

Modulation of Fatty Acid Oxidation and Glucose Uptake by Oxytocin in Adipocytes

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Abstract

Oxytocin (OT) is a hypothalamic neuropeptide synthesized and secreted by OT neurons. In addition to its conventional role in reproductive physiology, central OT also regulates various social behaviors, such as care, trust, and emotions. Central and subcutaneous OT infusions stimulate lipid metabolism in mice and rats when fed standard or high fat diets. Mice lacking the OT receptor (OTR) or OT peptide develop late-onset obesity with greater fat pad weights, larger adipocyte size and elevated plasma levels of leptin. To study the effects of OT on lipid metabolism, we examined the effects of serial OT doses (0, 10, 30, 100, 150, 300 nM) on 3T3L1 adipocytes, together with long (144 hours, 6 days) and short (24 hours, 1 day) term treatments. The short-term treatment with 150 nM OT increased triacylglycerol (TAG) accumulation and decreased mRNA expressions of carnitine palmitoyltransferase 1 α (CPT-1 α) and fatty acid binding protein 4 (FABP4). After long-term incubation with 150 nM OT, only the CPT-1 α mRNA was decreased. In differentiated adipocytes derived from pig adipose tissue stem cells, only hormone sensitive lipase mRNA was decreased after short- or long-term treatment with OT. To obtain further insight into the underlying mechanism of OT induction, we tested the involvement of the AKT/PKB pathway; however, AKT phosphorylation was decreased after treatment with 150 nM OT, suggesting that OT effects may be independent from the AKT/PKB pathway. Taken together, OT effects on adipocyte glucose and lipid metabolism are probably through mechanisms other than the AKT/PKB pathway.

Keywords

Oxytocin, Lipid Metabolism, Glucose Uptake

1. Introduction

Oxytocin (OT) is a neuropeptide hormone which is mainly produced as a large

precursor molecule in two hypothalamic nuclei, hypothalamic paraventricular (PVN) and supraoptic nuclei (SON) [1]. Plasma OT is increased with uterine contraction, milk ejection and maternal care in female mammals [2]. In addition, OT triggers learning and memory, social binding, regulation of emotional abilities and stress-related responses; OT may act as an endogenous antipsychotic in humans [3] [4] [5]. Furthermore, OT is a cardiovascular protective hormone for releasing atrial natriuretic peptide (ANP) to adjust blood volume and decrease heart rate of rats [6] [7] [8] [9]. Binding of OT to its receptor (OTR) results in the hydrolysis of phosphatidylinositol and release of cytosolic Ca^{2+} [10] leading to transcriptional activity by phosphorylation, activation of mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinases (ERKs) [11].

Increase of OT in human and rodents has been seen in obese population or diabetic animal models [12] [13] [14]. Animals developed obesity when they were deficient of OT or OT receptors [15] [16]. Peripheral OT treatment reduced adipose tissue proportion and also ameliorated food intake, fatty liver, as well as glucose intolerance in ob/ob mice [17] [18] [19]. Adipocyte size was smaller after treating with OT, the lipogenic related genes fatty acid binding protein 4 (FABP4), peroxisomal proliferator activated receptor gamma (PPAR γ), glucose transporter 4 (GLUT4), and leptin mRNA levels in adipose tissues were increased in rats, indicating that OT treatment improves nutrient provision in adipose tissue [20]. OT exerted insulin-like effects in isolated adipocytes, as stimulation of glucose oxidation, lipogenesis and pyruvate dehydrogenase activity [21] [22]. Glucagon and insulin were released by OT stimulations in isolated pancreas and islets of rodents [23] [24]. Daily peripheral injection and infusion of OT improved glucose tolerance test in mice with high-fat diet feeding [17] [25]. OT increased glucose uptake of cardiomyocytes through AKT/PKB phosphorylation in rodent [8] [26]. Therefore, it's believed that OT may have the function of anti-obesity. However, the detail regulated signaling pathway under OT stimulation has not been defined. Here, we used the adipocyte cell culture model to demonstrate how OT regulating gene expression is related to lipid and carbohydrate utilization.

We confirmed that the OT receptor and insulin receptor mRNAs are expressed in 3T3L1 adipocytes by real-time PCR analysis. We also demonstrated that OT influences both uptake and utilization of lipid and carbohydrates, as well as energy accumulation within adipocytes. The AKT/PKB signaling pathway did not seem to be involved in the OT regulation of lipid and glucose metabolism in adipocytes.

2. Materials and Methods

2.1. Cell Culture

The 3T3L1 cell line was purchased from ATCC (Manassas, VA, USA) and cultured by Dulbecco's Modified Eagle's Medium (DMEM, 12800-017, Gibco, ThermoFisher, Waltham, MA) with 10% calf serum (16010-159, Gibco, Ther-

moFisher, Waltham, MA), 1% Pen-Strep Ampho. Solution (PSA; Penicillin, 10^5 units/L; Streptomycin, 100 mg/L; Amphotericin B, 0.25 mg/L, #03-033-1B, Biological Industries, Cromwell, CT, USA) in the incubator at 37°C with 5% CO_2 in air and 100% of relative humidity. We seeded 3T3L1 cells ($2 \times 10^5/\text{cm}^2$) into 6-well plates and grew them until they reached 80% - 90% confluency. Cells were then incubated with 90% Dulbecco's Modified Eagle's Medium (DMEM) and 10% Fetal Bovine Serum (FBS, #04-001-1A, Biological Industries, Cromwell, CT, USA), 1.0 μM Dexamethasone, 0.5 mM Methylisobutylxanthine (IBMX, 3-isobutyl-1-methylxanthine, #I5879, Sigma-Aldrich, St. Louis, Missouri, USA), 1.0 $\mu\text{g}/\text{mL}$ insulin (#I9278-5ML, Sigma-Aldrich, St. Louis, Missouri, USA), and 1% PSA as start differentiation medium (SDM) for 3 days. The medium was then changed to DMEM with 1 $\mu\text{g}/\text{mL}$ insulin, 10% FBS, 1% PSA as maintained differentiation medium (MDM) until day 6. In order to investigate the OT effects on lipid and glucose metabolic genes, 3T3L1 adipocytes were treated with 0, 10, 30, 100, 150, 300 nM of OT during 6 days of differentiation (long-term) or for 24 hours after 6 days of differentiation (short-term). After OT treatments, triacylglycerol (TAG) deposition and specific RNAs and proteins were measured.

2.2. Glucose Uptake in 3T3L1 Adipocytes

The 3T3L1 adipocytes were treated with daily OT doses (0, 10, 30, 100, 150, and 300 nM) during differentiation for six days. At day 7, 3T3L1 adipocytes were incubated with fresh medium for 24 hours, and then cultured with glucose-free medium containing 2-NBDG (a fluorescent d-glucose analog, 2-[N-(7-nitrobenz-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose) for 10 mins. The levels of glucose uptake in cells were evaluated using the Glucose Uptake Fluorometric Assay Kit (Cat. K666-100, Biovision, Milpitas, CA), according to the manufacturer's instructions.

2.3. Synthesis of Triacylglycerol (TAG) in 3T3L1 Adipocytes

The 3T3L1 adipocytes were seeded in 6-well plates and treated with daily OT doses (0, 10, 30, 100, 150, and 300 nM) during differentiation for six days. Adipocytes were collected by rubber policeman at day 6 in 500 μL of cold diluted standard diluent (provided by TAG kit) for each well. After sonicating on ice and centrifugation at $10,000\times g$ for 10 mins at 4°C , the supernatant was collected. TAG concentration were analyzed and followed by triglyceride colorimetric assay kit (No. 10010303, Cayman Chemical, Ann Arbor, MI, USA). We added 10 μL of the diluted samples to the 96-well plates, and initiated the reaction by adding 150 μL of diluted enzyme buffer solution. After carefully shaking the plates to mix, we covered the plates and incubated for 15 mins at room temperature. The absorbance is measured at 540 nm by a plate reader.

2.4. Real-Time PCR for Measuring Gene Expressions

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA was

reverse-transcribed with random primers using the high capacity cDNA reverse-transcription reagents kit (#4368814, ThermoFisher, Waltham, MA, USA). The mRNA expression was determined using the SYBR green reagent (ThermoFisher, Waltham, MA, USA). Real-Time PCR was performed using the Step One Plus Real-Time PCR System (ThermoFisher, Waltham, MA, USA) with the following incubation protocol: initial 7 mins at 95°C, followed by 39 cycles of 10 secs at 95°C, 30 secs at 60°C, with a final extension for 1 min at 60°C. The β -actin (ACTB) mRNA in the same sample served as internal control. The sequences of primers were indicated in **Table 1**.

2.5. Preparation of Porcine Adipose Tissue Derived Stem Cells

The method was that of Chen et al., 2016 [27]. Briefly, cells were isolated from adipose tissue from 7 to 9-day old pigs using collagenase digestion. Cells were plated on six-well plates at a density of 6×10^5 cell per cm^2 . Cells were incubated until they reached 80 to 90% confluency. Cells were then differentiated in SDM for 3 days and then the medium was changed to MDM until day 6. For porcine adipocyte SDM, prepare 1 L of serum-free DMEM/F12 (with antibiotics of 1% PSA) containing the following: 1 ml insulin stock (10 mg/ml HEPES buffer, pH 8), final concentration = 10 $\mu\text{g/ml}$; 1 μl T3 stock (3,3',5'-Triiodo-L-thyronine, 1 mM in DMSO), final concentration= 1 nM; 200 μl transferrin stock (50 $\mu\text{g/ml}$

Table 1. Primer set

Genes	5' to 3'	Accession No.	Annealing Tem. (°C)
GLUT1-S	ACCATCTGGAGCTGTTCCG	NM_011400	60°C
GLUT1-A	GCCTTCTCGAAGATGCTCGT		
GLUT4-S	TTTGCACACGGCTTCCGAAC	NM_009204	60°C
GLUT4-A	AGTGTTCAGTCACTCGCTG		
INSR-S	CTACTGCTATGGGCTTCGGG	NM_001330056	60°C
INSR-A	TGGTCAGGTGTTCGGATG		
CPT1 A-S	GCAGCAGGTGGAAGTGTGTTG	NM_013495	58.2°C
CPT1 A-A	GTGTGAGTCTGTCTCAGGGC		
CPT1 B-S	GAATCCTCGACGACCTTCC	NM_009948	60°C
CPT1 B-A	TGAAGAAGGTCTGACGTGCC		
FABP4-S	TCACCATCCGGTCAGAGAGT	NM_024406	58.2°C
FABP4-A	CCAGCTTGTCACCATCTCGT		
CHREBP-S	GCTTCAGAAGACAGCGGAGT	NM_021455	60°C
CHREBP-A	TCTGGTCAAAGCGCTGATGT		
HSL-S	CCCCGAGATGTCACAGTCAAT	NM_001039507	60°C
HSL-A	GAATTCCCGGATCGCAGAA		
ACTB-S	GCCTTCTAGCACCATGAAGA	NM_007393	60°C
ACTB-A	AGCTGATCCGCCTTTAGCTC		

double distilled H₂O), final concentration= 10 µg/ml; 100 µl dexamethasone stock (10 mM in ethanol), final conc = 1 µM; 100 µl rosiglitazone stock (10 mM in DMSO), final concentration = 1 µM. Prepare adipocyte MDM with the same additions as the induction medium, but with omission of dexamethasone. Differentiated adipocytes were treated with 0, 10, 30, 100, 150, 300 nM OT during 6 days of differentiation or 24 hours after differentiation. After incubation with OT, adipocytes were collected for analysis of TAG concentration and mRNA expression.

2.6. Western Blotting

Cells were scraped from the plate and sonicated with diluted 1X RIPA Lysis Buffer (#9806, Cell signaling, Danvers, MA, USA) containing Pierce Protease and Phosphatase Inhibitor Tablets (#88668, ThermoFisher, Waltham, MA). The supernatant was collected at 4°C for 15 mins at 14,000 ×g. Protein levels were quantified by BCA protein assay (B6916, Sigma-Aldrich, St. Louis, Missouri, USA). For detection of the candidate proteins, cell aliquots (9 µg) were analyzed by electrophoresis for 75 mins at 100 V in 10% SDS-polyacrylamide gels using a Mini-PROTEAN Tetra Cell system (BioRad, Hercules, CA). Proteins were transferred to a polyvinylidene difluoride membrane at 400 mA for 75 mins on ice using a TE22 Mighty Small Transphor Unit (Hoefer, Holliston, MA). Non-specific binding sites were blocked with TBS (20 mM Tris-HCl, pH 7.4, 140 mM NaCl), containing 5% (w/v) non-fat dry milk and 0.1% Tween 20 for 1 hour at room temperature. Membranes were incubated with primary antibody overnight at 4°C. Then, membranes were incubated at room temperature for 1 hour with secondary antibody. Finally, the Immobilon™ Western Chemiluminescent HRP Substrate was applied (Millipore, Billerica, MA). Membranes were analyzed by densitometry using a BioSpectrum™ 500 Imaging System Motorized Platform (UVP, Upland, CA). The sizes of proteins were estimated using a broad range molecular mass standard (20 to 250 kDa) from Fermentas (#SM0671, ThermoFisher, Waltham, MA). Between every blocking step and antibody incubations, TBS containing 0.1% Tween-20 (TBST) was used for membrane washes every 15 mins with washes repeated three times. Glut4 (#2213, Cell signaling, Danvers, MA, USA), Phospho-AKT (Ser473) (#9271, Cell signaling, Danvers, MA, USA), and AKT (#9272, Cell signaling, Danvers, MA, USA) antibodies were prepared with 5% BSA in TBST (dilution 1/1000), and the secondary antibody goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz Biotechnology, Dallas, Texas, USA) was prepared with 5% nonfat dry milk in TBST (dilution 1/5000); and goat anti-mouse secondary antibody (sc-2005, Santa Cruz Biotechnology, Dallas, Texas, USA) was prepared with 5% gelatin in TBST (dilution 1/10,000).

2.7. Statistical Analysis

All data are presented as the mean ± SEM. Multiple comparisons were performed using ANOVA followed by the Turkey test using GraphPad Prism 5 (GraphPad software Inc., San Diego, CA, USA.).

3. Abbreviations and Acronyms

CHREBP: carbohydrate response element binding protein; CPT1 α : carnitine palmitoyltransferase-I α ; CPT1 β : carnitine palmitoyltransferase-I β ; FABP4: fatty acid binding protein 4; GLUT1: glucose transporters 1; GLUT4: glucose transporters 4; HSL: hormone sensitive lipase; INSR: insulin receptor; OT: oxytocin; SDM: start differentiation medium; TAG: triacylglycerol.

4. Results

4.1. Effects of Oxytocin on Adipocyte Differentiation and Lipid Accumulation

The 3T3L1 cell line underwent a differentiation program of 3 days in SDM followed by 3 days in MDM. Triacylglycerol (TAG) accumulation was not affected by short-term (24 hours) OT treatment (**Figure 1(a)**), but was increased by long-term (6 days) treatment with 100 and 150 nM OT treatment (**Figure 1(b)**). The mRNA expressions of CPT-1 α , the rate-limiting enzyme responsible for mitochondrial fatty acid oxidation, and FABP4, a fatty acid binding protein were both decreased by 100 and 150 nM OT after short-term incubation (**Figure 1(c)**). Only CPT-1 α was decreased after long term treatment with 150 nM OT (**Figure 1(d)**). CPT-1 β is a muscle isoform of CPT-1 α , and we have confirmed that there was no CPT-1 β expression in adipocytes (data not indicated). Expression of hormone sensitive lipase (HSL) mRNA was not altered by short-or long-term incubation with OT (**Figure 1(d)**). These results indicated that stimulation of OT may decrease fatty acid oxidation and attenuate the ability of binding fatty acids.

In pig adipose tissue derived stem cells (pADSCs), TAG was not changed after short-or long-term incubation with OT (**Figure 2(a)** and **Figure 2(b)**). The HSL mRNA was decreased with short-term incubation with 30 to 300 nM OT (**Figure 2(a)**) and by incubation with 150 and 300 nM OT after long-term incubation (**Figure 2(b)**). The mRNA levels of CPT-1 α and FABP4 were not changed (**Figure 2(a)** and **Figure 2(b)**). The data suggested OT treatment may affect lipid accumulation through an inhibitory effect on HSL mRNA expression in pig adipocytes.

4.2. The Effect of Oxytocin on Glucose Utilization in 3T3L1 Adipocytes

In differentiated 3T3L1 adipocytes, glucose uptake was significantly elevated in the presence of 10 to 300 nM OT (**Figure 3(a)**). The main insulin-sensitive glucose transporter responsible for glucose uptake in adipocytes is GLUT4, not GLUT1; 30 nM OT induced both GLUT4 mRNA (**Figure 3(b)**) and protein expression (**Figure 3(c)**). OT treatments may enhance glucose utilization through increase the expression of glucose transporters in adipocytes.

The carbohydrate response element binding protein (ChREBP), a transcription factor coupling hepatic glucose utilization and lipid synthesis, was not

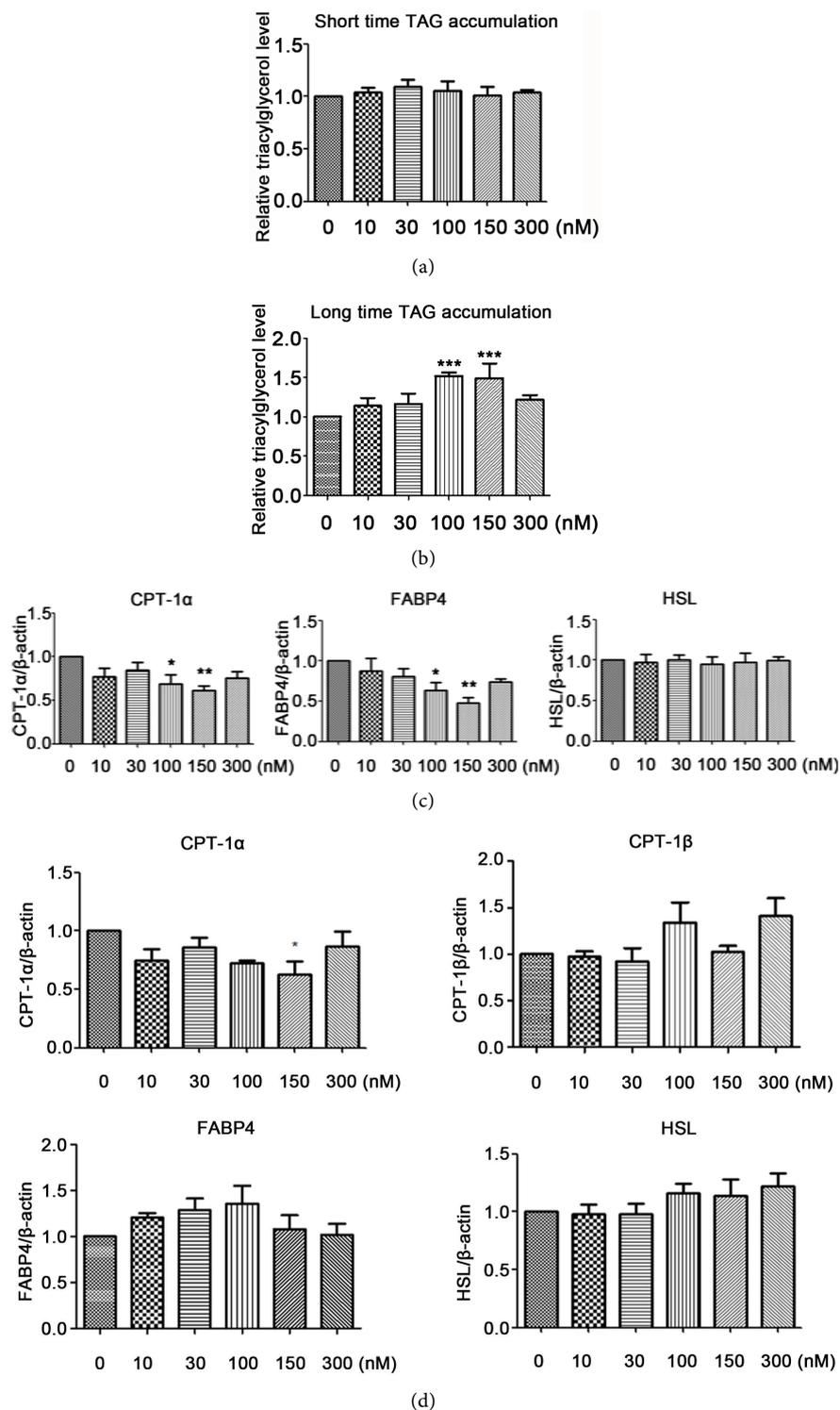


Figure 1. The short- and long-term effects of OT treatment on 3T3L1 adipocytes. The OT concentration was indicated on the x-axis. (A) The accumulation of TAG in 3T3L1 adipocytes after short-term (24 hours) treatment. (B) The accumulations of TAG in 3T3L1 adipocytes after long-term (144 hours) treatment. (C) The mRNA expressions in 3T3L1 adipocytes after short-term treatment (24 hours). (D) The mRNA expressions in 3T3L1 adipocytes after long-term (144 hours) treatment. Values were expressed as mean \pm SEM. One-way ANOVA with the Tukey post hoc test was used to examine the treatment effect. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, $N \geq 6$.

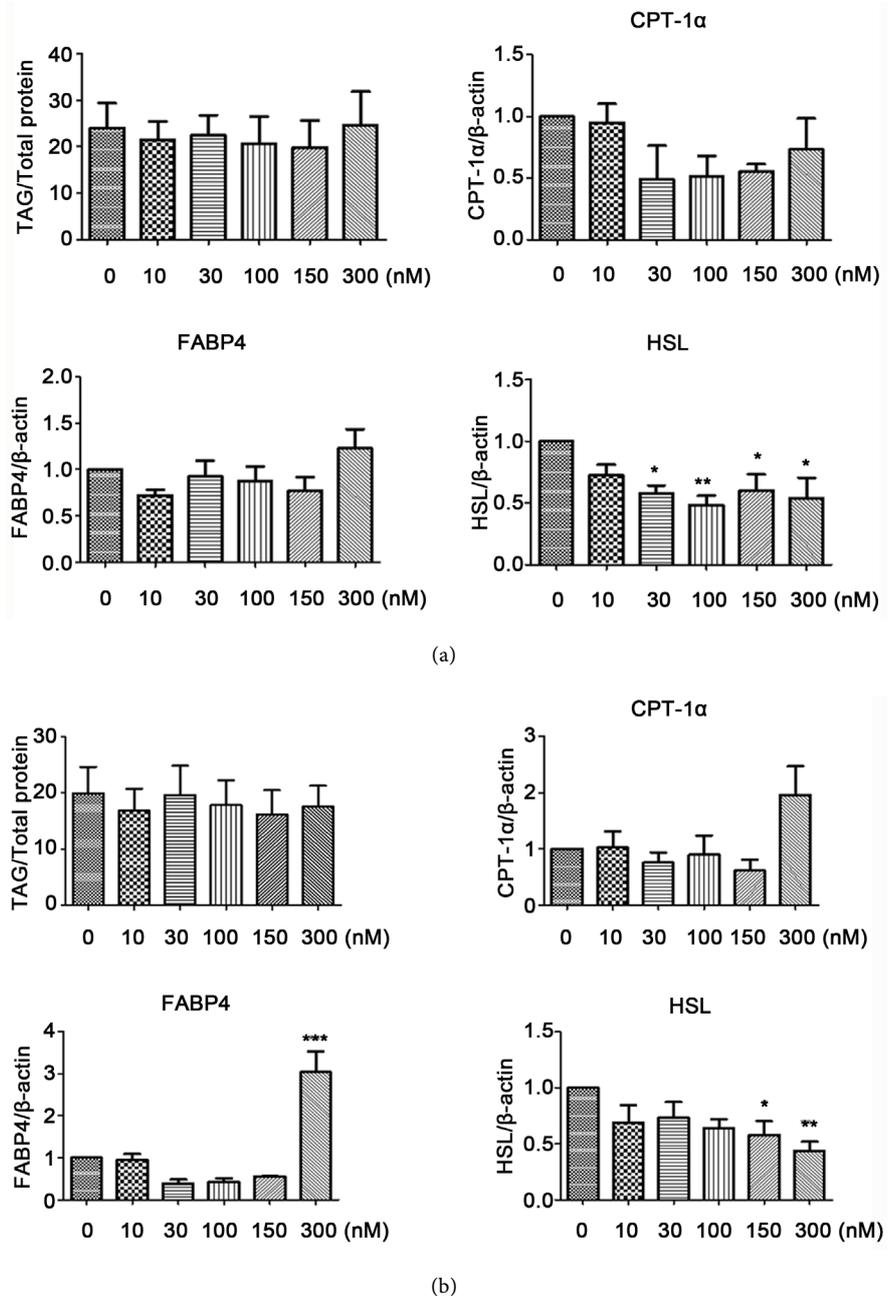


Figure 2. TAG accumulation and mRNA expressions in pig adipocytes after short-term (24 hours, A) and long-term (144 hours, B) treatment with OT. Values were expressed as mean \pm SEM. One-way ANOVA with the Tukey post hoc test was used to examine the treatment effect. * $P \leq 0.05$, ** $P \leq 0.01$. $N \geq 6$.

changed after OT treatment (**Figure 3(b)**). For determining whether insulin downstream pathway is activated by OT treatments, the insulin receptor mRNA (INSR) was examined. The result showed that INSR mRNA was not affected by OT (**Figure 3(b)**).

In addition, there was significantly less AKT phosphorylation at 150 nM OT in 3T3L1 adipocytes (**Figure 4**). Thus, OT did not affect the mRNA concentration of the INSR or the upstream insulin signaling pathway (AKT/PKB).

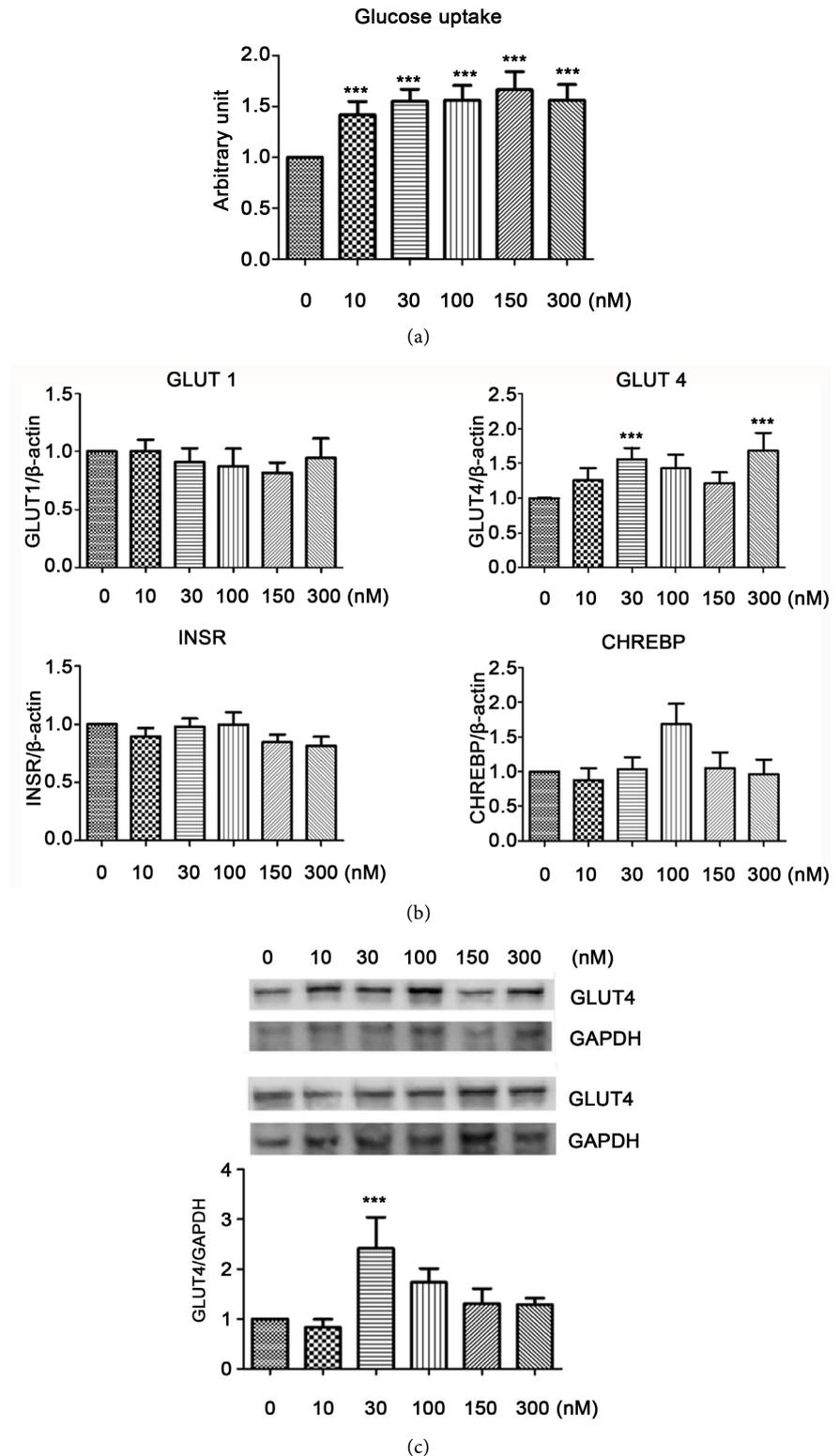


Figure 3. Long-term treatment (6 days) of 3T3L1 cells with OT. (a) Glucose uptake; (b) The glucose utilization related gene expressions; (c) The protein expression levels of GLUT4. The upper panel was the western blotting results. GAPDH (glyceraldehyde 3 phosphate dehydrogenase) was used as loading control. Values were expressed as mean \pm SEM. One-way ANOVA with the Tukey post hoc test was used to examine the treatment effect. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. $N \geq 4$.

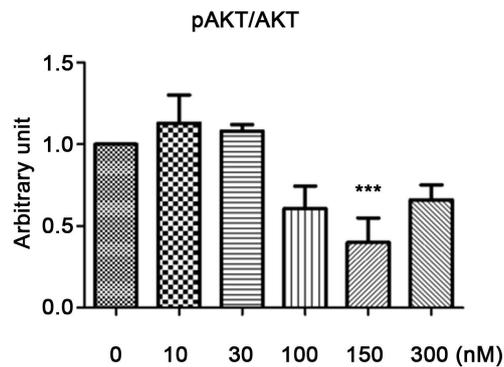


Figure 4. The phosphorylation of AKT/PKB was in-activated by 150 nM OT at day 6 in 3T3L1 adipocytes. Values were expressed as mean \pm SEM. One-way ANOVA with the Tukey post hoc test was used to examine the treatment effect. *** $P \leq 0.001$. $N \geq 4$.

5. Discussion

Short-term treatment of 3T3L1 cells with OT decreased expression of CPT-1 α and FABP4 mRNA, but did not change the TAG concentration. Long-term treatment with OT increased TAG concentration in adipocytes and decreased CPT-1 α expression, suggesting decreased fatty acid oxidation to increase TAG deposition. The HSL mRNA expression was inhibited by OT both in short- and long-term treatment in porcine differentiated adipocytes suggesting that OT increased TAG concentration by decreasing fatty acid oxidation and inhibiting lipolysis. Incubation with OT enhanced the absorption of glucose in adipocytes and up-regulated GLUT4 transporter mRNA expression, but did not affect the mRNA concentrations of the INSR or ChREBP and did not modulate the upstream insulin signaling pathway, AKT/PKB.

Previous studies indicated very mixed results for OT effects in adipocytes. For example, OT increased not only lipogenesis in *ex vivo* experiments with adipose tissue or cell culture systems [28] [29], but also lipolysis in other cells as well [30] [32]. It was demonstrated that OT suppressed adipocyte differentiation [31], whereas the OTR mRNA expression was increased during adipocyte differentiation. It was also shown that the OTR expressions in 14-week-old mice were significantly increased in adipose tissues (subcutaneous and epididymal) compared to those of 7-week-old mice [32]. However, our results suggested OT increased TAG content in adipocytes through decreasing the expression of genes related to fatty acid oxidation and lipolysis. Such effects are similar to the biological effects of insulin surges.

The glucose oxidation was increased along with increased pyruvate dehydrogenase activity under OT treatment [33] [34]. Treatment of OT induced AMP protein kinase (AMPK) pathway on mouse skeletal muscle cells [35], and OT-induced glucose uptake was blocked by either STO-609 or Compound C, indicating AMPK and calcium play important roles in the function of OT in mediating glucose uptake. In consistency with these observations, our data showed that long-term OT treatment increased the expression of GLUT4 which would result in increase of the glucose uptake in adipocytes.

There are mixed evidences for several pathways to influence the insulin-like effects of OT. Modulation of Ca^{2+} and the AMPK pathway were involved in skeletal muscle [35] and in cardiomyocytes [8]. Inositol phosphate did not appear to be involved [36]. In myometrial cells, protein synthesis was stimulated, apparently via an increase in protein kinase C activity [37] [38]. OT was bound to OT receptors and activated the MAP kinases ERK1/2 pathway through *Gaq/Gai*, enhancing the novel and atypical PKC isoforms in hTERT-C3 cells [39]. The present experiments with OT treatments *in vitro* suggested participation of other pathways besides the AKT/PKB signaling pathway was to modulate fatty acid metabolism, glucose uptake and oxidation and energy homeostasis.

There is no direct evidence for OT regulating pathways on lipid or carbohydrate metabolisms in adipocytes. The mechanisms for OT effects on adipocytes may be species or even tissue specific. Thus, extensive exploration of various mechanisms is required to discern mechanisms for OT effects in adipocytes.

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