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The Mechanistic Links between Insulin and Human Organic Anion Transporter 4

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Abstract

Human organic anion transporter 4 (hOAT4) belongs to a class of organic anion transporters that exert critical function in the secretion, absorption, and distribution of numerous drugs in the body, such as anti-viral drugs, anti-cancer therapeutics, antibiotics, antihypertensive medicine, and anti-inflammatory drugs. hOAT4 is richly existent in the kidney and placenta. We previously established that serum- and glucocorticoid-inducible kinases (sgk) stimulate hOAT4 expression and transport activity by abrogating the inhibitory effect of a ubiquitin ligase Nedd4–2. Insulin is one of the upstream signaling molecules for sgk. We therefore investigated the effect of insulin on hOAT4 function. We showed that insulin stimulated hOAT4 expression and transport activity, and the action of insulin was abolished in cells overexpressing Nedd4–2-specific siRNA to knockdown the endogenous Nedd4–2. We further showed that insulin phosphorylated serine 327 on Nedd4–2 and weakened the interaction between hOAT4 and Nedd4–2. Interestingly, in cells overexpressing sgk2, the stimulatory effect of insulin on hOAT4 was diminished. In addition, the stimulatory effect of insulin on hOAT4 was blocked by wortmannin and buparlisib, two PI3K inhibitors. In conclusion, our study demonstrated that insulin stimulates hOAT4 expression and transport activity by abrogating the inhibition effect of Nedd4–2 on the transporter. Moreover, insulin regulates hOAT4 by competing with sgk2 rather than through sgk2.

Graphical Abstract

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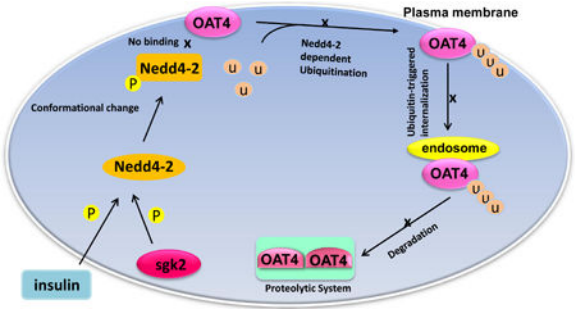
Participated in research design: Wang, You. Conducted experiments: Wang, Zhang. Performed data analysis: Wang, Zhang, You. Wrote or contributed to the writing of the manuscript: Wang, You.

Conflict of interest

The authors have declared that there is no conflict of interest.



Keywords

Organic Anion Transporter; Drug Transport; Regulation; Insulin; Serum and Glucocorticoid-Inducible Kinase; Ubiquitin Ligase

1 Introduction

Human organic anion transporter 4 (hOAT4) belongs to a class of organic anion transporters, that exert critical function in the body secretion, absorption, and distribution of numerous drugs, such as anti-viral drugs, anti-cancer therapeutics, antibiotics, antihypertensive medicine, and anti-inflammatory drugs. The activity of these membrane proteins can be regulated at many levels such as transcriptional, translational, and posttranslational modulation (Ahn and Nigam, 2009; Dantzler and Wright, 2003; Srimaroeng et al., 2008; Terada and Inui, 2007; VanWert et al., 2010; You, 2002). OAT4-mediated transport was suggested to be bidirectional (Babu et al., 2002) and sodium-independent (Cha et al., 2000). hOAT4 is richly existent in the kidney and placenta (Cha et al., 2000). In the kidney, hOAT4 is expressed at the apical membrane of the proximal tubule cells and participates in renal excretion and reabsorption of endogenous substances in addition to numerous drugs and xenobiotics. In the placenta, hOAT4 is expressed at the basolateral membrane of syncytiotrophoblasts (Ugele et al., 2003). Placenta utilizes dehydroepiandrosterone sulfate (DHEAS), a precursor generated by the fetal adrenals, to synthesize estrogen. Buildup of extra DHEAS is linked with intrauterine growth retardation (Rabe et al., 1983). DHEAS is a hOAT4 substrate. Therefore, hOAT4 may exert a significant role in placental uptake of DHEAS for making estrogens and for protecting fetus from the DHEAS cytotoxicity. OAT4 has also been found to be expressed in choroid plexus epithelial cells and brain microvessel endothelial cells through RT-PCR assay (Kusch-Poddar et al., 2005; Xu et al., 2005). However, whether OAT4 protein is expressed in these cells are not known.

Due to such a critical role, delineating how hOAT4 is regulated has deep clinical significance. We formerly established that members of OAT family constitutively internalizes from and recycles back to plasma membrane and the transport activity of OAT can be modulated by changing the trafficking kinetics of these transporters (Zhang et al., 2008; Zhang et al., 2010; Zhang et al., 2012). An important event preceding OAT internalization is the ubiquitin conjugation to the transporter, catalyzed by a ubiquitin ligase Nedd4–2. Enhancement of Nedd4–2-dependent OAT ubiquitination leads to an acceleration of OAT internalization/removal from plasma membrane and subsequent degradation (Xu et

al., 2017). As a result, OAT expression at the plasma membrane, and therefore OAT transport activity is reduced.

The serum- and glucocorticoid-inducible kinases (sgk) participate in governing many cellular processes including sodium Na^+ balance, renal transport, and cell proliferation (Buse et al., 1999; Chen et al., 1999; Leong et al., 2003; Naray-Fejes-Toth et al., 1999; Rozansky et al., 2002; Waldegger et al., 1998). The family of the sgk protein kinases has three members: sgk1, sgk2 and sgk3. Sgk1 and sgk3 are richly present in every tissue, whereas sgk2 is expressed mainly in the liver, brain, kidney, and pancreas. We have recently demonstrated that sgk stimulated OAT transport activity by abrogating the inhibitory effect of Nedd4–2 on the transporter (Wang et al., 2016; Wang and You, 2017).

Insulin has been shown to regulate the cellular processes through multiple signaling pathways (Barros et al., 2009; Lee et al., 2007; Lungkaphin et al., 2014). One of the mediators for insulin is sgk. Therefore, in the current study, we investigated the effect of insulin on hOAT4. Interestingly, we discovered that insulin regulates hOAT4 by competing with sgk rather than through sgk.

2. Materials and Methods

2.1 Materials

[^{3}H]-labeled estrone sulfate was acquired from PerkinElmer (Waltham, MA). COS-7 cells were acquired from American Type Culture Collection (Manassas, VA). Membrane-impermeable biotinylation reagent NHS-SS-biotin, streptavidin-agarose beads and protein G-agarose beads were acquired from Pierce (Rockford, IL). cDNA for human Nedd4–2 was kindly provided by Dr. Peter M. Snyder of the College of Medicine, University of Iowa (Iowa City, IA). cDNAs for mouse sgk2 (wild-type sgk2, inactive sgk2 (IN-sgk2) and constitutive active sgk2 (CA-sgk2) were kindly provided by Dr. Alan C. Pao from Department of Medicine, Stanford University (Stanford, CA). Mouse anti-Myc antibody (9E10) was acquired from Roche (Indianapolis, IN). Rabbit anti-Nedd4–2, was purchased from Abcam (Cambridge, MA). Rabbit anti-P-Nedd4–2 (Serine 327) antibody was acquired from Cell Signaling (Danvers, MA). Mouse anti-β-actin was acquired from Santa Cruz (Santa Cruz, CA). Nedd4–2-specific siRNA oligonucleotides (Silencer® Select, identification number s23570) and negative control siRNA oligonucleotides (Silencer® Select, catalog number 4390843) were acquired from Ambion (Grand Island, NY). Insulin and all other reagents were acquired from Sigma-Aldrich (St. Louis, MO).

2.2 Culturing of the Cells and cDNA Transfection

Parental COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. Transfection of cDNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was performed for 48 hours, following the manufacturer's instructions. Cells stably expressing hOAT4 were kept in DMEM medium supplemented with 0.2mg/ml G418 (Invitrogen, Carlsbad, CA), 10% fetal bovine serum.

2.3 Transport Measurements

Uptake of [^{3}H]-estrone sulfate (100 nM) was carried out, following standard protocol previously established in our lab (Wang et al., 2016). Uptake solution (phosphate-buffered saline with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4 and 0.3μM [^{3}H]-estrone sulfate) was added to 48-well plates in which cells were plated. The uptake process was terminated by removing the uptake solution and washing the cells with ice-cold PBS twice at indicated time points. 0.2 N NaOH was used for cell lysis and then 0.2 N HCl was used for neutralization. Liquid scintillation counter (Beckman LSC LS6500) was used for detection of [^{3}H]-estrone sulfate. Uptake activity was expressed as a percentage of the uptake measured in control cells. Data were corrected for nonspecific background measured in mock cells (parental cells).

2.4 Cell Surface Biotinylation

The measurement of the expression level of hOAT4 at the plasma membrane was performed using a biotinylation strategy, following the standard protocol previously established in our lab (Wang et al., 2016). Sulfo-NHS-SS-biotin (0.5 mg/ml in phosphate-buffered saline with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 8.0) was added to 6-well plates in which cells were plated. After biotinylation and quenching of the unreacted sulfo -NHS-SS-biotin by rinsing with 100 mM glycine in PBS/CM, the cells were lysed on ice for 30 min. After 20 min centrifugation at 16000g at 4 °C, each sample supernatant of cell lysates was added to 40 μl of streptavidin-agarose beads to purify the cell membrane proteins. hOAT4 (tagged with Myc at its carboxyl terminus) was detected in the pool of surface proteins by SDS-PAGE and immunoblotting using an anti-Myc antibody 9E10 (Roche, Indianapolis, IN).

2.5 Immunoprecipitation

We followed the procedure previously established in our laboratory (Wang et al., 2016; Zhang et al., 2018). Cells were lysed with lysis buffer, containing 1% of proteinase inhibitor cocktail. For the experiment in which anti-Myc was used to pull-down hOAT4, the lyzed cells were precleared with protein G-agarose beads. Anti-Myc antibody (1:100) was incubated with protein G-agarose beads at 4 °C for 1.5 hours. The precleared cell lysates were then mixed with antibody-bound protein G-agarose beads with rotating at 4 °C overnight. For the experiment in which Anti-Flag M2 affinity gel (Sigma–Aldrich, St. Louis, MO) was used to pull-down Nedd4–2, the precleared cell lysates were mixed with Anti-Flag M2 affinity gel with rotating at 4 °C overnight. Proteins bound to the protein G-agarose beads or Anti-Flag M2 affinity gel were eluted with Urea buffer containing β-mecaptoethanol and examined by immunoblotting with appropriate antibodies.

2.6 Electrophoresis and Immunoblotting

We followed the procedure previously established in our laboratory (Wang et al., 2016). Protein samples were separated on 7.5% SDS-PAGE minigels and electroblotted on to PVDF membranes. The blots were incubated with 5% nonfat dry milk in PBS-0.05% Tween 20, washed, and incubated with appropriate primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The SuperSignal West Dura Extended Duration Substrate kit (Pierce, Rockford, IL) was used to detect the signals. The scanning

densitometry with the FluorChem 8000 imaging system (Alpha Innotech Corp., San Leandro, CA) was used to quantify the nonsaturating, immunoreactive protein bands.

2.7 Data Analysis

We repeated each experiment a minimum of three times, and multiple experiments were used for statistical analysis. Statistical analysis was performed using Student's paired t-tests between two groups or GraphPad Prism software (GraphPad Software Inc., San Diego, CA), one-way ANOVA, multiple comparisons among multiple treatments. A p-value of <0.05 was considered significant.

3 Results

3.1 Effect of insulin on hOAT4 transport activity

To examine the role of insulin in hOAT4 function, we treated hOAT4-expressing COS-7 cells with insulin, followed by the measurement of hOAT4-mediated uptake of [^{3}H]-estrone sulfate (ES), a prototypical substrate for hOAT4. As shown in Fig. 1, insulin induced a dose-, and time-dependent rise in the uptake in compared with that in control cells with a ~ 180% stimulation at insulin concentration of 100 nM for 12 hr. treatment. To investigate the mechanism of insulin-induced stimulation of hOAT4 activity, we measured hOAT4-mediated uptake of [^{3}H]-estrone sulfate at various substrate concentrations. Using an Eadie-Hofstee analysis (Fig. 2), we showed that treatment with insulin caused an increased maximal transport velocity V_{max} of hOAT4 (129.77 ± 19.82 pmol·mg^{-1}·3min^{-1} with control cells and 230.07 ± 22.21 pmol·mg^{-1}·3min^{-1} with cells treated with insulin) without notable alteration in the substrate-binding affinity K_m of the transporter (3.27 ± 0.30 μM with control cells and 3.45 ± 0.26 μM with cells treated with insulin).

3.2 Effect of insulin on hOAT4 Expression

Two possibilities could be responsible for a rise in the maximal transport velocity V_{max} of hOAT4 shown in Fig. 2: either the number of the transporter at the plasma membrane could be increased or the transporter turnover rate could be enhanced. We carried out experiments that dissect these likelihoods by examining transporter expression both at the plasma membrane and in the total cell lysates. We showed that treatment with insulin led to an increase of hOAT4 expression at the plasma membrane (Fig. 3a), and in total cell lysates (Fig. 3c, top panel). Such a change in hOAT4 expression was not because of the overall perturbation of the cellular proteins since the expression of cellular protein marker β-actin was not affected under these situations (Fig. 3c, bottom panel).

3.3 Relationship between insulin and sgk2 on hOAT4 transport activity

One of the downstream mediators for insulin is sgk. We therefore investigated the relationship between insulin and sgk2 on hOAT4 transport activity. We transfected hOAT4-expressing cells with control vector, inactive form of sgk2 (IN-sgk2), wild-type of sgk2 (WT-sgk2), or the constitutive active form of sgk2 (CA-sgk2). Transfected cells were then treated with or without insulin (100nM, 12h), followed by [^{3}H]-estrone sulfate uptake (3min, 0.3 μM). As shown in Fig. 4a, in the absence of insulin, hOAT4-mediated uptake was not significantly changed in IN-sgk2-transfected cells, was moderate in WT-sgk2-transfected

cells, and was highest in CA-sgk2-transfected cells as compared to that in control cells, consistent with activity of the sgk2. However, when these cells were treated with insulin (Fig. 4b), we observed a maximum stimulation of hOAT4-mediated uptake in control or in IN-sgk2-transfected cells, suggesting that overexpression of IN-sgk2 failed to block insulin's stimulatory effect. However, in cells transfected with WT-sgk2, the stimulatory effect of insulin became smaller as compared to that in cells transfected with IN-sgk2. In cells transfected with CA-sgk2, the stimulatory effect of insulin became diminished as compared to that in cells transfected with IN-sgk2. Similar results were observed in the cells overexpressing Nedd4–2 (Fig. 4c). These results indicate that the sgk2-stimulated hOAT4 uptake and insulin-stimulated uptake were not additive. When cells expressed highest level of sgk2 activity (CA-sgk2), insulin was unable to further stimulate hOAT4 transport activity.

3.4 Effect of Nedd4–2 on insulin-stimulated hOAT4 expression

We previously demonstrated that the ubiquitination of OAT catalyzed by ubiquitin ligase Nedd4–2 leads to the internalization of the transporter from cell surface and subsequent degradation. In this experiment, we investigated the role of Nedd4–2 in insulin-stimulated hOAT4 expression (Fig. 5). In control cells, insulin significantly enhanced hOAT4 expression at the plasma membrane (Fig. 5a), whereas in cells transfected with Nedd4–2-specific siRNA to knock down the expression of endogenous Nedd4–2, insulin was unable to enhance hOAT4 expression at the plasma membrane (Fig. 5c). These results suggest that insulin stimulated hOAT4 expression through Nedd4–2.

3.5 Effect of Nedd4–2 on insulin-stimulated hOAT4 transport activity

We next investigated the role of Nedd4–2 in insulin-stimulated hOAT4 transport activity (Fig. 6). In control cells (Fig. 6a) and cells transfected with Nedd4–2 (Fig. 6b), insulin significantly enhanced hOAT4-mediated uptake of [^{3}H]-estrone sulfate, whereas in cells transfected with Nedd4–2-specific siRNA to knock down the expression of endogenous Nedd4–2, insulin was unable to enhance hOAT4 transport activity (Fig. 6c), demonstrating that insulin stimulated hOAT4 transport activity through Nedd4–2.

3.6 Effect of insulin on the interaction of Nedd4–2 with hOAT4

Nedd4–2 regulates OAT ubiquitination by directly interacting with the transporter. We assessed the effect of insulin on the interaction of Nedd4–2 with hOAT4 through co-immunoprecipitation assay. hOAT4-expressing cells were transfected with Nedd4–2. Transfected cells were incubated with or without insulin. hOAT4 was then immunoprecipitated, followed by immunoblotting with anti-Nedd4–2 antibody to detect hOAT4-associated Nedd4–2. As shown in Fig. 7a top panel, in the absence of insulin, a significant quantity of Nedd4–2 was found in hOAT4 immunoprecipitates (lane 1), indicating a direct association between these two proteins. Yet, in cells treated with insulin (lane 2), the quantity of Nedd4–2 found in hOAT4 immunoprecipitates was much reduced. These data suggest that insulin hindered the interaction between hOAT4 and Nedd4–2. The difference in the amount of Nedd4–2 associated with hOAT4 was not due to the difference in the amount of hOAT4 pulled down because the same quantity of hOAT4 was immunoprecipitated under these conditions (Fig. 7a bottom panel). Similar results were obtained using a reciprocal approach, where Nedd4–2 was pulled down first, followed by

immunoblotting with anti-Myc antibody (epitope Myc was tagged to hOAT4) to detect Nedd4–2-associated hOAT4 (Fig. 7c, top panel).

3.7 Effect of insulin on Nedd4–2 phosphorylation

We previously showed that sgk stimulates OAT activity by phosphorylating the amino acid residue serine 327 on Nedd4–2 (Wang and You, 2017). In this experiment, we examined whether insulin stimulates hOAT4 activity by phosphorylating Nedd4–2 at the same site. We transfected Nedd4–2 into hOAT4-expressing cells. The transfected cells were treated with or without insulin, followed by immunoblotting with phosphor-Nedd4–2 (Ser327)-specific antibody. As shown in Fig 8a, top panel, the phosphor-Nedd4–2-specific antibody detected a band at the size of Nedd4–2 in control cells, suggesting that Nedd4–2 was certainly phosphorylated at Ser327. The phosphorylation signal was enhanced by 70% in cells treated with insulin. The total expression of Nedd4–2 was not affected by insulin (Fig 8a, middle panel). Furthermore, the difference in the amount of Nedd4–2 phosphorylated was not due to an overall change in cellular proteins as the amount of cellular protein marker β-actin was not affected under this condition (Fig. 8a, bottom panel). Therefore, insulin enhanced the phosphorylation of Nedd4–2 at serine 327.

3.8 Effects of PI3K inhibitors on insulin-stimulated hOAT4-mediated estrone sulfate uptakes

PI3K has been shown to be the key regulator in insulin pathway (Avramoglu et al., 2006; Fry, 1994; Song et al., 2018) and PI3K inhibitors have been utilized to block insulin-mediated effect (MacKay et al., 2012; Zhou et al., 2014). To examine the effects of PI3K inhibitors on insulin-stimulated hOAT4-mediated estrone sulfate uptakes, we employed two selective PI3K inhibitors wortmannin and buparsilib. As shown in Fig. 9, both inhibitors significantly blocked the stimulatory effect of insulin on hOAT4 transport activity (wortmmanin or buparsilib alone did not show non-specific cytotoxicity). Together these results provide evidence that PI3K plays an essential role in the regulation of insulin stimulated hOAT4 activity.

4 Discussion

Organic anion transporters (OATs) is a major deciding factor of the effects of therapeutics and toxic chemicals. Thus, delineating the regulation of OATs at the molecular and cellular levels is clinically and pharmacologically important. In the present study, we investigated the mechanistic link between insulin and hOAT4-mediated drug transport.

Our present studies were performed in a heterologous cell system - COS-7 cells, which have been proven to be an excellent model system for investigation of the cloned organic anion transporter (Chioukh et al., 2014; Duan et al., 2010; Hagos et al., 2015; Zhang et al., 2008; Zhang et al., 2013). First, these cells are originated from the kidney. Examinations in such system have revealed other renal transport processes. Secondly, this cell line does not endogenously express OATs. Thus, expression of hOAT4 in these cells will permit us to characterize this transporter without the difficulty of being interfered by other organic anion transporters. Thirdly, the many signaling pathways are intact in these cells, providing a good

exploratory model system for understanding the regulation of many transport processes. Lastly, the previous work revealed that the transport properties of OATs in these cells were consistent with those displayed in other systems. Our investigation in COS-7 cells will pave the path for the next stage of work concentrating on evaluating whether the similar mechanisms are operational in native epithelia.

Our lab has previously established that members of OAT family constitutively internalize from and recycle back to the plasma membrane and OAT transport activity can be modulated by changing the rates of either the internalization or the recycling of these transporters (Zhang et al., 2008; Zhang et al., 2010; Zhang et al., 2012). Ubiquitin conjugation (ubiquitination) of OAT, catalyzed by a ubiquitin ligase Nedd4–2 is an important mechanism which modulates the rate of OAT internalization. Enhancement of Nedd4–2-dependent OAT ubiquitination leads to an accelerated OAT internalization and degradation (Xu et al., 2016a; Xu et al., 2016b; Xu et al., 2017). As a result, OAT expression at the cell surface, and therefore OAT transport activity is decreased.

Nedd4–2 is under the control of many protein kinases (Debonneville et al., 2001; Garcia-Tardon et al., 2012; Lee et al., 2007; Snyder et al., 2004; Wang and You, 2017). It is possible that differential regulations of OAT by various protein kinases are exerted through the dynamic phosphorylation at different sites on Nedd4–2, in doing so, bringing about diverse conformational change in Nedd4–2 and altering its binding to OAT, which results in a change in OAT ubiquitination, trafficking and transport activity (Lee et al., 2007; Snyder et al., 2004; Wang and You, 2017). Our lab has recently demonstrated that the serum- and glucocorticoid-inducible kinases (sgks) stimulated OAT transport activity by selectively phosphorylating serine residue 327 on Nedd4–2 (Wang and You, 2017), which weakens the binding of Nedd4–2 to OAT, leading to a reduced ubiquitin-triggered OAT internalization and degradation (Wang et al., 2016; Wang and You, 2017). Here our results further indicate that insulin increases hOAT4 expression and activity by inducing phosphorylation of the same serine on Nedd4–2.

Insulin is a peptide hormone that regulates the activity of many membrane proteins (e.g. epithelial sodium channel (ENaC), Glucose Transporter type 4 (Glut4) and Na+/H+ exchanger 3) (Brewer et al., 2014; Klisic et al., 2002; Lee et al., 2007). The regulation of insulin in cellular processes could be through multiple signaling pathways. Several lines of evidence indicate that insulin was an upstream activator of sgks (Lee et al., 2007; Liu et al., 2014; Mansley et al., 2016). In the current study, we showed that insulin stimulated hOAT4 expression and transport activity (Figs. 1–3). We further showed that regulation of hOAT4 activity by insulin was mediated by ubiquitin ligase Nedd4–2. Knocking down endogenous Nedd4–2 by Nedd4–2-specific siRNA abolished the effect of insulin on hOAT4 (Figs. 5 and 6). The effect of insulin on hOAT4 expression and activity was paralleled with the phosphorylation of serine 327 on Nedd4–2 and a weakened association of Nedd4–2 to hOAT4 (Figs. 7 and 8). However, interestingly, the effect of insulin on hOAT4 was diminished in cells overexpressing sgk2 (Fig. 4), suggesting that the effect of insulin and sgk2 were not additive, and that insulin competes with sgk2, rather than acting through sgk2, in the regulation of hOAT4. PI3K has been shown to be the key regulator in insulin pathway (Avramoglu et al., 2006; Fry, 1994; Song et al., 2018). In our current study, we observed that

two selective PI3K inhibitors wortmannin and buparlisib significantly blocked the stimulatory effect of insulin on hOAT4 transport activity (Fig. 9). PI3K has been reported to be a target for anti-tumor therapeutics. Wortmannin is a widely used model inhibitor for PI3K in both in vitro and in vivo studies (MacKay et al., 2012; Ng et al., 2000; Zhou et al., 2014). Buparlisib is currently under late-stage clinical development. Multiple clinical trials have been initiated to investigate the anti-tumor effect of Buparlisib in the patients with a variety of cancers (Criscitiello et al., 2018).

In addition to its role in the kidney, OAT4, specifically expressed in higher primates, is localized to the basolateral membrane of syncytiotrophoblast of the placenta and responsible for the elimination of steroid sulfates, xenobiotics, and clinically important drugs from the fetal compartment and therefore reducing the toxicity for the developing fetus. Gestational diabetes mellitus (GDM) is a condition in which a pregnant woman develops any degrees of glucose intolerance due to the lack of insulin. GDM has already become a major health concern with a growing prevalence. Further studies investigating the activity and expression of OAT4 in GDM placenta would be particularly interesting.

In conclusion, our current study demonstrated that insulin stimulates hOAT4 expression and transport activity through modulating the ubiquitin ligase Nedd4–2. Interestingly, the action of insulin is in competition with that of sgk2 rather than through sgk2 (Fig. 10).

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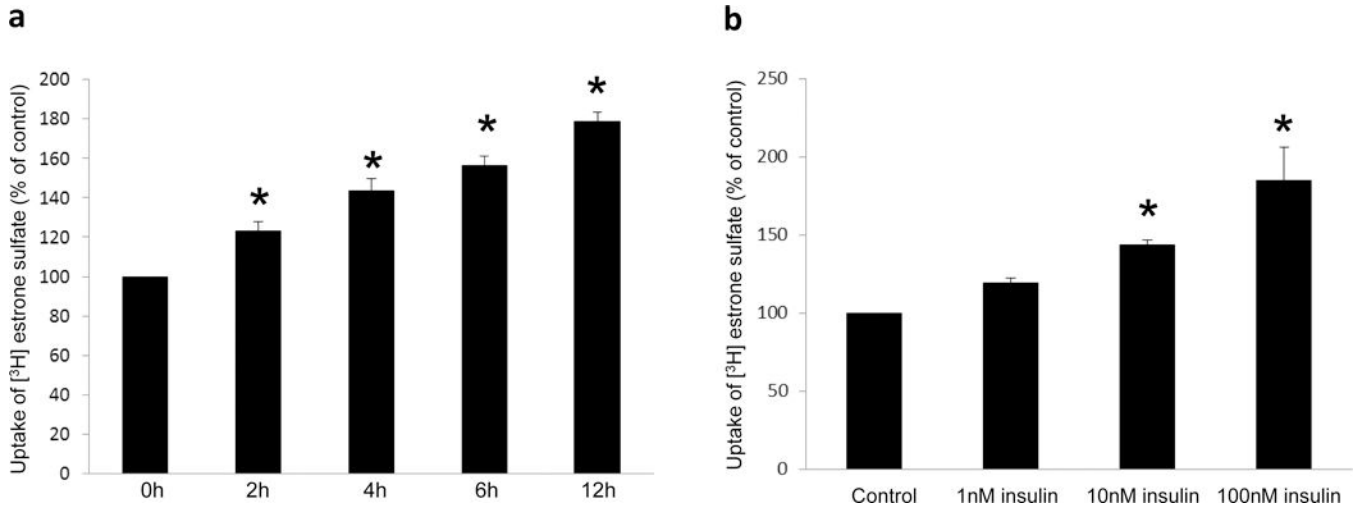

Fig. 1. Effect of insulin on hOAT4 transport activity.

(a) Time-dependent stimulation of hOAT4 transport activity. hOAT4-expressing COS-7 cells were treated with insulin (100nM) for 2h, 4h, 6h and 12h. 3-min uptake of [^{3}H]-estrone sulfate (0.3 μM) was then measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. The data represent uptake into hOAT4-expressing cells minus uptake into mock cells (parental COS-7 cells). Values are mean ± SD (n = 3). *P<0.05. (b) Dose-dependent stimulation of hOAT4 transport activity. hOAT4-expressing COS-7 cells were treated with insulin at varies doses in 12h-treatment. 3-min uptake of [^{3}H]-estrone sulfate (0.3 μM) was then measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. The data represent uptake into hOAT4-expressing cells minus uptake into mock cells (parental COS-7 cells). Values are mean ± SD (n = 3). *P<0.05.

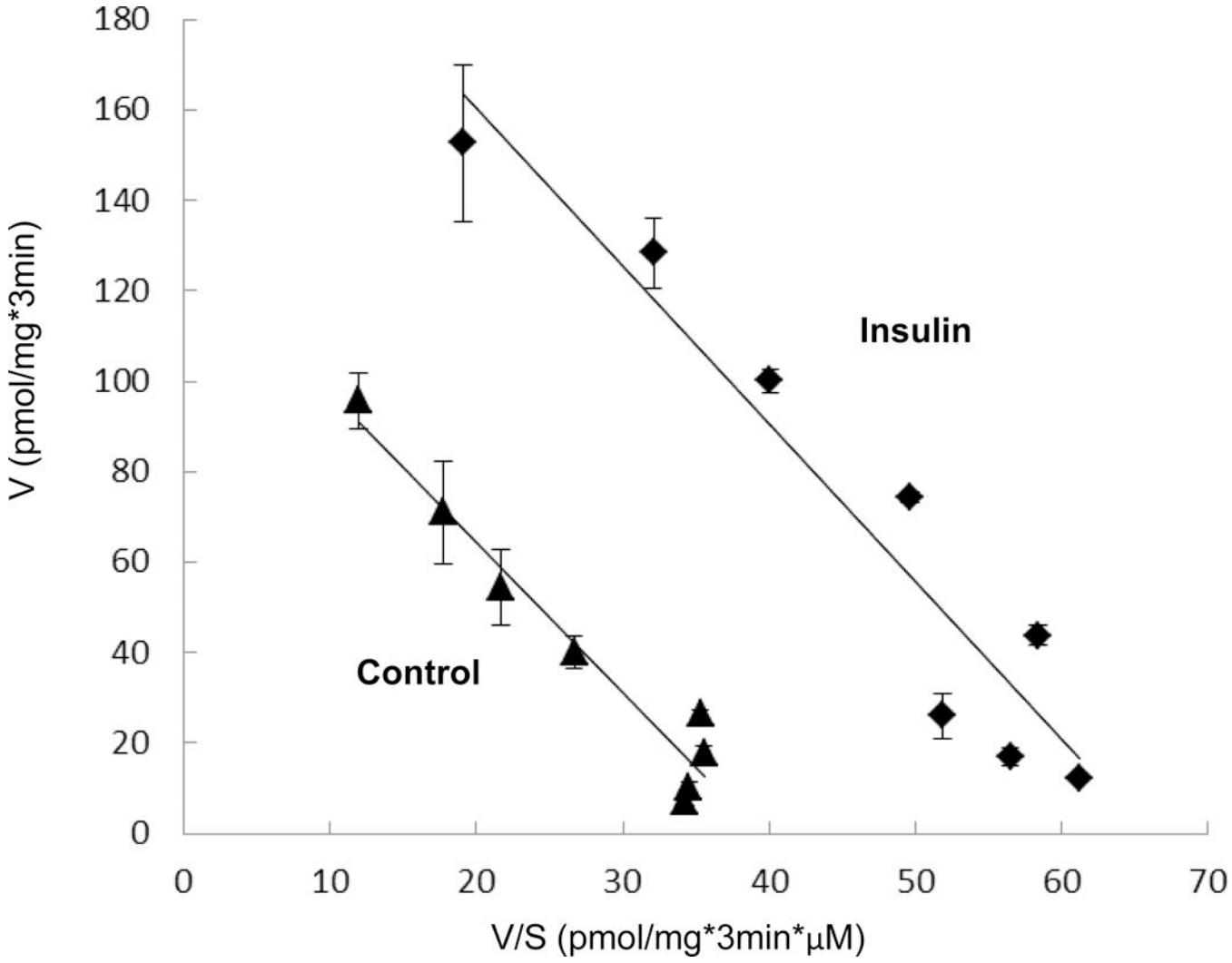

Fig. 2. Effect of insulin on the kinetics of hOAT4-mediated estrone sulfate transport.

COS-7 cells expressing hOAT4 were treated with the insulin (100nM, 12h), and initial uptake (3 min) of [^{3}H]-estrone sulfate was measured at the concentration of 0.1–10 μM. The data represent uptake into hOAT4-transfected cells minus uptake into mock cells (parental COS-7 cells). Values are means ± SD (n = 3). *V*, velocity; S, substrate concentration.

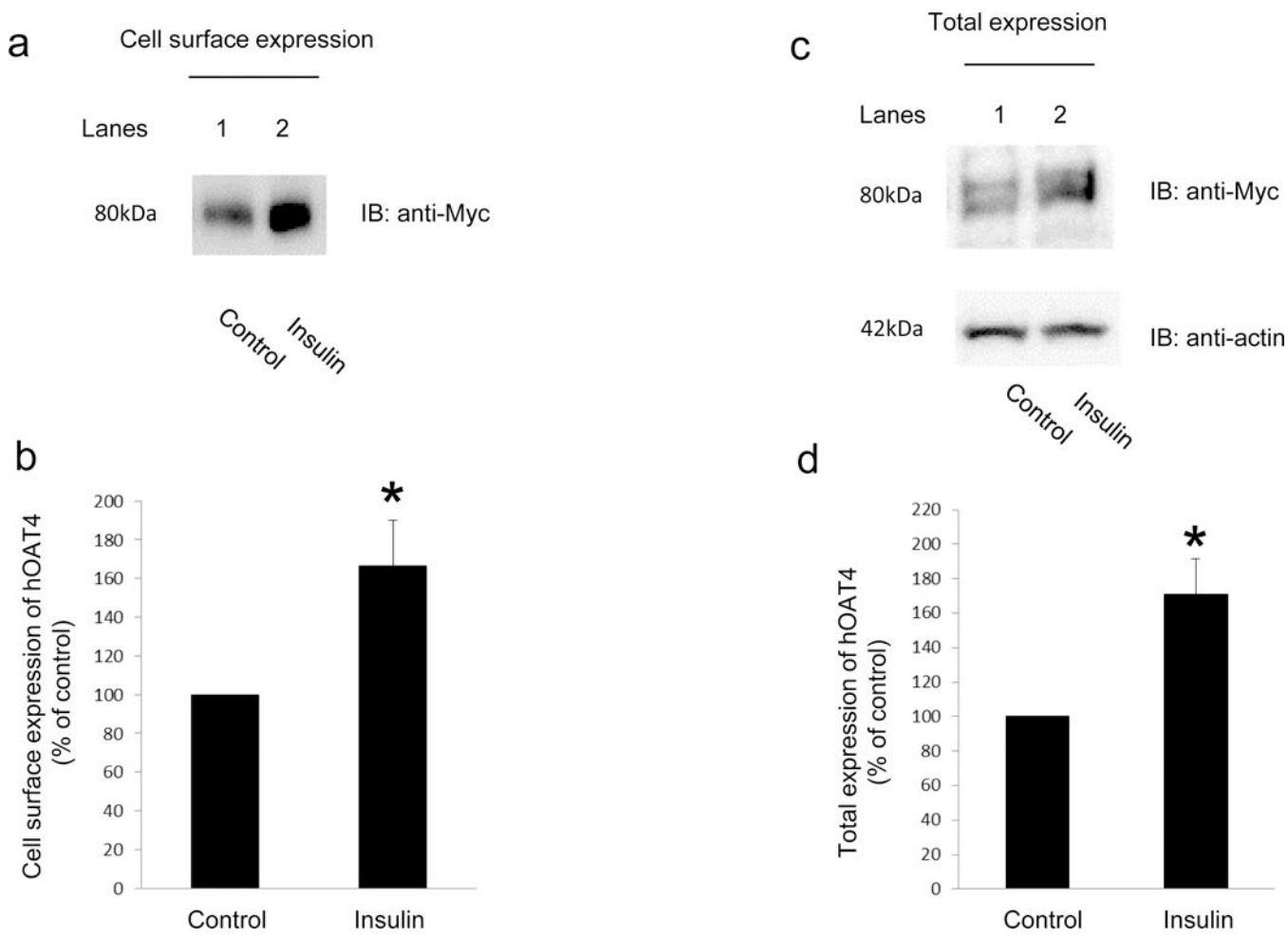

Fig. 3. Effect of insulin on hOAT4 expression.

(a). Cell surface expression of hOAT4. hOAT4-expressing COS-7 cells were treated with the insulin (100nM, 12h). Cells were labeled with biotin. Biotinylated/cell surface proteins were separated with streptavidin beads, followed by immunoblotting (IB) with an anti-Myc antibody. Epitope Myc was tagged to hOAT4 for immune-detection. (b). Densitometry plot of results from Fig. 3a, as well as from other experiments. The values are mean ± SD (n = 3). *P<0.05. (c). Top panel: Total expression of hOAT4. hOAT4- expressing COS-7 cells were treated with the insulin (100nM, 12h). Cells were lysed, followed by immunoblotting (IB) with an anti-Myc antibody. Epitope Myc was tagged to hOAT4 for immune-detection. Bottom panel: the same blot from Fig. 3c was re-probed with anti β-actin antibody. β-actin is a cellular protein marker. (d). Densitometry plot of results from Fig. 3c, top panel as well as from other experiments. The values are mean ± SD (n = 3). *P<0.05.

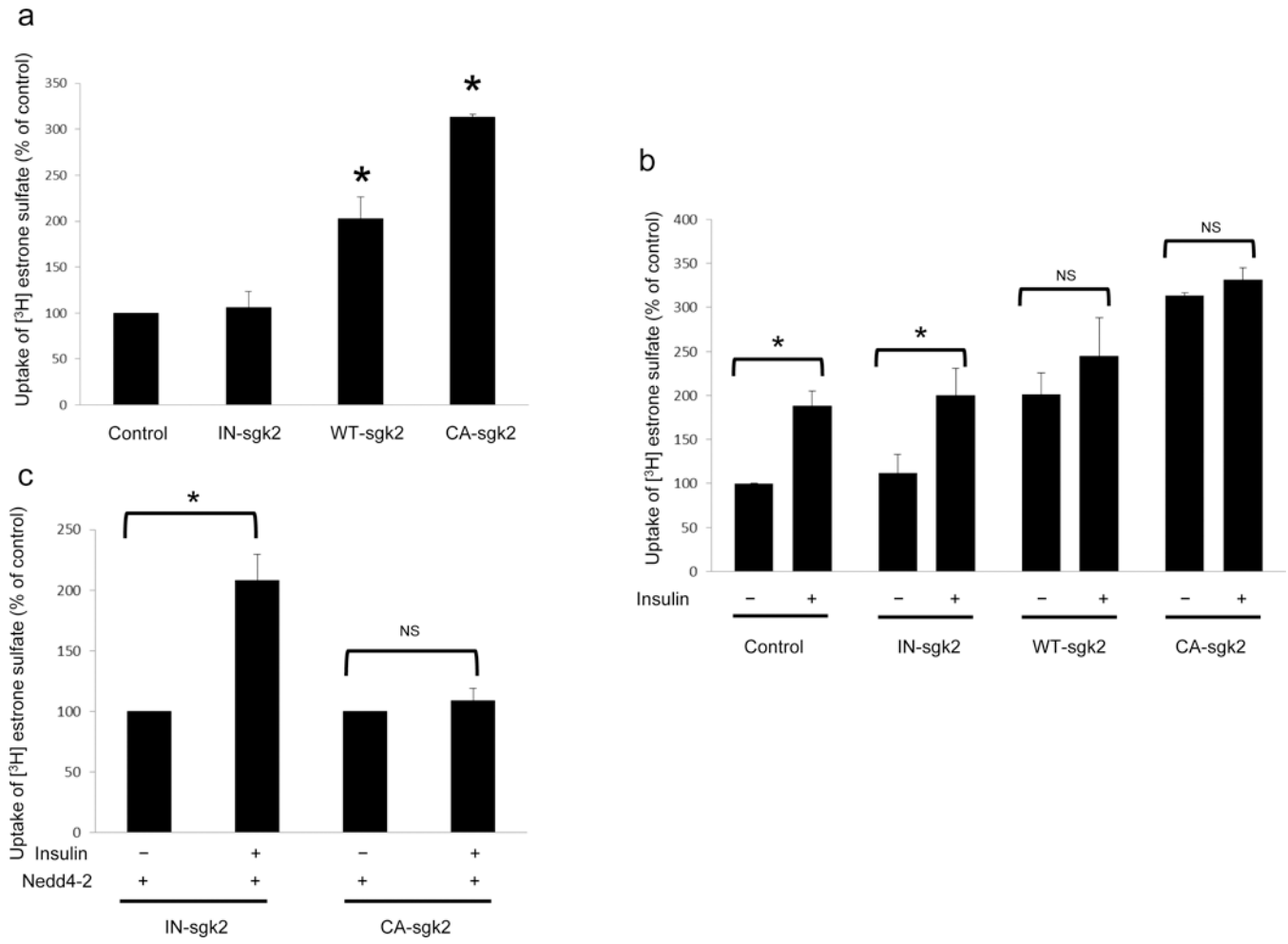

Fig. 4. Relationship between insulin and sgk2 on hOAT4 transport activity.

(a) The effect of sgk2 on hOAT4 transport activity. COS-7 cells were transfected with hOAT4 and control vector, or with hOAT4 and the inactive form of sgk2 (IN-sgk2), or with hOAT4 and the wild-type of sgk2 (WT-sgk2), or with hOAT4 and the constitutive active form of sgk2 (CA-sgk2). After 48h transfection, cells were starved for 12h. [^{3}H]-estrone sulfate uptake was then measured (3min, 0.3 μM). Uptake activity was expressed as a percentage of the uptake measured in control cells. The data represent uptake into hOAT4-expressing cells minus uptake into mock cells (parental COS-7 cells). Values are mean ± SD (n = 3). *P<0.05. (b) Relationship between insulin and sgk2 on hOAT4 transport activity. COS-7 cells were transfected with hOAT4 and control vector, or with hOAT4 and the inactive form of sgk2 (IN-sgk2), or with hOAT4 and the wild-type of sgk2 (WT-sgk2), or with hOAT4 and the constitutive active form of sgk2 (CA-sgk2). Transfected cells were then treated with or without insulin (100nM, 12h), followed by the measurement of [^{3}H]-estrone sulfate uptake (3min, 0.3 μM). Uptake activity was expressed as a percentage of the uptake measured in control cells. The data represent uptake into hOAT4-expressing cells minus uptake into mock cells (parental COS-7 cells). Values are mean ± SD (n = 3). *P<0.05. NS: statistically not significant. (c) Relationship between insulin and sgk2 on hOAT4 transport activity in the cells overexpressing Nedd4–2. COS-7 cells were transfected with Nedd4–2,

hOAT4 and the inactive form of sgk2 (IN-sgk2), or with Nedd4–2, hOAT4 and the constitutive active form of sgk2 (CA-sgk2). Transfected cells were then treated with or without insulin (100nM, 12h), followed by the measurement of [^{3}H]-estrone sulfate uptake (3min, 0.3 μM). Uptake activity was expressed as a percentage of the uptake measured in control cells. The data represent uptake into hOAT4-expressing cells minus uptake into mock cells (parental COS-7 cells). Values are mean ± SD (n = 3). *P<0.05. NS: statistically not significant.

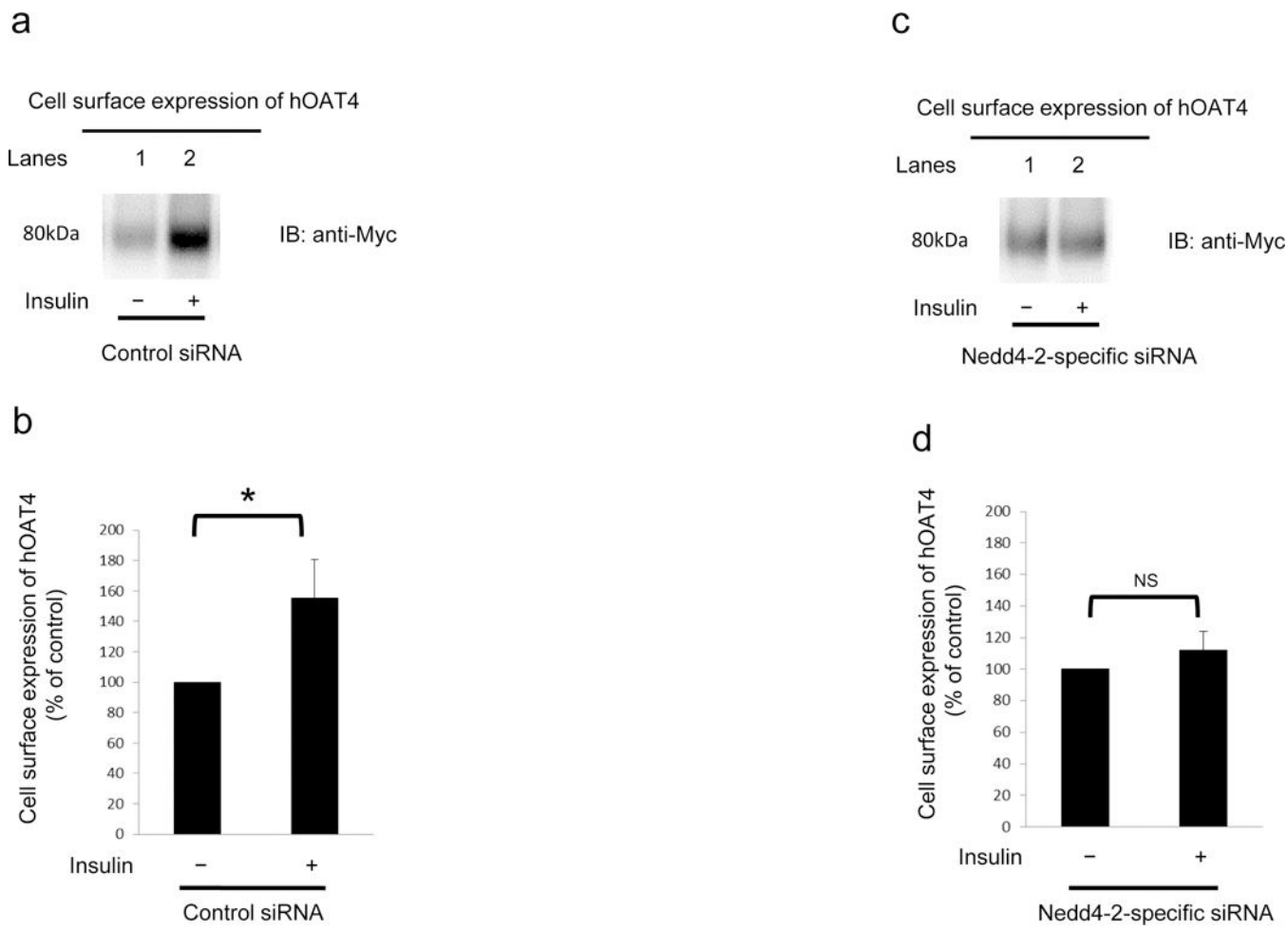

Fig. 5. Role of Nedd4–2 in insulin-stimulated hOAT4 expression.

(a) COS-7 cells were co-transfected with hOAT4 and scrambled control siRNA. Transfected cells were treated with or without insulin (100nM) for 12h and then were labeled with biotin. Biotinylated/cell surface proteins were separated with streptavidin beads, followed by immunoblotting (IB) with an anti-Myc antibody. Epitope Myc was tagged to hOAT4 for immune-detection. (b). Densitometry plot of results from Fig. 5a, as well as from other experiments. The values are mean ± SD (n = 3). *P<0.05, (c) COS-7 cells were co-transfected with hOAT4 and Nedd4–2-specific siRNA. Transfected cells were treated with or without insulin (100nM) for 12h and then were labeled with biotin. Biotinylated/cell surface proteins were separated with streptavidin beads, followed by immunoblotting (IB) with an anti-Myc antibody. Epitope Myc was tagged to hOAT4 for immune-detection. (d). Densitometry plot of results from Fig. 5c, as well as from other experiments. The values are mean ± SD (n = 3). NS: statistically not significant.

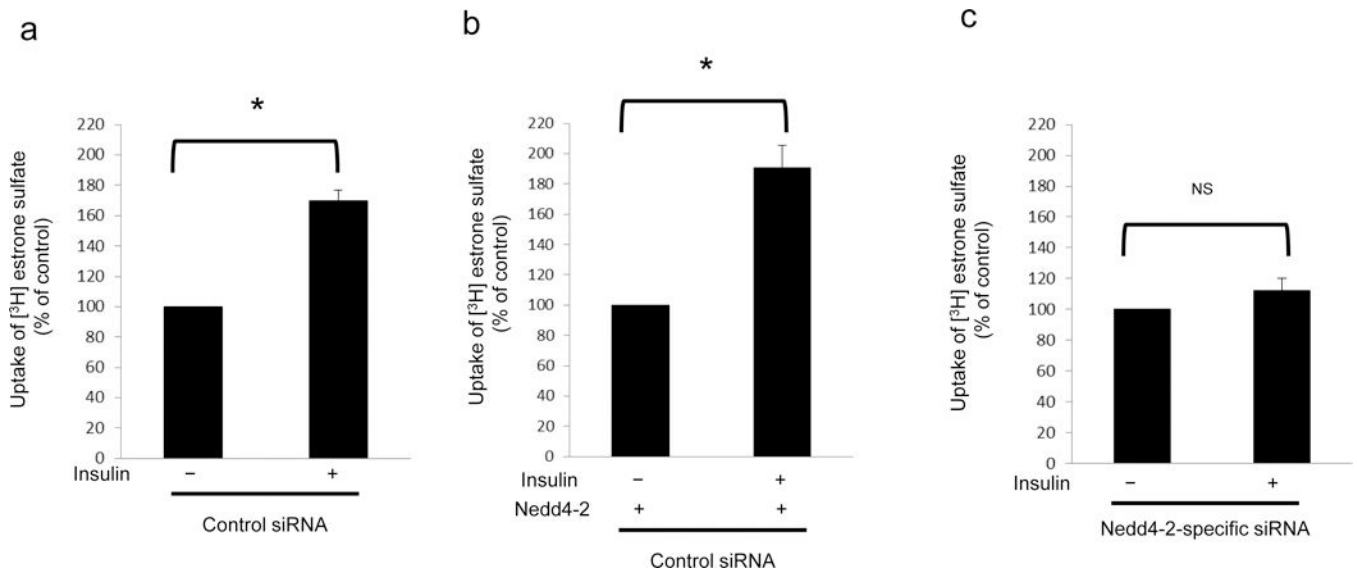

Fig. 6. Role of Nedd4–2 in insulin-stimulated hOAT4 transport activity.

(a) COS-7 cells were co-transfected with hOAT4 and scrambled control siRNA. Transfected cells were treated with or without insulin (100nM) for 12h and then measured for the uptake of [^{3}H]-estrone sulfate (3-min uptake and 0.3 μM estrone sulfate). The data represent uptake into hOAT4-transfected cells minus uptake into mock cells (parental COS-7 cells). Values are mean ± SD (n = 3). *P<0.05 (b) COS-7 cells were triple-transfected with hOAT4, Nedd4–2 and scrambled control siRNA. Transfected cells were treated with or without insulin (100nM) for 12h and then measured for the uptake of [^{3}H]-estrone sulfate (3-min uptake and 0.3 μM estrone sulfate). The data represent uptake into hOAT4-transfected cells minus uptake into mock cells (parental COS-7 cells). Values are mean ± SD (n = 3). *P<0.05. (c) COS-7 cells were co-transfected with hOAT4 and Nedd4–2-specific siRNA. Transfected cells were treated with or without insulin (100nM) for 12h and then measured for the uptake of [3H]-estrone sulfate (3-min uptake and 0.3 μM estrone sulfate). The data represent uptake into hOAT4-transfected cells minus uptake into mock cells (parental COS-7 cells). Values are mean ± SD (n = 3). NS: statistically not significant.

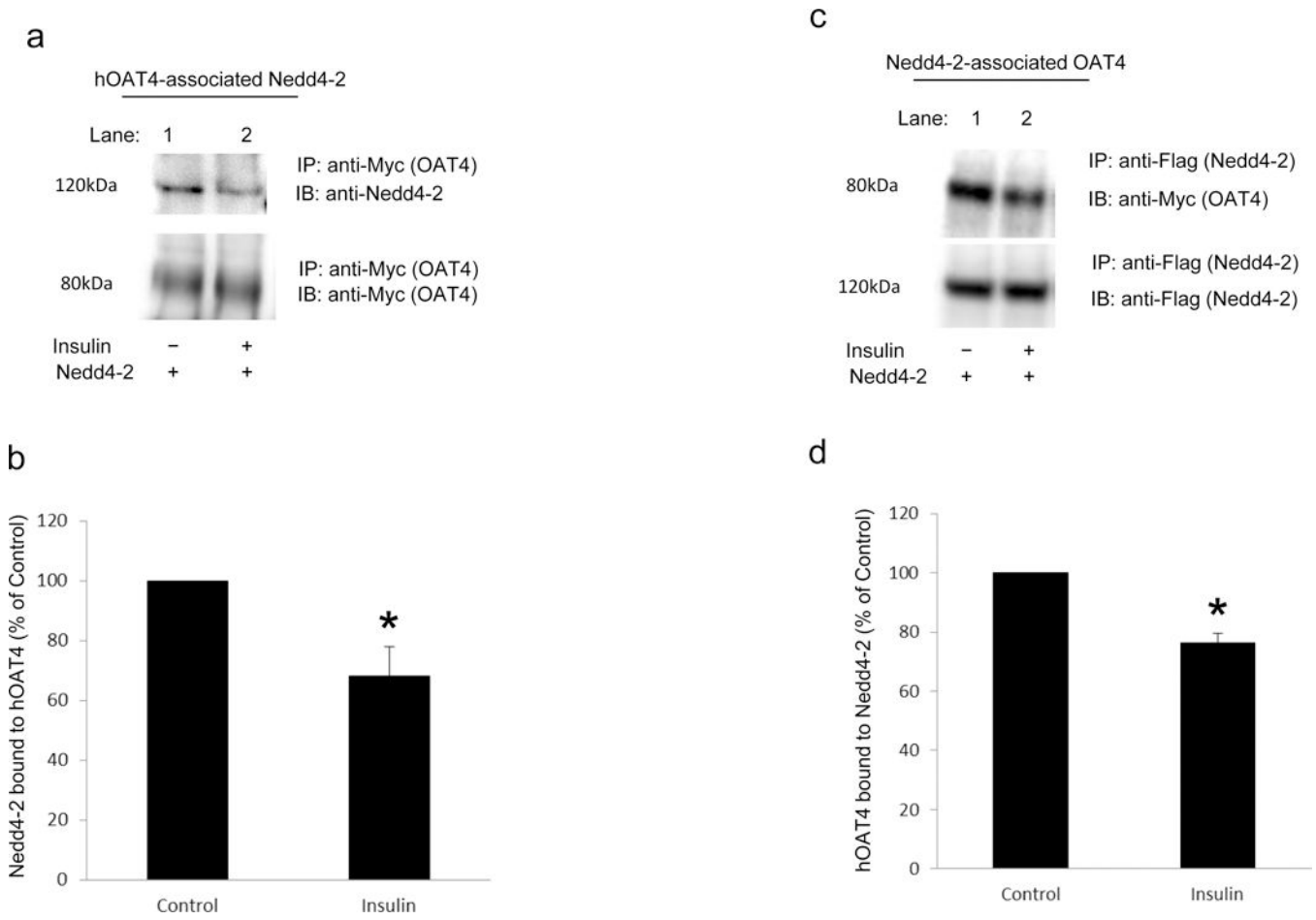

Fig. 7. Role of insulin in the interaction between Nedd4–2 and hOAT4.

(a) COS-7 cells were transfected with hOAT4 and Nedd4–2. *Top panel:* Transfected cells were treated with or without insulin (100nM, 12h) and then lysed, and hOAT4 was immunoprecipitated (IP) with anti-Myc antibody, followed by immunoblotting (IB) with the anti-Nedd4–2 antibody. Epitope Myc was tagged to hOAT4 for immune-detection. *Bottom panel:* The same immunoblot from Fig. 7a top panel was reprobed by anti-Myc antibody to detect the total amount of hOAT4 pulled down. (b) Densitometry plot of results from Fig. 7a as well as from other experiments. The values are mean ± SD (n = 3). *P<0.05. (c) *Top panel:* Transfected cells were treated with or without insulin (100nM, 12h) and then lysed, and Nedd4–2 (tagged with epitope Flag for immunodetection) was immunoprecipitated (IP) with anti-Flag M2 affinity gel, followed by immunoblotting (IB) with the anti-Myc antibody. Bottom panel: The same immunoblot from Fig. 7c top panel was reprobed by anti-Flag antibody to detect the total amount of Nedd4–2 pulled down. (d) Densitometry plot of results from Fig. 7c as well as from other experiments. The values are mean ± SD (n = 3). *P<0.05.

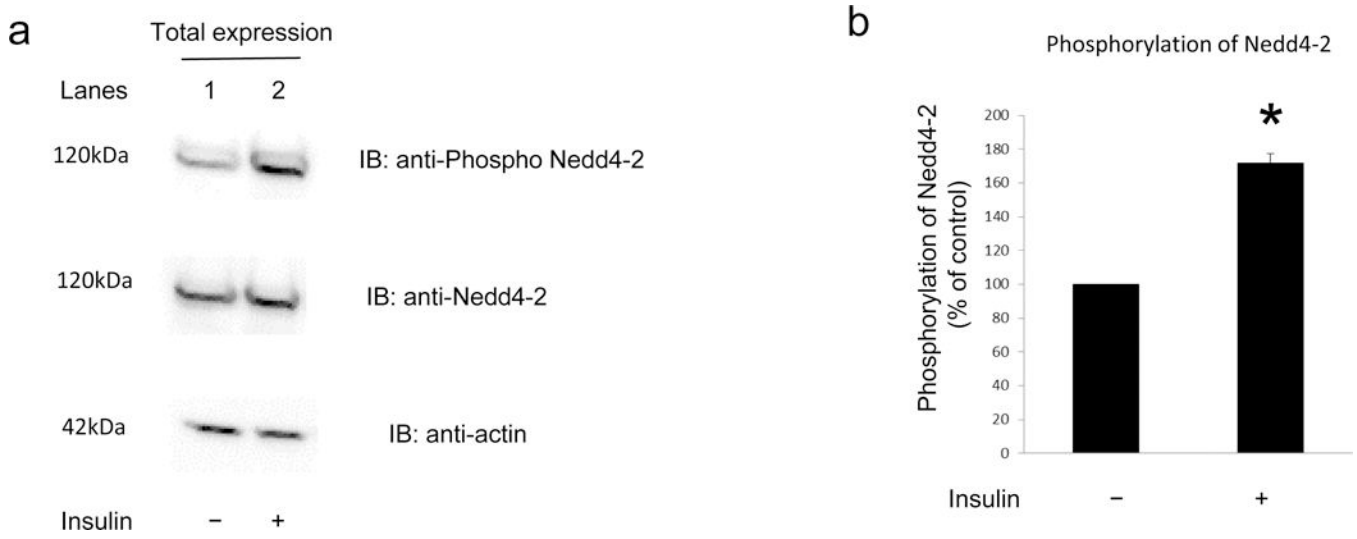

Fig. 8. Effect of insulin on hOAT4 phosphorylation.

(a) COS-7 cells were co-transfected with hOAT4 and Nedd4–2. Transfected cells were treated with or without insulin (100nM) and then lysed, followed by immunoblotting with the anti-Phospho-Nedd4–2 antibody (Top panel), anti-Nedd4–2 antibody (middle panel), or anti-β-actin antibody (bottom panel) respectively. β-actin is a cellular protein marker. (b). Densitometry plot of results from Fig. 8a, the top panel as well as from other experiments. The values are mean ± SD (n = 3). *P<0.05.

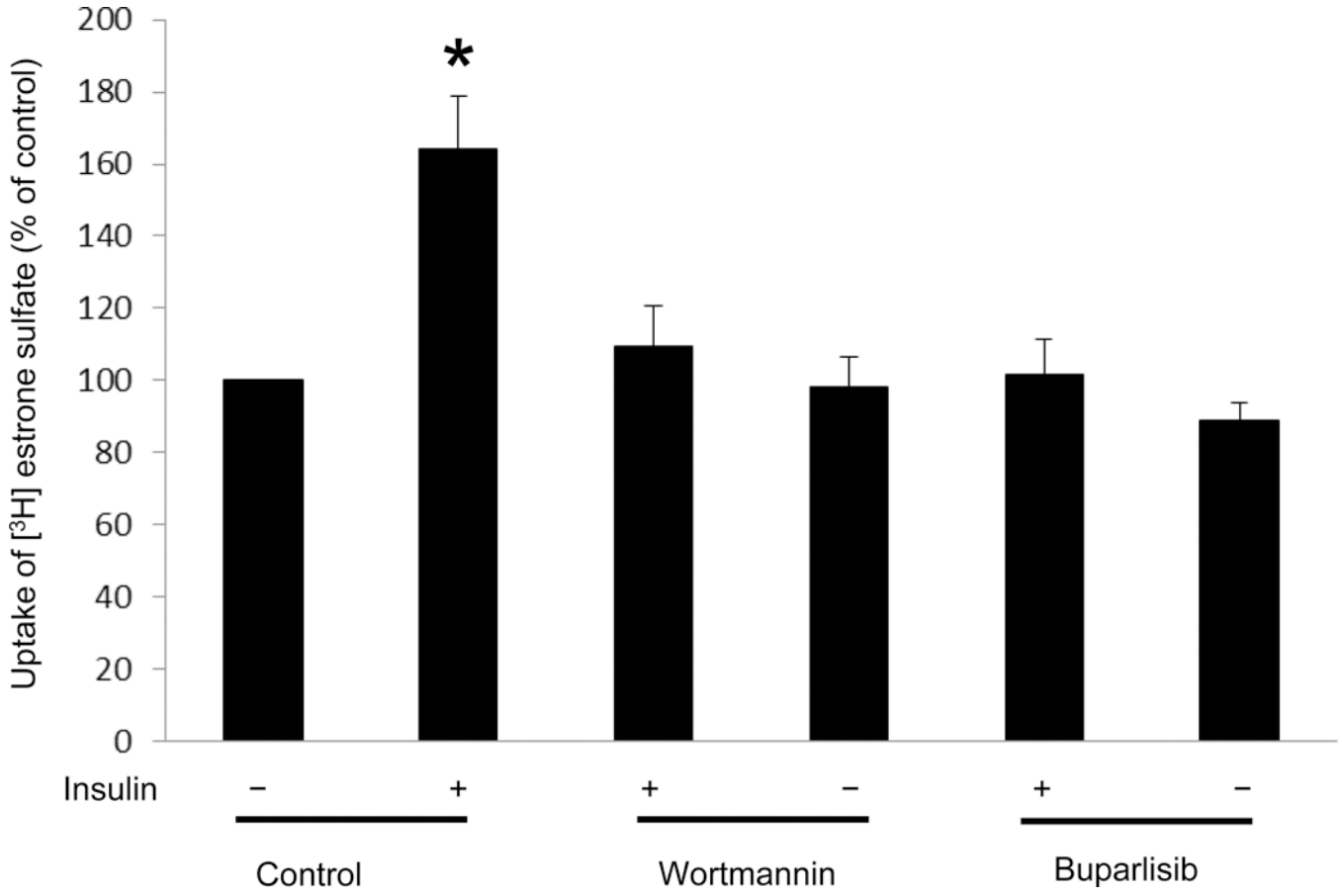

Fig. 9. The effects of wortmannin and buparlisib on insulin stimulation of hOAT4-mediated transport.

hOAT4-expressing cells were treated with insulin (100nM, 12h) with or without PI3K inhibitors (wortmannin (100nM, 12h) or buparlisib (250nM, 12h)) or PI3K inhibitors alone, followed by measuring the uptake [^{3}H] estrone sulfate (ES, 3min, 0.3 μM). Uptake activity was expressed as a percentage of the uptake determined in control cells. The data represent uptake into hOAT4-expressing cells minus uptake into mock cells. Values are mean ± SD (n = 3).*P<0.05.

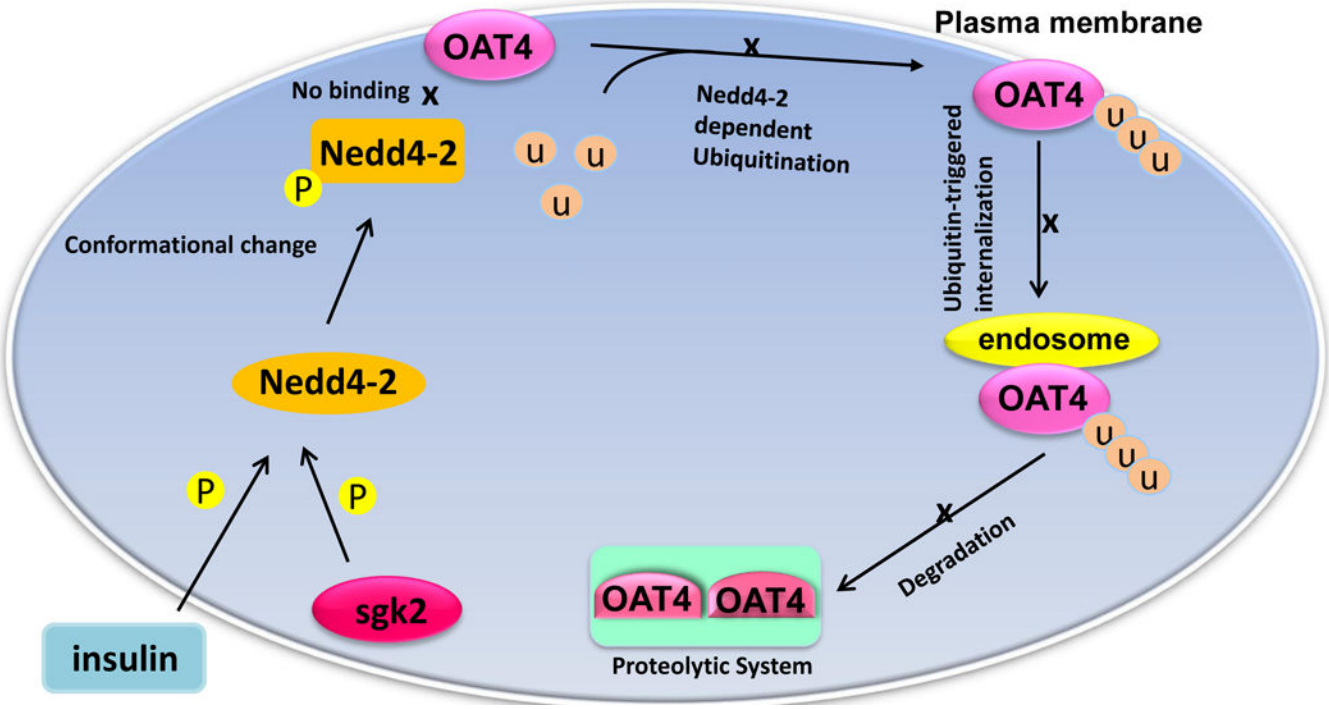

Fig. 10. The Mechanistic Links between Insulin, sgk, and hOAT4.
U: ubiquitin. P: phosphorylation