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Oxytocin modulates mTORC1 pathway in the gut

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ABSTRACT

Our recent findings of a weaning-related pattern of oxytocin (OT) and OT receptor (OTR) expression in the rat enteric nervous system and in villus-crypt enterocytes, together with the known high level and stability of OT in breast milk support that OT may play a role in gut function and development. We previously described a biphasic dose–response of the PI3K/Akt pathway in gut cells treated with OT. Activation peaked at 62.5 nM OT (30 min) and coincided with OTR internalization. Here we use automated Western blotting to further explore OT-elicited changes in Akt and $pAkt^{T308}$, as well as in downstream substrates p70 S6 kinase-1 (S6K1) and eIF-4E binding protein 1 (4E-BP1). Relative to fresh growth medium (FGM) alone, our results showed OT in FGM reduced the abundance and phosphorylation of S6K1 and the phosphorylation of 4E-BP1, both substrates of mammalian target of rapamycin complex 1 (mTORC1). Phosphorylation of mTORC1 regulator, $Raptor^{S792}$, was increased by high and low OT concentrations, with predicted inhibitory effects on mTORC1. OT thus downregulates anabolic effects induced by FGM activity catalyzed by mTORC1. OT is a regulator of the PI3K/Akt/mTORC1 pathway in $Caco_2BB$ cells and may modulate translation in gut cells.

1. Introduction

The known high level and stability of oxytocin (OT) in breast milk [1] in conjunction with the weaning-related pattern of OT and OT receptor (OTR) expression that we have observed in the rat enteric nervous system and in villus-crypt enterocytes [2] raises intriguing questions about the role that OT plays in gut function and development. Our group found that when combined with secretin OT reduced rat colonic inflammation [3]. This finding is consistent with studies showing that OT can reduce inflammatory and cellular oxidative stress [4,5]. We have also found that mice lacking the OTR have altered gastrointestinal motility, inflammation, macromolecular permeability, and mucosal maintenance. Our discoveries suggest that, in addition to its role in the brain, OT may also have an important neuromodulatory role in the gut. However, the relevant cell signaling mechanism associated with OT in the gut remains to be elucidated.

We recently demonstrated that OT activates the phosphoinositide 3-kinase (PI3K)/Akt pathway in a dose and time-dependent manner in $Caco_2BB$ cells (*in vitro* model of enterocytes). We further established that the activation peaked at 62.5 nM (high) OT [6]. In the present study, we extend our investigation of the PI3K/Akt pathway by looking at mammalian target of rapamycin complex-1 (mTORC1) and its substrates. mTORC1 is important in protein synthesis through its modulation of ribosomal biogenesis [7], cell proliferation and cell size [8] by way of sensing nutrient sufficiency signals [9] and cellular responses to stressors [10].

The relationship between Akt and mTORC1 is very important and most certainly involves crosstalk, although a full understanding of this complex relationship is just beginning to emerge. Increased pAkt activity increases phosphorylation of hamartin/tuberin complex (TSC1/TSC2), which attenuates its inhibitory effect on mTORC1 (i.e., increases mTORC1 activity) [11,12]. Modulation of mTOR can also have upstream effects. A recent study demonstrated that chronic rapamycin treatment, which inhibits mTORC1, differentially phosphorylates Akt on residues T308 vs. S473 and impairs insulin action and glucose tolerance [13]. Interestingly, disruption of the negative feedback loop upon mTORC1

Abbreviations: 4E-BP1, eIF-4E binding protein 1; Akt, serine/threonine protein kinase (B); mTORC1, mammalian target of rapamycin complex 1; OT, oxytocin; OTR, oxytocin receptor; PI3K, phosphoinositide 3-kinase; pAkt, phosphorylated Akt; pS6, phosphorylated S6; pS6K1, phosphorylated S6K1; PDK1, 3-phosphoinositide-dependent kinase 1; S6, ribosomal S6 protein; S6K1, P70 ribosomal S6 kinase-1; TSC1/TSC2, hamartin/tuberin complex.

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mediated by S6 kinase, a substrate of mTORC1, and results in increased insulin sensitivity [14].

The present study investigates a possible role for OT in regulating mTORC1 and its substrates. Because of their known roles in downstream signaling pathways, we examined Raptor, part of the mTORC1 complex, as well as mTORC1 substrates S6K1 and eIF4E binding protein 1 (4E-BP1). pS6K1 enhances downstream translation activity [15] while 4E-BP1 functions as a natural inhibitor of translation initiation factor 4E (eIF4E) in protein synthesis [16,17]. The phosphorylation of 4E-BP1^{S65} is a signaling marker for disrupted inhibition of eIF4E; the less 4E-BP1^{S65} is phosphorylated, the more it inhibits eIF4E translation activity [18].

Here, we show that OT has an overall dampening effect on the PI3K/Akt/mTORC1 pathway. We also show that OT increases the phosphorylation of Raptor^{S792} while downregulating both the abundance and phosphorylation of S6K1 and 4E-BP1^{S65}.

2. Materials and methods

2.1. Cells and culture reagents

$Caco_2$BB cells (C2BBe1 clone; American Type Culture Collection, Manassas, VA) were grown (5% CO_2 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).

2.2. Reagents

Human OT (Phoenix Pharmaceuticals Inc., Burlingame, CA). OTR antagonist (OTA; desGly-NH_2-d$(CH2)_5$[D-Tyr^2,Thr^4]OVT (ST-11-61); donated by Dr. Maurice Manning, University of Toledo, OH [19]).

2.3. Antibodies

Studies used: mouse anti-αtubulin (mAb) (T6074, Sigma–Aldrich, St. Louis, MO), goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate, goat anti-mouse IgG HRP conjugate (ProteinSimple Santa Clara, CA), rabbit mAb anti-pAkt^{S473} (XP, 4060; Cell Signaling Technology (CST), Inc., Danvers, MA), rabbit anti-pAkt^{T308} (9265; CST), rabbit mAb anti-(pan)Akt (4691;CST), rabbit anti-p70S6 kinase (mAb) (2708; CST), mouse anti-pS6K1 (mAb-Thr389; 9206; CST), rabbit anti phospho-Raptor (Ser792, 2083; CST), rabbit mAb anti GAPDH (2118; CST), rabbit anti-phospho-4E-BP1 Ser65 (9451; CST), rabbit anti-4E-BP1 (9452; CST).

2.4. OTR stimulation and protein extraction

OT stimulation experiments were performed in cell cultures 24 h after seeding of 25×10^4 cells/cm^2. Continuous stimulation times (10–60 min, as indicated) were terminated by placing the cultures on ice. The cultures were washed twice with ice cold phosphate-buffered saline (PBS) and cold wash buffer provided by the kit described below. Subsequently 0.1 ml of ice cold protein extraction cocktail prepared from the Cell Lysis kit Bicine/Chaps (p/n CBS403, ProteinSimple, Santa Clara, CA) was added for 15 min. The extraction cocktail, containing protease inhibitors and phosphatase inhibitors, was used according to the supplier instructions. The protein extracts were scraped, cooled on ice for 5 min 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 were 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.

2.5. Simon™ automated Western blotting and analysis

All reagents were prepared and used according to manufacturer's recommendations for use on Simon™ (ProteinSimple, San Jose, CA, www.proteinsimple.com/simon.html). Reagents included: 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, capillaries, containing a proprietary UV-activated chemical linked reagent, and antibody diluent and antibodies (goat-anti rabbit secondary antibody, and goat-anti mouse secondary antibody).

Samples were diluted to adjust protein concentration to 3–4 μg in 2.5 μl with sample buffer and further diluted 1:2 by adding 2.5 μl of the 2× master mix (containing 80 mM DTT, 2× sample buffer and 2× fluorescent standards). The final samples of 5 μl each were boiled 5 min, placed on ice for 5 min, briefly centrifuged and 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. Aliquots were stored at −20 °C and removed for each run on Simon™.

Simon™ Instrumentation. The instrument was prepared by adding 2 ml of matrix removal buffer to trough 1, 2 ml of wash buffer to trough 2, and 0.8 ml of running buffer to trough 3. Capillaries and the 384-well plate containing samples, antibodies, and matrices were then placed inside the instrument. The simple Western was run with capillaries (12) filled with separation matrix for 100 s, stacking matrix for 16 s and protein extracts for 12 s. The samples were then separated with 250 V for 40 min and then immobilized to the capillary wall using default immobilization conditions and washed with matrix removal buffer for 140 s to remove the separation matrix. Capillaries were then washed with wash buffer for 150 s and blocked with antibody diluent for 15 min. Next, capillaries were incubated with primary antibody (3 h), washed, and incubated with HRP conjugated secondary antibodies 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™.

2.6. Statistical analysis

Band density differences at each time point or each reagent concentration were computed against controls using a paired Student's *t*-test (2-tailed or 1-tailed in the absence or presence of expectation, respectively; $\alpha = 0.05$). Data were collected from 3 to 5 replicates per condition and analyzed using SPSS Base 9.0 (SPSS, Chicago, IL). All plots present mean ± standard error.

3. Results

3.1. Downregulation of pAkt in presence of medium by OT is time dependent

We have previously shown that high (62.5 nM) OT has a differential effect on PI3K compared with Akt, especially after 30 min stimulation of $Caco_2$BB cells (a sub-line of the $Caco_2$ colon

adenocarcinoma human cell line selected for its brush border structures) [6]. Our preliminary experiments suggested that fresh growth medium (FGM) (rich in growth factors) may enhance phosphorylation of Akt. In this experiment we stimulated $Caco_2BB$ cells with OT in the presence of FGM and, with FGM alone, at different concentrations and timepoints.

Low OT (7.8 nM) attenuated phosphoisoform induction compared with FGM alone, with the maximum effect at 30 min (Fig. 1). We observed that levels of $pAkt^{T308}$ and $pAkt^{S473}$ were enhanced in the presence of FGM in a time-dependent manner. This result suggests that OT decreases the anabolic effects of the medium. FGM by itself also elicited phosphoisoform changes over time.

Like low OT, high OT (62.5 nM) attenuated phosphoisoform induction, albeit with a different time course (Fig. 2A). Furthermore, the data suggested that high OT has a differential effect on kinases that target residues S473 (mTOR complex 2; mTORC2) and T308 (3-phosphoinositide-dependent kinase 1; PDK1). While high OT had a minimal effect on mTORC2, significant effects were observed for PDK1. Total Akt levels were also reduced at 10 min by high OT. We next showed Akt phosphoisoform induction was dependent upon the OTR. Partial recovery of $pAkt^{T308}$ after 30 min of high OT stimulation was inhibited by the potent OTR antagonist OTA. This implies significant dependence upon OTR activation for phosphoisoform induction beyond that stimulated by the addition of FGM (Fig. 2B and C).

3.2. Effects of OT on substrates of mTORC1

We explored downstream effects of OT upon the mTORC1 pathway. With FGM alone, phosphorylation of S6K1 was observed at 20 and 30 min, but not at 60 min. Low OT attenuated pS6K1 levels at 20 and 30 min and resulted in some S6K1 phosphorylation at 60 min (Fig. 1). We further assessed the induction and phosphorylation of S6K1 and translation initiation factor 4E-binding protein (4E-BP1) because reduced phosphorylation of 4E-BP1 is associated with greater inhibition of eIF4E translation activity [16].

Treatment with FGM alone resulted in increased abundance of S6K1 at all times (peaking at 20 min), and increased pS6K1 levels at 10 and 60 min (Fig. 3A). High OT reduced the expression of S6K1 at all times and attenuated pS6K1 levels at 10 and 60 min, but increased pS6K1 relative to control at 20 and 30 min of stimulation. This inconsistency during the 20–30 min time period may be the consequence of OTR internalization as observed in our previous study [6]. 4E-BP1 and phosphorylated 4E-BP^{S65} (p4E-BP^{S65}) showed slight similarity to the biphasic progression of S6K1 and pS6K1. Low OT significantly decreased pS6K1 while high OT brought it closer to the levels observed with FGM alone, and the OT receptor antagonist (OTA) prevented this high OT-induced increase (Fig. 3B and C), which suggests that OTA interferes with the interaction of high OT with the OTR such that it resembles

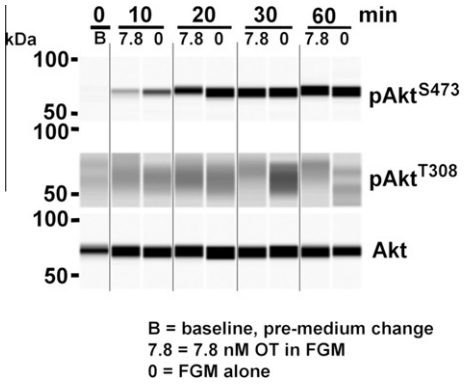

Fig. 1. Timecourse of Akt and S6K1 phosphorylation response to low OT. $Caco_2BB$ cultures were stimulated with low OT (7.8 nM) in fresh growth medium (FGM) and FGM alone (0). Electrophoretic bands were developed with anti-$pAkt^{T308}$, with anti $pAkt^{S473}$ and anti-total Akt antibodies. Note that low OT downregulates $pAkt^{T308}$, the maximal effect occurring at 30 min. FGM also has a time-dependent effect on $pAkt^{T308}$. Different time dependence is seen for $pAkt^{S473}$. The phosphorylation of S6K1 observed after FGM was attenuated by OT.

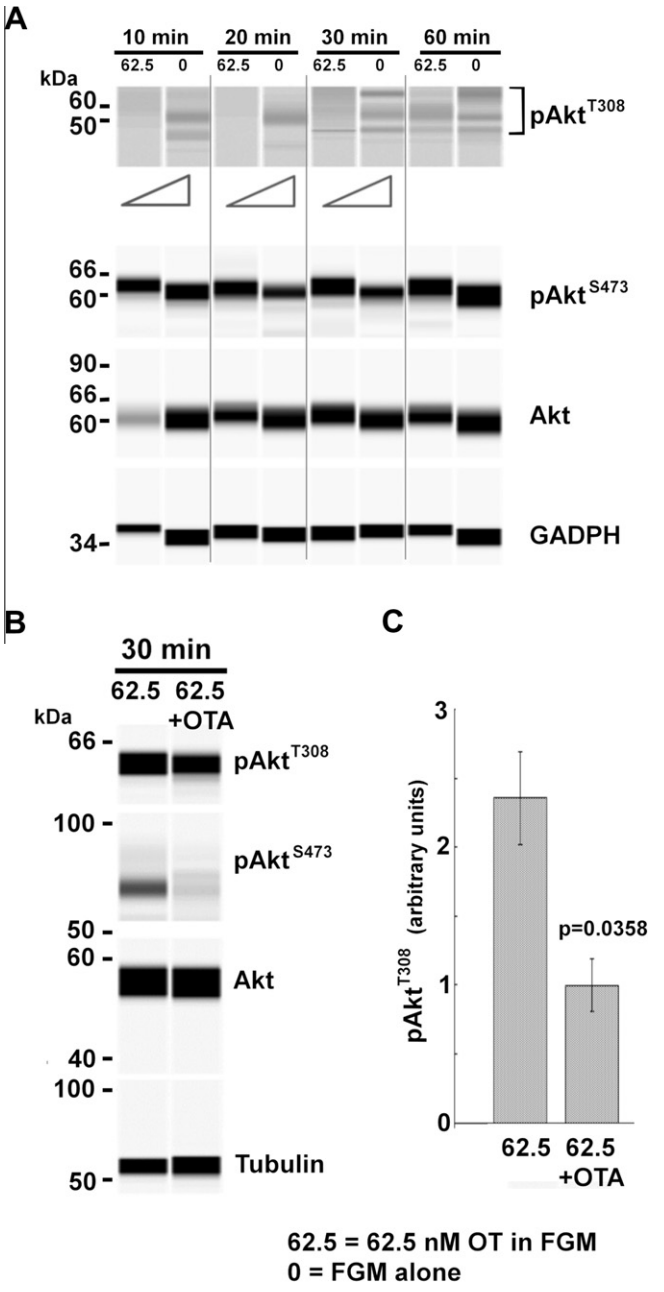

Fig. 2. Timecourse of Akt phosphorylation response to high OT and dependence upon the OTR. (A) $Caco_2BB$ cultures were stimulated with high OT (62.5 nM) in FGM vs. FGM alone (0) at indicated timepoints. Representative electrophoretic bands developed with anti-$pAkt^{T308}$, anti $pAkt^{S473}$ and anti-total Akt antibodies are shown. Triangles denote differential pT308 band densities detected by chemiluminescence. OT attenuated the effect of growth factors in the medium on pAkt and total Akt. The time course of FGM's effect is complex and varies from that of OT. (B and C) Cell cultures were stimulated for 30 min with 62.5 nM OT in the absence or presence of 5 nM of the OTR antagonist, OTA. In Western blot (B) and with quantification of data (C) a differential decrease in Akt^{T308} phosphorylation was observed in the presence of OTA ($p = 0.0358$, $n = 3$).

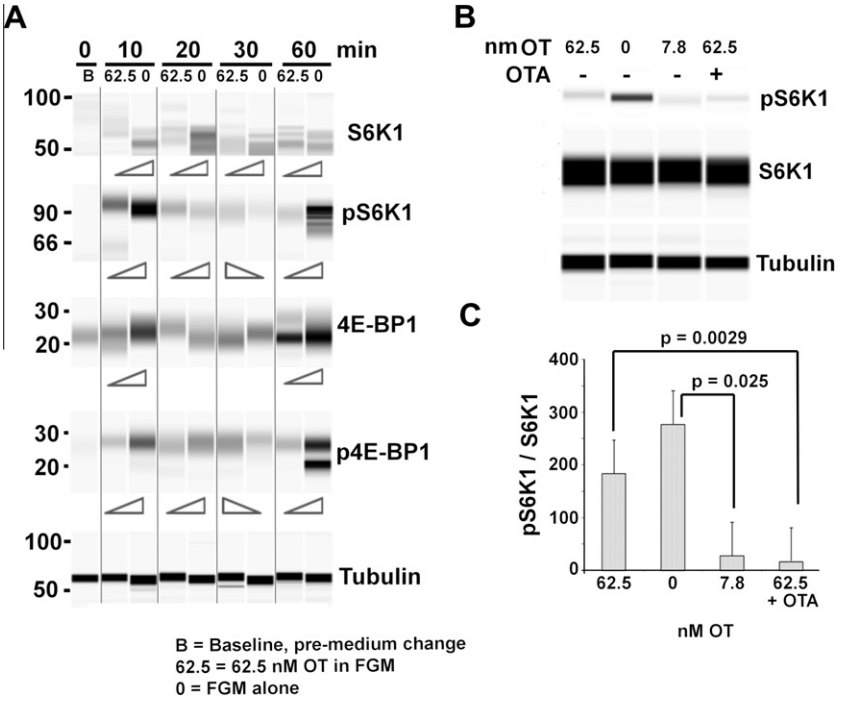

Fig. 3. Timecourse of S6K1 and 4E-BP1 expression and phosphorylation in response to high OT and dose-dependent effect of OT on pS6K1 at 30 min. (A) Automated Western blots showing $Caco_2BB$ cultures stimulated with high OT (62.5 nM) in FGM vs. FGM alone (0). Electrophoretic bands were developed with anti S6K1, $pS6K1^{T389}$, 4E-BP1, and $p4E\text{-}BP1^{S65}$. Notice that S6K1 is downregulated by OT at all timepoints. However, the other three were all downregulated at 10 and 60 min. The apparent inconsistency during the 20–30 min time period may result from OTR internalization that takes place during this time interval. (B and C) $Caco_2BB$ cells were stimulated in FGM; FGM alone (0), low OT (7.8 nM), high OT (62.5 nM) and high OT in the presence of the OTR receptor antagonist, OTA (5 nM). Note the decrease of pS6K1 under low OT and its increase under high OT in Western blot (B) and when normalized to total S6K1 levels (C). High OT in presence of OTA has an effect similar to low OT.

the effect of low OT. This implies that high OT-elicited reductions in pS6K1 are mainly OTR-dependent.

3.3. OT induces $pRaptor^{S792}$, a marker of mTORC1 downregulation

$pRaptor^{S792}$ disrupts mTORC1 functioning in complex with Raptor protein [20]. In this study, both high and low OT significantly increased $pRaptor^{S792}$ abundance after 30 min relative to FGM ($p = 0.02$ and 0.038, respectively; $n = 5$) (Fig. 4). This implies that mTORC1 activity may be inhibited by both an upstream decrease in pAkt and by Raptor phosphorylation.

4. Discussion

This investigation is part of a larger group effort to reveal the mechanisms underlying mother-infant nurture [2,21]. As aforementioned, we wish to elucidate the importance of OT in gut function given the exposure of neonates and infants to OT in breast milk, which is tightly correlated with OT and OTR expression in the rat gut pre- and post-weaning, as well as the well-established role of OT in nurture [1,2,22]. Furthermore, because of the newly reported association between mTOR and autism [23,24], demonstrated effectiveness of OT nasal spray in alleviating the symptoms of autism [25], and, gut pathology observed in a subset of autistic children [26], we are particularly interested in the implications of our work for understanding and treating autism. Recently we reported a differential induction of pAkt isoforms in response to high vs. low OT in FGM in $Caco_2BB$ cells [6]. Additional experiments indicated that OT attenuates the response to FGM alone. The current study extends our previous findings and elucidates possible mechanism(s). Our findings demonstrate that OT, via the OTR, inhibits the mTOR pathway, alters pAkt induction and stimulates

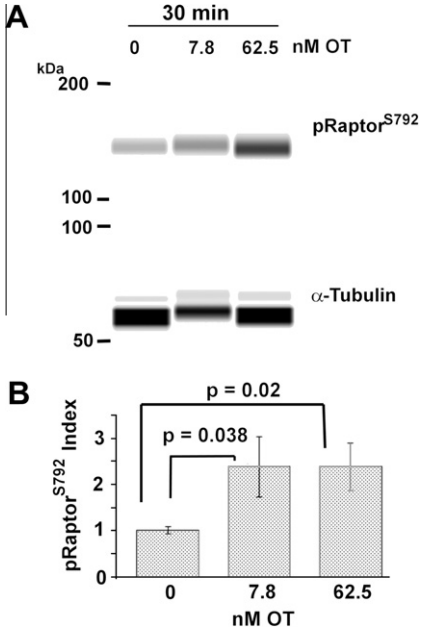

Fig. 4. Both low and high OT increase $pRaptor^{S792}$. $Caco_2BB$ cultures were suspended in FGM with and without low and high OT as indicated. Western blot analysis (A), with quantification of data (B) showed that both high and low OT increased the abundance of $pRaptor^{S792}$.

$Raptor^{S792}$ phosphorylation. At the same time, OT reduces phosphorylation and alters the timecourse of induction of mTOR substrates S6K1 and 4E BP1.

We previously described increased pAkt in response to OT concentrations ranging from 7.8 nM (low) to 62.5 nM (high) in

$Caco_2BB$ cells; notably, the $pAkt^{T308}$ isoform was more abundant than $pAkt^{S473}$ [6]. In the present study we determined that the effect of OT is actually an attenuation of the positive effect of FGM upon pAkt and, which is more pronounced upon $pAkt^{T308}$ than $pAkt^{S473}$. In addition, we found that OT decreased pS6K1 levels in concert with its effects on $pAkt^{T308}$. We infer from this that OT modulates S6K1 activity, since pS6K1 serves as a S6K1 activation marker. We also found that OT induces $Raptor^{S792}$ phosphorylation, which inhibits mTORC1 [20]. Since S6K1 is a substrate of mTORC1 [27,28], we infer from our $Raptor^{S792}$ finding that reductions in S6K1 activation evoked by OT are a direct consequence of mTORC1 inhibition. In support, we show that OT also decreased phosphorylation of 4E-$BP1^{S65}$ (another mTORC1 substrate) in parallel with reductions in S6K1 phosphorylation. Altogether, these results support a role for high OT in negatively regulating the mTORC1 pathway.

Negative regulation of mTORC1 has the potential to slow translation. 4E-BP1 is the binding protein of translation initiation factor 4E (eIF4E), a rate-limiting factor of cap-dependent translation. Dephosphorylation of 4E-$BP1^{S65}$, like that observed with OT, inhibits translation by permitting 4E-BP1 to bind to eIF4E [29–31]. Interestingly, low OT treatment (but not high) of myometrial cells was shown to inhibit phosphorylation of translation elongation factor (eEF2) [32]. This implies OT modulation of eEF2 downstream of rate-limiting, cap-dependent translation initiation by eIF4E (and therefore, eEF2 function in response to OT is negligible). We suggest that OT slows translation initiation by decreasing fully active pAkt ($pAkt^{T308–S473}$) by greater reductions of $pAkt^{T308}$ than $pAkt^{S473}$ and, that this results in less TSC2 inhibition, which reduces Rheb GTPase activity, decreasing the phosphorylation of S6K1 and 4E-BP1 as a result of reduced mTORC1 complex activation.

mTORC1 is actively inhibited by TSC1/TSC2 under basal (non-stimulating) conditions and also inhibited by $Raptor^{S792}$ phosphorylation [33,34]. While we do not know if the enhanced $Raptor^{S792}$ phosphorylation observed after OT is directly catalyzed by the pAkt/mTORC1 pathway, nutrient insufficiency can activate the energy sensor AMPK (AMP dependent protein kinase). This in turn increases $Raptor^{S792}$ phosphorylation, downregulates translation, and prepares the cell for autophagy [33,34]. OT may thus be involved in the cellular response to nutrient and energy sufficiency via the Akt/mTORC1/S6K1-4E-BP1 pathway. OT may also affect insulin sensitivity, by downregulation of pS6K1, which upregulates the insulin receptor and its substrate [35,36], and effects inflammation, proliferation and other cellular processes in the gut.

These findings support our prior study showing that OT dampens translation machinery in the PI3K/Akt/mTORC1 pathway in gut cell cultures under certain *in vivo*-like conditions. Our findings are highly novel and suggest that OT modulates important functional processes in the gut and may extend to other sites of OT signaling, including the brain. Altogether, our work supports the need for physiological assessment of OT function in the gut, which may have compelling implications for interventions that affect OT exposure in neonates and infants and autism treatment.

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