^{S724} is increased by low, but not high OT. **d** Ratio of pIRE1a^{S724}/ Total IRE1a shows differential response to high and low OT. Note significant increase of pIRE1a^{S724} with low OT. **e** Blot showing robust increase of XBP1s with low but not high OT under longer incubation time. **f** Ratio of XBP1s /tubulin shows similarity of pattern to that observed in (**d**). This is consistent with coordinate active enzyme (pIRE1a ^{S724})-substrate (XBP1s) activity

(ATF4) downstream of PERK/eIF2a. ATF4 is induced and thus, evades stress-induced, global inhibition of translation and activates a transcription program expressing autophagy proteins (B'Chir et al. 2013). The TRIB3 gene is part of the autophagy program activated by ATF4/CHOP (C/EBP binding protein) (Ohoka et al. 2005) that can inhibit Akt activation by direct binding and blocking of T308 phosphorylation (Du et al. 2003). To examine the ability of OT to increase the cytoplasmic abundance of TRIB3, we stimulated Caco2BB cultures with 62.5 nM OT for 30 min and analyzed changes of TRIB3 abundance. The pAkt^{T308}/total Akt ratio was downregulated by OT while the abundance of TRIB3/GAPDH was upregulated by OT (Fig. 5). The *Z* test for two different population proportions was significant ($p < 0.01$). The correlation coefficient between FGM and OT effects on pAkt^{T308} was significant (0.93) and, for

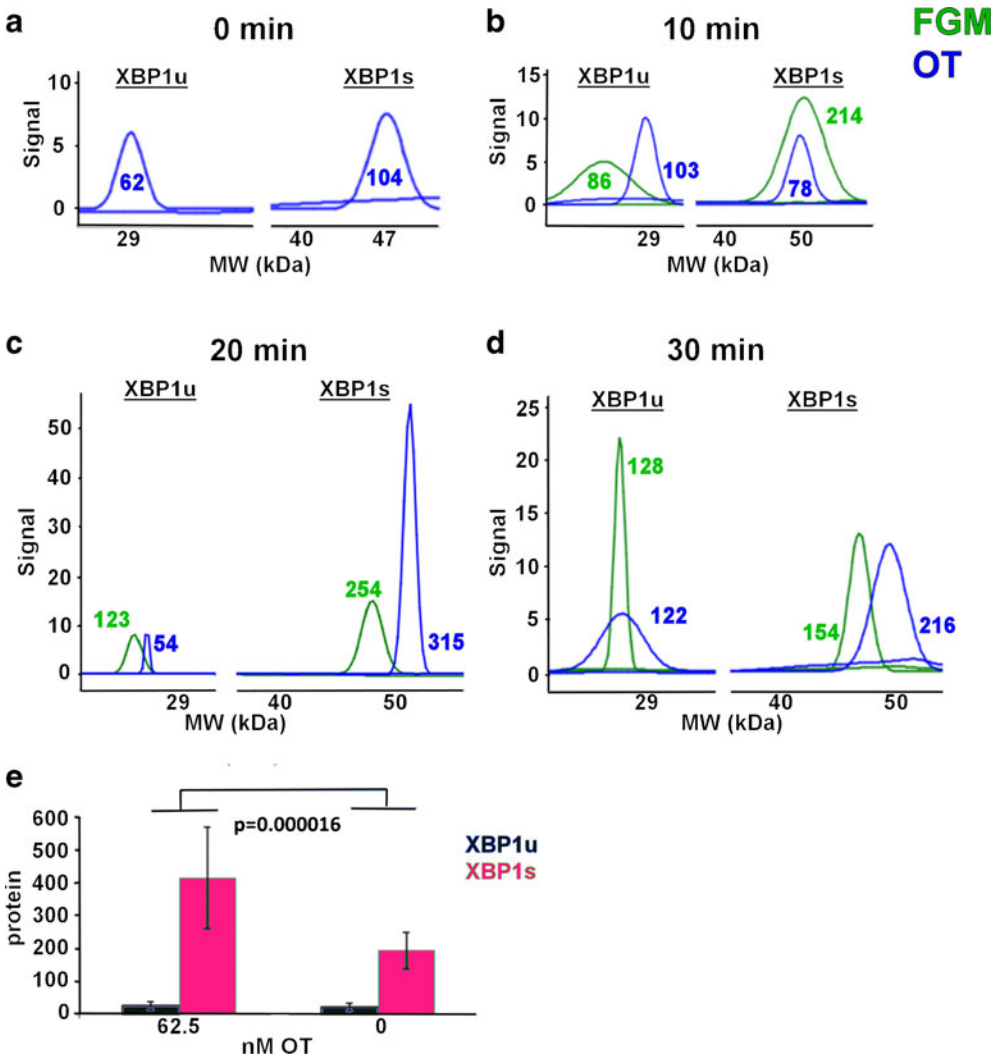

Fig. 4 OT decreases, at 10 min, and increases at 20 and 30 min, XBP1s relative to XBP1u. Protein extracts of Caco2BB cultures stimulated with 62.5 nM OT in fresh growth medium (FGM) from 0 to 30 min, compared with FGM alone (0 nM OT), were analyzed by immunocapillary electrophoresis. (**a**–**d**) Color coded curve peak areas represent quantities of XBP1s and XBP1u of OT+FGM treated vs FGM alone detected by rabbit-antibodies targeting the shared antigenic domain of both XBP1 isoforms. XBP1u and XBP1s were measured at baseline (**a**) and 10 min (**b**) 20 min (**c**) or 30 min (**d**) after the addition of OT. (**e**) The mean abundance of XBP1 spliced and unspliced protein in response to OT or FGM alone after 30 min. A χ^2 test was computed for two treatment groups of 2 isoform categories, $n=4$ pairs of each group. * The numbers within the peaks represent arbitrary units of the area under the curve

TRIB3, while relative abundance was weaker but negative (−0.43), the r value differences were significant ($p=0.0088$). These results indicate that OT may activate UPR signals and thus, increase cytoplasmic TRIB3, which may lead to a negative feedback loop that downregulates Akt activation.

Induction of BiP by oxytocin

BiP (GRP78) is an ER-resident chaperone involved in clearing misfolded proteins from stressed ER and, it belongs to a set of gene products responsive to the XBP1 transcription factor during ER stress (Lee et al. 2003). Since OT increased the abundance of XBP1s (Figs. 3 and 4), we examined BiP levels in response to OT. Low and high OT increased BiP significantly 30 min after stimulation of Caco2BB cultures with both OT doses (Fig. 6). Thus, OT stimulates the signaling markers of UPR transducers and also increases the actual product of UPR in the cells.

Discussion

The rationale for studying OT signaling in gut cells stems from our prior work showing that the OTR is highly expressed in enterocytes of newborn rats during their suckling period (Welch et al. 2009). Those studies showed that after weaning, OTR expression declined in the fully developed villi and appeared at the crypt villus junction and at the newly developed crypts, as well as in cell adherens junctions. This suggests OT may be important to gut development, including regulation of cellular function of enterocytes and maintenance of crypt functions. At the cellular level, we reported that Caco2BB gut cells stimulated with OT modulated $pAkt^{T308}$ levels (Klein et al. 2011), and that this response is expressed as attenuation of the overall anabolic stimulation of FGM, including mTORC1 and its downstream targets: OT modulation of Akt led to increased $pRaptor^{S792}$, which inhibits mTORC1 and its substrates S6K1and pS6K1 (Klein et al. 2013).

In our current study, we asked: by what mechanism does OT attenuate the activity of mTORC1? Since it is known that

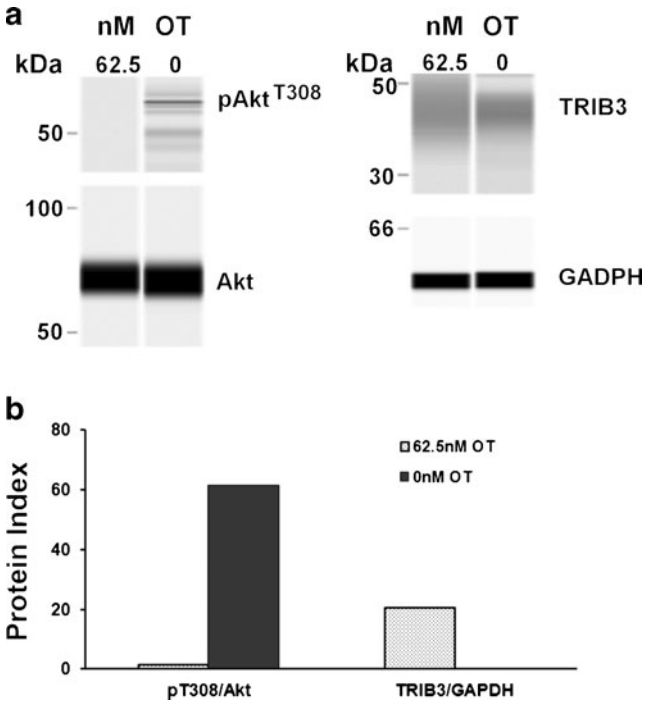

Fig. 5 Stimulation of TRIB3 by OT is inversely related to decreased $pAkt^{T308}$. Caco2BB cells were stimulated for 30 min with fresh growth medium containing 62.5 nM OT and compared with 0 nM OT cultures 30 min after the addition of FGM. **a** Fractionated protein extracts were analyzed for $pAkt^{T308}$ immunoreactivity in reference to total Akt, and for TRIB3 immunoreactivity in reference to GAPDH. **b** The mean immunoreactivity ratios of both pAkt/total Akt and TRIB3/GAPDH ($N=6$, $p<0.01$) show significance ($p<0.01$) using a Z test for two population proportions. The direct correlation coefficient between control and OT-treated pAkt and respective total Akt is strong ($r=0.93$). A weak, but inverse correlation coefficient exists between treated and untreated TRIB3/GAPDH ratios ($r=-0.45$); the difference between these two opposing correlations is significant ($p=0.0088$)

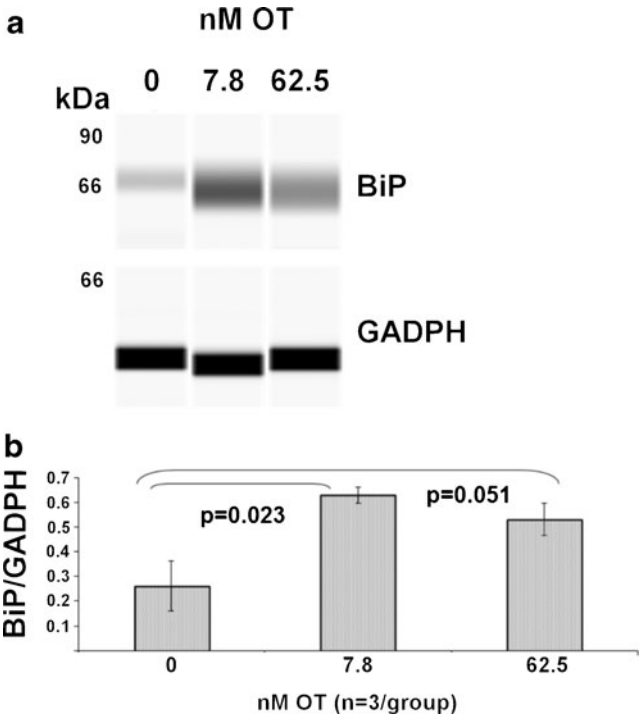

Fig. 6 OT increases immunoglobulin binding protein (BiP). Caco2BB cultures exposed to low (7.8 nM) and high (62.5 nM) OT in fresh growth medium for 30 min were compared with cultures treated with fresh growth medium alone. Protein extracts where analyzed for BiP abundance in reference to GAPDH. **a** Band density and (**b**) ratio of BiP/GAPDH ($n=3$) were increased

TRIB3 binds to Akt at the activation loop and directly blocks T308 phosphorylation (Du et al. 2003), we tested the hypothesis that TRIB3 may be involved in mediating the OT effect on $pAkt^{T308}$, thereby modulating the activity of mTORC1. We found that during OT stimulation the mean decrease of $pAkt^{T308}$ was inversely proportional to the mean increase of TRIB3. Downregulation of $pAkt^{T308}$ (but not $pAkt^{S473}$) by OT resulted from the induction of TRIB3. The TRIB3 gene is targeted by ATF4, an ER stress mediator. OT may stimulate ER stress transducers, thus inducing TRIB3 via ATF4 activation. TRIB3's blockade of T308 phosphorylation maintains mTORC1 attenuation.

It should be noted that TRIB3 is a component of the autophagy system. Since we have observed that OT modulates $pAkt^{T308}$ indirectly by TRIB3 negative feedback loop, we suggest that Akt may act in two different ways. On the one hand, in case of chronic stress, low Akt activity can join other apoptotic factors to kill the stressed cells. On the other hand, in the case of successful recovery from stress by chaperones and/or nutritional recovery by autophagy, low Akt activity can contribute to cell survival. This survival may be possible because of temporary delay of translation until amino acids become available for protein synthesis and/or until the UPR can catch up with synthesis.

OT stimulation of Caco2BB gut cells modulates several UPR pathway markers, including eIF2a, PERK, IRE1, XBP1, and the ER-resident chaperone BiP. In this study, we investigated markers of UPR in OT-stimulated gut cells because the mTORC1 pathway is also linked to cellular stress. Our studies demonstrate that OT is highly involved in regulating this system, since several molecules that are components of different branches of the UPR are regulated by OT. We hypothesized that OT can modulate the UPR and protein translation via the mTORC1 pathway. As summarized in Table 1, our results show that OT treatment stimulates UPR markers in a dose-dependent manner, although this dose-dependency is U-shaped for some of the responsive elements.

Our observation that PERK and IRE1a are activated under different OT levels is consistent with the known functional complexity of the three branches that regulate UPR in the cell. For example, PERK is not the only kinase that targets eIF2a. Adding to the complexity is that OT itself is known to serve multiple functions. OT may be acting upon the UPR in two independent ways that are consistent with other OT findings. OT has been shown to reduce cellular stress (Onaka et al.

2012), and it has been shown to inhibit apoptosis (Noiseux et al. 2012). Thus, it is conceivable that on the one hand OT helps the cell to cope with ER stress, and that on the other hand OT inhibits apoptosis mediated by other stress sensors (Martinon et al. 2010).

IRE1, another pathway of the UPR, is associated with XBP1 mRNA splicing. Our data show that OT can activate the IRE1/XBP1 pathway separately from the other two branches. It is interesting to note that the UPR sensing branches can be regulated collectively or individually. For example, the IRE1-XBP1 pathway was separately activated from the canonical cooperative UPR branches, in an inflammatory signaling pathway via Toll-like receptors (TLR), while isolated XBP1s activity silenced the other UPR branches (Martinon et al. 2010). This was shown to occur in macrophages where TLR2/4 stimulation induced XBP1 by a selective transcription program giving rise to inflammatory cytokines that facilitate the handling of microbial cargo (Martinon et al. 2010). This XBP1 function in macrophages is distinct from ER stress associated with extensive misfolded proteins, which can lead to death by apoptosis upon clearance failure of the stressed ER. It is not clear whether, in enterocytes, TLR-induced XBP1 ("UPR-light" branch), similarly to canonical ER stress, can slow the rate of translation while chaperone expression is increased to clear excess unfolded proteins from the ER and Golgi compartments. Nonetheless, we have shown that low and high OT increase the levels of the UPR protein BiP. This demonstrates that OT induces not only signaling markers of UPR transducers but also induces expression of a UPR chaperone.

IRE1 and XBP1 are proteins essential for the differentiation of secretory cells and regulation of secretion by their management of ER protein loads (Martinon and Glimcher 2011). OT increased the abundance XBP1s, and with high OT this response was sensitive to OTA. When cells were exposed to FGM for 90 min, before the 30-min-long stimulation with OT, both IRE1 and XBP1s responded in the same manner to low and high OT, implying that they were coordinated. This is in contrast to the response by pPERK and e-IF2a described above. This is also in contrast with simultaneous sensing activation of all three UPR branches due to ER overload that results from extensively misfolded and/or under-glycosylated proteins, which can lead to apoptosis (Zhang and Kaufman 2004; Gonz lez et al. 2002; Tu et al. 2000).

As substrate of mTORC1 kinase, 4E-BP1 negatively regulates the cap-dependent rate-limiting translation initiation factor eIF4E (Rong et al. 2008). mTORC1 inhibition results in low phosphorylation of 4E-BP1^{S65}. This confirms our earlier finding that treatment with high OT resulted in low phosphorylated S6 kinase 1 (pS6K1) levels, an indicator of mTORC1 inhibition (Klein et al. 2013). Interestingly, although p4E-BP1$^{T37/46}$ is expected to prime 4E-BP1^{S65} phosphorylation (Gingrass et al. 1999), their levels were inversely related in our assays. This suggests that with high OT the inhibition

Table 1 Summary of the effects of 30 min OT upon the UPR markers and translation factors examined in this study

Experimental findings					References
Proteins	Effect of oxytocin				
	High[a] (62.5 nM)	Control (0 nM)	Low[a] (7.8 nM)	High+OTA[b] vs high	
4E-BP1	↑↑	Low	↑	–	Total unphosphorylated 4E-BP1 inhibits eIF4E (Gingras et al. 1999)
p4E-BP1^{S65}	↓	Low	↑	–	pSer65 decreases inhibition of eIF4E (Gingras et al. 1999)
p4E-BP1$^{T37/46}$	↑↑	Low	↓	–	pThr37/46 primes 4E-BP1 for Ser65 phosphorylation (Gingras et al. 1999)
eIF2a	↑↑	Medium	↑	–	eIF2a is the active form of this translation factor (Kimball et al. 1998)
p-eIF2a^{S51}	↑↑	Medium	↔	–	pSer51 inhibits eIF2a, marks UPR to ER stress (Harding et al. 2001)
pPERK^{T980}	↑↑	Low	↑	↓	pThr980 marks sensing of ER stress, phosphorylates Ser51 of eIF2a (Harding et al. 2001)
XBP1s	↑↑	Low	↑↑	↓↓↓	XBP1s induces expression of UPR genes, marks activation of IER1 ER stress sensing (Lee et al. 2003)
XBP1s[c]	↔	Low	↑↑	–	XBP1s is the product of unconventionally spliced mRNA of XBP1u by IRE1a (Hetz et al. 2011)
IRE1a[c]	↑↑	Low	↑	–	IRE1a is ER membrane anchored sensor of ER stress (Hetz et al. 2011)
pIRE1a[c]	↔	Low	↑↑	–	Autophosphorylation on Ser724 activates its endoribonuclease that splices XBP1u mRNA. (Hetz et al. 2011)

[a] Vs control

[b] High (62.5 nM OT)+OTA (5 nM) for experiments where $n \geq 3$

[c] Pre-stimulated by FGM

of p4E-BP1^{S65} via mTORC1 overrides the priming effect of p4E-BP1$^{T37/46}$.

eIF2a is another protein involved in translation. Low OT increased phosphorylation of eIF2a, but predominantly increased total eIF2a. And therefore the ratio of the total active eIF2a to p-eIF2a increased, indicating that eIF2a may be active at low OT. In contrast, high OT stimulation increased phosphorylation of eIF2a. Thus, the phosphorylation marker that indicates inhibited translational activity was induced only by high OT. This is similar to and coincident with the response of the ER stress sensor PERK to high OT. Unlike pPERK, however, levels of p-eIF2a induced by high OT were sensitive to OTR antagonism. Therefore, high OT exerted its effect on PERK via an OTR independent mechanism and on eIf2a via and OTR-dependent mechanism. Further studies are required to identify the kinase (other than PERK) responsible for eIF2a phosphorylation with high OT.

Modulation of translation factors by high OT downstream of mTORC1 and at the level of the ER stress sensor PERK suggests that OT may act as a regulator of protein translation during states of cellular stress, and as a regulator of inflammation. In addition, OT may act differently at various stages of development. In the context of a transient high level OTR expression in enterocytes of postnatal rat pups (Welch et al. 2009), OT may be instrumental in protection of enterocytes against the cellular ER stress involved in intense developmental differentiation of colonic surface and small intestine villus structure. Later in life, these biological mechanisms may be redirected to regulate inflammatory responses in the gut. It is reasonable to assume in the rat model that during the first two to three postnatal weeks enterocytes are exposed to a vast repertoire of organic molecules (including OT), mainly from mother's milk. At this stage, enterocyte signaling pathways may be stimulated by a variety of food-derived molecules. This stimulation could stress the ER, and trigger concomitant "new" bacteria-elicited inflammatory reactions. The transiently elevated OTR expression in newborn enterocytes may be advantageous in its regulation of separate UPR functions during postnatal gut development and maturation. These findings demonstrate that OT modulates several sensors of ER stress, supporting our hypothesis that transiently elevated OTR expression in neonatal gut may protect this developing organ during a critical postnatal period.

The mTORC1 pathway is a main responder to nutrient availability. Nutrient insufficiency has been shown to activate this pathway (Ma and Blenis 2009), modulating translation factors and sub-pathways that influence cell growth and cell size (Dunlop and Tee 2009), apoptosis and autophagy (Dunlop et al. 2011), and response to cell stressors (Corradetti and Guan 2006). OT's modulation of the mTORC1 pathway suggests that the OT supplied by milk to the gut cells and nutrient levels may share a common mechanism in determining cell response and cell fate. The level of nutrient and level of hormone may work in tandem to modulate mTORC1 activity.

The mTORC1 pathway links three areas of our research; gut function, OT and ASD. ASD-related mutations in genes coding for TSC1/2, NF_1, PTEN, and Fragile X (FMR) proteins cause hyperactivation of the mTORC1-eIF4E pathway (Kelleher and Bear 2008). A connection between GI disorders and ASD was previously hypothesized based on clinical observations (Horvath and Perman 2002a), and histological evidence (Horvath et al. 1999). Reported GI signs in ASD included defects in secretion of secretin and disaccharide digestive enzymes, as well as hypertrophic Paneth cells that are secretors of two families of molecules of major importance: Wnt molecules on which crypt stem cell proliferation depends(Pinto and Clevers 2005), and bactericidal oligopeptides (defensins) that are thought to regulate intestinal microbiota populations. Changes in microbiome composition have been shown to exist in children with ASD (Kang et al. 2013), the mechanistic significance of which is not yet understood. A mouse model of severe gut inflammation demonstrated impaired UPR secondary to XBP1 deficiency (Kaser et al. 2008). Paneth cell hypertrophy in ASD may reflect an inflammatory response to microbial and other environmental factors (Kaser et al. 2010) associated with impaired UPR (Hodin et al. 2011). We therefore believe that this function may be relevant to gastrointestinal disorders in general and in autism in particular.

Taken together, the results of our previous and current studies strongly support a role for OT in stress modulation. These findings support our hypothesis that transiently elevated OTR expression in neonatal gut may serve a protective function during a critical postnatal developmental period.

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