Acta Pharmaceutica Sinica B 2020;10(1):186–194

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

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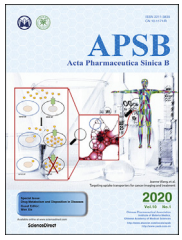

ORIGINAL ARTICLE

Insulin-like growth factor 1 modulates the phosphorylation, expression, and activity of organic anion transporter 3 through protein kinase A signaling pathway

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Received 8 February 2019; received in revised form 10 April 2019; accepted 9 May 2019

KEY WORDS

Organic anion transporter;
Drug transport;
Regulation;
IGF-1;
PKA;
Phosphorylation

Abstract Organic anion transporter 3 (OAT3) plays a vital role in removing a broad variety of anionic drugs from kidney, thus avoiding their possible toxicity in the body. In the current study, we investigated the role of insulin-like growth factor 1 (IGF-1) in the regulation of OAT3. We showed that IGF-1 induced a dose- and time-dependent increase in OAT3 transport activity, which correlated well with an increase in OAT3 expression. The IGF-1-induced increase in OAT3 expression was blocked by protein kinase A (PKA) inhibitor H89. Moreover, IGF-1 induced an increase in OAT3 phosphorylation, which was also blocked by H89. These data suggest that the IGF-1 modulation of OAT3 occurred through PKA signaling pathway. To further confirm the involvement of PKA, we treated OAT3-expressing cells with PKA activator Bt2-cAMP, followed by examining OAT activity and phosphorylation. We showed that OAT3 activity and phosphorylation were much enhanced in Bt2-cAMP-treated cells as compared to that in control cells. Finally, linsitinib, an anticancer drug that blocks the IGF-1 receptor, abrogated IGF-1-stimulated OAT3 transport activity. In conclusion, our study demonstrated that IGF-1 regulates OAT3 expression and transport activity through PKA signaling pathway, possibly by phosphorylating the transporter.

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Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

https://doi.org/10.1016/j.apsb.2019.05.005

1. Introduction

Organic anion transporter 3 (OAT3) is a member of the organic anion transporter family, which plays vital parts in the removal of many drugs from the kidney, such as anti-viral drugs, anti-tumor therapeutics, antibiotics, antihypertensive and anti-inflammatory drugs, and thereby avoiding their possible toxicity in the body[1−6].

A biology model of transporters called remote sensing and signaling has recently attracted a lot attention[7−9]. In this model, OATs play a key role in interorgan communication and in regulating local and whole-body homeostasis. The intercellular and inter-organ communication is carried out by hormones, small molecules and cell signaling. Hormones produced in the original organ under stimuli and released into blood stream regulate target organ transporters through activating cell signaling.

Hormones regulate OATs through the activation of various protein kinases. A protein kinase modifies its substrate molecules, typically proteins, by chemically conjugating phosphate groups to them, a post-translational process called phosphorylation. Phosphorylation frequently results in a functional change of the target protein by changing its three-dimensional conformation, activity, cellular distribution, protein stability or its interaction with other proteins. Protein kinases regulate various membrane proteins including channels, transporters and receptors. For example, parathyroid hormone down-regulates NaPi-IIc transporter involving protein kinase C (PKC)-induced phosphorylation of the transporter[10]. Glucose transporter 4 is up-regulated by PKC-induced phosphorylation[11], and PMA, a PKC activator, increases P-glycoprotein (P-gp) phosphorylation, which is correlated with increased P-gp transporter activity[12].

Previous studies from our laboratory demonstrated that OAT transport activity is subjected to the regulation by several physiological stimuli such as angiotensin II, peptide hormone bradykinin, and progesterone[13−15]. We showed that these hormones down-regulate OAT activity through the activation of PKC. Interestingly, our laboratory demonstrated that activation of PKC inhibits OAT transport activity without directly phosphorylating the transporter itself[16].

IGF-1 is produced primarily by the liver under the stimulation of growth hormone (GH) and plays significantly roles in growth, development, and metabolism[17−19]. IGF-1 exerts its effect on its substrates through IGF-1 receptor. IGF-1 is involved in various renal physiological processes including renal development, glomerular functions, and tubular handling. In addition, GH/IGF-1 axis contributes to various kidney diseases including renal cancer, acute kidney failure, diabetic nephropathy and polycystic kidney disease[17,20].

PKA, also known as cAMP-dependent protein kinase, exists in a physiological tetrameric complex which consists of two regulatory subunits and two catalytic subunits[21]. PKA is one of the most widely studied protein kinases and is activated following the release of the catalytic subunits in response to the second messenger cAMP[22]. We previously demonstrated that activation of PKA by Bt2-cAMP enhanced OAT3 transport activity, stimulated SUMOylation and suppressed ubiquitination[23]. IGF-1 regulates physiological and pathological processes through various signaling pathways including the activation of PKA. For example, IGF-1 enhanced cell survival *via* protein kinase A pathway[24]. IGF-1/PKA pathway plays a vital role in regulating stem cell protection, self-renew Guofengal, and regeneration[25], and IGF-1 stimulated OAT3 SUMOylation involving PKA signaling[23].

The abnormalities in the IGF-1 have been reported to be related to the development of several diseases, such as Laron syndrome and acromegaly[26,27]. Mecasermin, a synthetic analog of IGF-1, has been used to treat patients with growth failure and short stature caused by IGF-1 deficiency[28]. IGF-1 receptor is a transmembrane protein activated by IGF-1 binding. The mutation of IGF-1 receptor causes pre and postnatal growth retardation[29].

In the current study, we investigated the effect of IGF-1 on OAT3 phosphorylation, expression and transport activity as well as its downstream signaling pathway.

2. Materials and methods

2.1. Materials

COS-7 cells were obtained from ATCC (Manassas, VA, USA). [^{3}H]-labeled estrone sulfate (ES) was obtained from PerkinElmer (Waltham, MA, USA). Membrane-impermeable biotinylation reagents, Sulfo-NHS-SS-biotin, streptavidin agarose beads, and protein G agarose beads were purchased from Pierce (Rockford, IL, USA). Mouse anti-myc antibody (9E10) was obtained from Roche (Indianapolis, IN, USA). Mouse anti-E-cadherin, anti-GAPDH and anti-phospho-Ser/Thr antibodies were obtained from Abcam (Cambridge, MA, USA). Horseradish peroxidase-conjugated anti-mouse antibody was bought from Santa Cruz (Santa Cruz, CA, USA). Dibutyryl cyclic-AMP sodium salt (Bt2-cAMP), H89 dihydrochloride hydrate (H89), insulin-like growth factor-I human (IGF-1) and anti-myc agarose affinity gel were bought from Sigma−Aldrich (St. Louis, MO, USA). IGF-1 receptor inhibitor, linsitinib, was purchased from Selleck Chemicals (Houston, TX, USA).

2.2. Cell culture

Parental COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Corning, Corning, NY, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 °C with 5% CO_2. Cells stably expressing human OAT3 (hOAT3) were established in our laboratory as previously described[14,30]. Cells stably expressing hOAT3 were maintained in DMEM containing 0.2 mg/mL G418 (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum.

2.3. Transport measurements

The transport activity of OAT3 was determined by measuring [^{3}H]-ES uptake into stable OAT3-expressing COS-7 cells. The uptake solution consisted of phosphate-buffered saline (pH 7.4) with 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$ (PBS/CM) and [^{3}H]-ES (300 nmol/L). The uptake solution was added to the cells. After an indicated period of time, uptake was ended by aspirating the uptake solution and rapidly washing the cells with ice-cold PBS solution. The cells were then lysed in 0.2 mol/L NaOH, neutralized in 0.2 mol/L HCl, and collected for liquid scintillation counting by using a liquid scintillation counter (Beckman LSC LS6500). Uptake activity was expressed as the percentage of the uptake value measured in control cells.

2.4. Cell surface biotinylation

The amount of OAT3 at the cell surface was determined using the membrane-impermeable biotinylation reagent, Sulfo-NHS-SS-biotin, as described in our previous publications[9,26]. The cells in culture were washed two times by PBS/CM pH 8.0 and incubated with Sulfo-NHS-SS-biotin (0.5 mg/mL in PBS/CM, pH 8.0) for two consecutive 20 min. After biotinylation, the cells were washed and quenched with 100 mmol/L glycine to remove the unreacted Sulfo-NHS-SS-biotin. Then the cells were lysed on ice for 45 min and centrifuged at 16,000×g at 4 °C. The supernatant of cell lysates was added to 40 μL of streptavidin-agarose beads to pull down the cell membrane proteins. Cell surface OAT3 protein was detected by SDS-PAGE and immunoblotting using an anti-myc antibody (9E10) (myc was tagged to OAT3 for immune detection).

2.5. Protein phosphorylation

Cells were lysed with lysis buffer. Protein concentration for each sample was measured and same amount of proteins were precleared at 4 °C for 2 h and incubated with anti-myc agarose affinity gel (Sigma−Aldrich) at 4 °C overnight. On next day the beads carrying immunoprecipitated proteins were washed with lysis buffer three times, denatured with urea denature buffer containing β-mercaptoethanol, and analyzed by SDS-PAGE and immunoblotting with anti-phospho-Ser/Thr antibody.

2.6. Electrophoresis and immunoblotting

We followed the procedure previously established in our laboratory[31,32]. Protein samples were separated on 7.5% SDS-PAGE mini-gels (Bio-Rad, Hercules, CA, USA) and electroblotted onto PVDF membranes (Invitrogen). The membranes were treated with 5% nonfat dry milk in PBST (0.05% Tween-20 in PBS) for 1 h at room temperature and incubated overnight at 4 °C with appropriate primary antibodies. Then the blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, followed by detection with SuperSignal West Dura Extended Duration Substrate kit (Pierce). FluorChem 8800 imaging system (Alpha Innotech Corp., San Leandro, CA, USA) was used to quantify the non-saturated, immunoreactive protein bands.

2.7. Data analysis

Each experiment was repeated a minimum of three times. The statistical analysis was from multiple experiments. Between two groups, statistical analysis was performed using Student's paired t-tests. Among multiple treatments, one-way ANOVA was applied by using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). A P-value of <0.05 was considered significant.

3. Results

3.1. Effect of IGF-1 on OAT3 transport activity

To explore the role of IGF-1 in OAT3 transport function, we treated OAT3-expressing COS-7 cells with IGF-1 for 4 h, then measured the OAT3-mediated uptake of [^{3}H]-ES, a prototypical substrate for OAT3. As shown in Fig. 1A, IGF-1 induced a dose-dependent increase in the ES uptake. When compared with non-treated group, treatment with 100 nmol/L IGF-1 resulted in a significant increase of 60% in the ES uptake. OAT3-expressing COS-7 cells were also treated with IGF-1 for various time periods. As seen in Fig. 1B and 100 nmol/L IGF-1 increased the ES uptake in a time-dependent manner, with ∼15%, 30% and 75% increase at treatment time points of 1, 2, and 4 h.

3.2. Effect of IGF-1 on OAT3 expression

The change in transport activity of OAT3 may reflect a change in its three-dimensional structure or in its level of expression. We therefore examined the effect of IGF-1 on OAT3 expression. Our results revealed that treatment of OAT3-expressing cells with IGF-1 led to an increase of OAT3 expression both at the cell surface (Fig. 2A top panel and 2B), and in its total cell extract

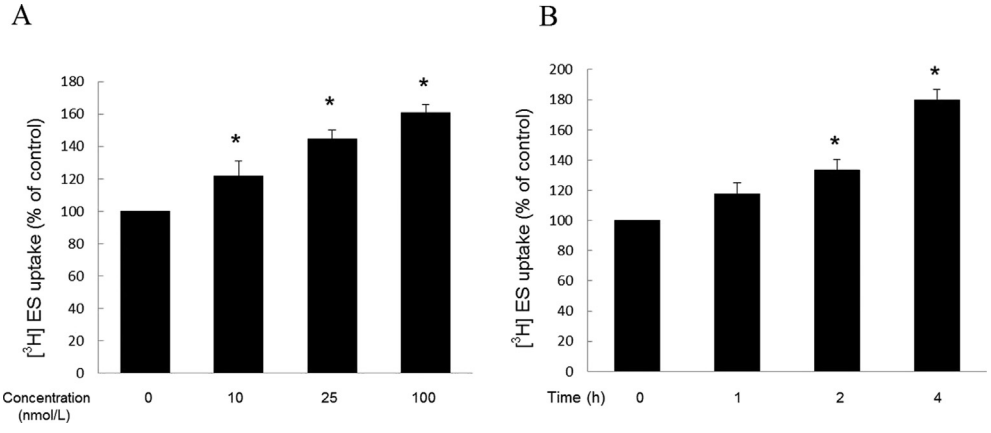

Figure 1 Effect of IGF-1 on OAT3 transport activity. (A) OAT3-expressing cells were treated with IGF-1 at various doses for 4 h. 4-min uptake of [^{3}H]-estrone sulfate (ES, 0.3 μmol/L) was then measured. Transport activity was expressed as percentage of the uptake in control cells. (mock cells: ∼400 CPM and OAT3-expressing cells without treatment: ∼4000 CPM). Values are mean ± SD (n = 3); $^*P < 0.05$. (B) OAT3-expressing cells were treated with 100 nmol/L IGF-1 for various time points. 4-min uptake of [^{3}H]-estrone sulfate (ES, 0.3 μmol/L) was then measured. Transport activity was expressed as percentage of the uptake in control cells. Values are mean ± SD (n = 3); $^*P < 0.05$. Statistical analysis was performed using one-way ANOVA by using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

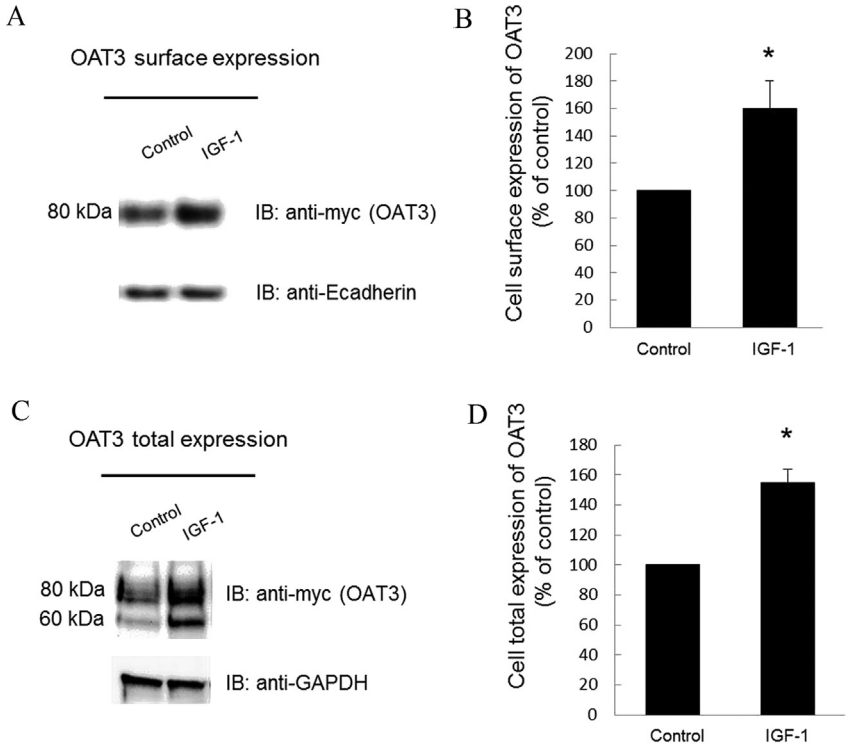

Figure 2 Effect of IGF-1 on OAT3 expression. (A) Cell surface expression of OAT3. Top panel: OAT3-expressing cells were treated with IGF-1 (100 nmol/L, 4 h). Biotinylated cell surface proteins were separated with streptavidin beads, followed by immunoblotting (IB) with anti-myc antibody (OAT3 was tagged with epitope myc for immunodetection). Bottom panel: The identical blot of the top panel was re-probed with anti-E-cadherin antibody. E-cadherin is a marker for cell membrane proteins. (B) Densitometry analysis of blot results from Fig. 2A, top panel as well as from other experiments. The values are mean ± SD ($n = 3$); $^{*}P < 0.05$. (C) Total protein expression of OAT3. Top panel: OAT3-expressing cells were treated with IGF-1 (100 nmol/L, 4 h). After treatment, cells were lysed, followed by immunoblotting with anti-myc antibody. Bottom panel: The identical blot of the top panel was re-probed with anti-GAPDH antibody. GAPDH is a marker for total cell proteins. (D) Densitometry analysis of blot results from Fig. 2C, top panel as well as from other experiments. The values are mean ± SD ($n = 3$); $^{*}P < 0.05$. Statistical analysis was performed using Student's paired *t*-tests.

(Fig. 2C top panel and 2D). Such a change in OAT3 expression was not because of the overall disturbance of membrane and cellular proteins since the expression of plasma membrane protein marker E-cadherin and cellular protein marker GAPDH were not affected under these situations (Fig. 2A, bottom panel and Fig. 2C, bottom panel). OAT3 at the cell surface displayed a single band at 80 kDa (Fig. 2A, top panel), whereas OAT3 showed two bands at 60 and 80 kDa in total cell extracts (Fig.2C, top panel). Our laboratory previously illustrated that OAT undergoes glycosylation as a maturation process in the endoplasmic reticulum (ER)−Golgi complex[33,34]. The immature form is a non-glycosylated form of 60 kDa, which matures in ER−Golgi complex to a glycosylated form of 80 kDa. Only the mature form (80 kDa) can target to cell surface. Furthermore, IGF-1-induced increase in OAT3 expression was blocked by PKA inhibitor H89 (Fig. 3), suggesting that IGF-1 modulates OAT3 through PKA signaling pathway.

3.3. *Effect of IGF-1 on OAT3 phosphorylation*

As protein kinases regulate their substrate proteins, by phosphorylating these substrates, IGF-1 may regulate OAT3 through PKA-dependent OAT3 phosphorylation. We therefore examined OAT3 phosphorylation in IGF-1-treated cells (Fig. 4). OAT3 was immunoprecipitated (IP) with anti-myc antibody affinity gel (OAT3 was tagged with epitope myc for immunodetection) or with control IgG−agarose (as negative control), followed by immunoblotting with anti-phospho-Ser/Thr antibody. As shown in Fig. 4A, top panel, phosphorylated OAT3 was only detected in sample that was immunoprecipitated with anti-myc antibody but not in sample that was immunoprecipitated with negative control IgG, demonstrating the specificity of the phosphorylation band for OAT3. In Fig. 4B, we showed that IGF-1 significantly enhanced OAT3 phosphorylation as compared to that in control cells, and such enhancement of OAT3 phosphorylation was blocked by PKA inhibitor H89, once again indicating that IGF-1 modulates OAT3 through PKA signaling pathway.

3.4. *Effect of PKA activator Bt2-cAMP on OAT3 transport activity and phosphorylation*

To confirm the direct involvement of PKA in OAT3 function and phosphorylation, we treated OAT3-expressing cells with PKA activator Bt2-cAMP, followed by the measurement of OAT3-mediated uptake of [^{3}H]-estrone sulfate (ES) and OAT3 phosphorylation. Our results revealed that OAT3 transport activity (Fig. 5A) and phosphorylation (Fig. 5B, top panel) was much more enhanced in Bt2-cAMP-treated cells as compared to that in control cells. The change in OAT3 phosphorylation (Fig. 5B, top panel) was not due to the difference in the amount of OAT3

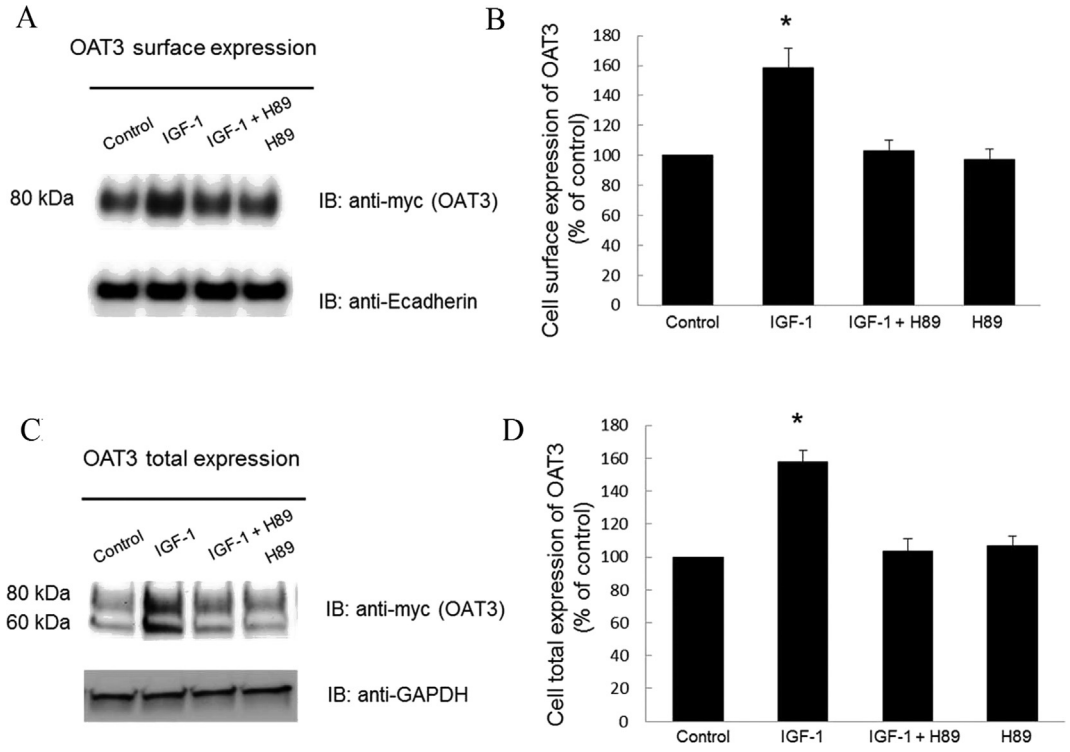

Figure 3 IGF-1 regulates OAT3 expression through PKA pathway. (A) Top panel: OAT3-expressing cells were treated with IGF-1 (100 nmol/L) with or without PKA inhibitor H89 (10 μmol/L) for 4 h. Cells were labeled with membrane impermeable biotin. Biotinylated cell surface proteins were separated with streptavidin beads, followed by immunoblotting (IB) with anti-myc antibody (OAT3 was tagged with epitope myc for immunodetection). Bottom panel: The identical blot of the top panel was re-probed with anti-E-cadherin antibody. E-cadherin is a marker for cell membrane proteins. (B) Densitometry analysis of blot results from Fig. 3A top panel as well as from other experiments. The values are mean ± SD ($n = 3$); $^{*}P < 0.05$. (C). Top panel: OAT3-expressing cells were treated with IGF-1 (100 nmol/L) with or without H89 (10 μmol/L) for 4 h. The cells were collected and lysed, followed by immunoblotting with anti-myc antibody. Bottom panel: The identical blot of the top panel was re-probed with anti-GAPDH antibody. GAPDH is a marker for total cell proteins. (D). Densitometry analyses of blot results from Fig. 3C top panel as well as from other experiments. The values are mean ± SD ($n = 3$); $^{*}P < 0.05$. Statistical analysis was performed using one-way ANOVA by using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

immunoprecipitated because similar amount of OAT3 was pulled down in all samples (Fig. 5B, bottom panel).

3.5. *Effect of linsitinib on OAT3 transport activity*

Linsitinib is an anti-cancer drug and an inhibitor for IGF-1 receptor, which has been investigated in a phase III clinical trial. We treated OAT3-expressing cells with IGF-1 in the presence and absence of linsitinib, then measured OAT3-mediated uptake of [^{3}H]-estrone sulfate (ES). Our results (Fig. 6) showed that IGF-1 significantly stimulated OAT3 transport activity, and such stimulation was blocked by linsitinib in a dose-dependent manner, suggesting that IGF-1 and linsitinib have antagonistic roles in the regulation of OAT3 transport activity.

4. Discussion

Active transport of organic anion carried out by organic anion transporters (OATs) is a major determining factor of the outcomes of therapeutic and toxic chemicals[1–6]. Thus, it is of clinical and pharmacological importance to understand the mechanisms governing OAT regulation. Our current study investigated the regulatory mechanism of OAT3 by IGF-1 and revealed that this hormone modulates OAT3 expression and transport activity possibly through PKA-dependent phosphorylation of the transporter.

Our current work was conducted in COS-7 cells, an excellent cell model for investigating organic anion transporters and other renal transporters[31,35–38]. COS-7 cells are derived from kidney tissue of the African green monkey. Many features of OATs in COS-7 cells are consistent with those observed in other systems such as animal models[39]. Therefore, studies conducted in COS-7 cells will pave the way for future work exploring the mechanisms in native epithelia.

Protein kinases exert their roles through phosphorylating their substrate proteins. Introducing a negatively charged phosphate group to a substrate protein (phosphorylation) may change the three-dimensional conformation of the substrate, its activity, cellular distribution, stability or the interaction of the substrate protein with its interacting partner. Many membrane proteins such as organic cation transporter type 1, glucose transporter GLUT1 and dopamine transporter are regulated by PKC-dependent phosphorylation[40–42]. Previous studies from our lab demonstrated that OAT transport activity is subjected to the down-regulation by several physiological stimuli through the activation of PKC[13–15]. Among those physiological stimuli are angiotensin II, peptide hormone bradykinin, and progesterone. We established that activation PKC accelerates the rate of OAT internalization from cell

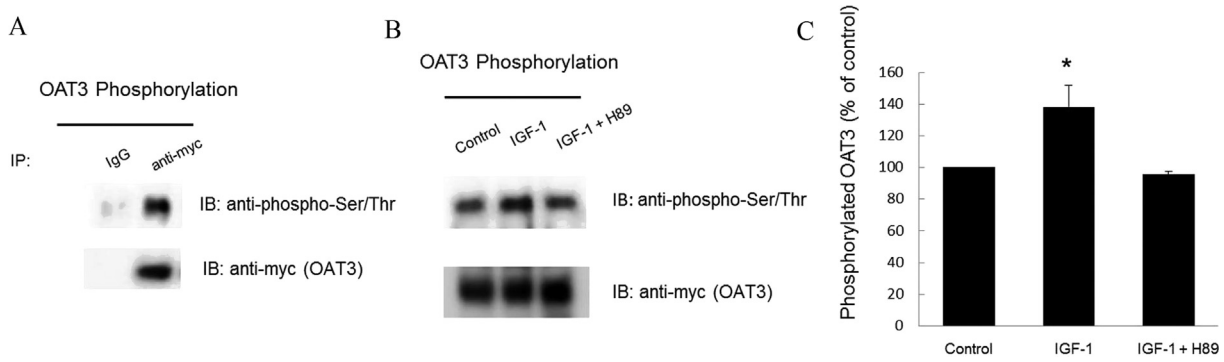

Figure 4 IGF-1 regulates OAT3 phosphorylation through PKA pathway. (A) Top panel: OAT3-expressing cells were lysed, pre-cleared and immunoprecipitated with control IgG-agarose (as negative control) or anti-myc agarose affinity gel, followed by immunoblotting (IB) with anti-phospho-Ser/Thr antibody. Bottom panel: The identical blot of the top panel was re-probed with anti-myc antibody to determine the amount of OAT3 immunoprecipitated. (B) Phosphorylation of OAT3. Top panel: OAT3-expressing cells were treated with IGF-1 (100 nmol/L) with or without PKA inhibitor H89 (10 μmol/L) for 4 h. After treatment, cells were lysed, pre-cleared and immunoprecipitated with anti-myc agarose affinity gel, followed by immunoblotting (IB) with anti-phospho-Ser/Thr antibody. Bottom panel: The identical blot of the top panel was re-probed with anti-myc antibody to determine the amount of OAT3 immunoprecipitated. (C) Densitometry analysis of blot results from Fig. 4B, top panel as well as from other experiments. The values are mean ± SD ($n = 3$); $^{*}P < 0.05$. Statistical analysis was performed using one-way ANOVA by using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

surface to intracellular endosomes and the rate of OAT degradation, a process catalyzed by an ubiquitin ligase Nedd4-2[31,35,43].

Interestingly through the course of our investigation, we demonstrated that activation of PKC down-regulates OAT expression and transport activity without directly phosphorylating the transporters. Site-directed mutagenesis of potential PKC sites by Wolff et al.[44] further confirmed our observation. In contrast to peptide hormone bradykinin/PKC and angiotensin II/PKC pathways, our interesting results in the current study showed that IGF-1/PKA pathway did involve direct phosphorylation of OAT3. We showed that both IGF-1 and activation of PKA induced significant increase in OAT3 phosphorylation (Figs. 4 and 5), which correlated well with an increase in OAT3 expression and transport activity (Figs. 1 and 5). Adding a phosphate group on a target protein may change protein's structural properties, its stability and dynamics[45–47]. Furthermore, the cross-talk among various post-translational modifications occurs when the phosphorylation modification on the target protein influences other modifications including SUMOylation and ubiquitination. It has been reported that phosphorylation of the protein could stimulate or inhibit its SUMOylation[48–51]. In addition, the interplay between phosphorylation and ubiquitination has also been reported[46,47,52]. Recently, we demonstrated that activation of PKA stimulates SUMOylation and inhibits ubiquitination of OAT3[23], and ubiquitination of OAT3 leads to the internalization of OAT3 from cell surface to intracellular compartment and subsequent degradation. Therefore, it would be an interesting future direction for us to explore whether OAT3 phosphorylation promotes OAT3 SUMOylation, which in

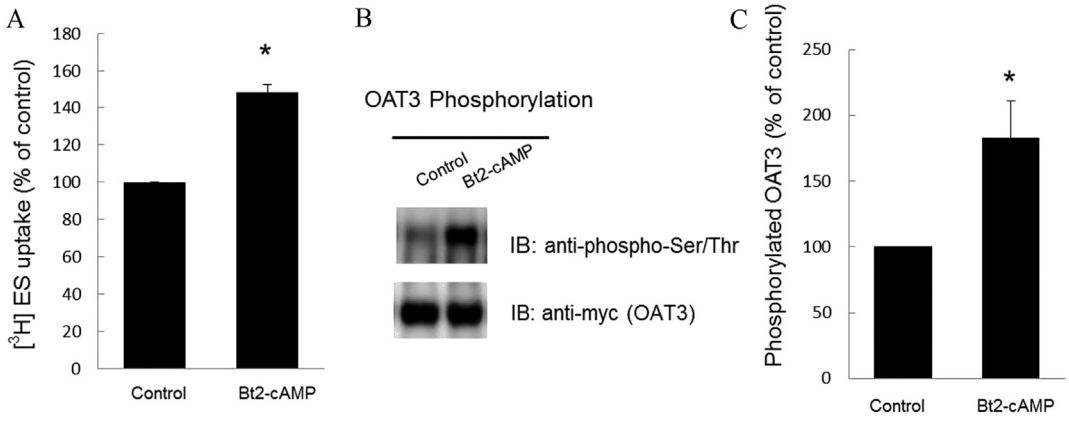

Figure 5 PKA activator Bt2-cAMP up-regulates OAT3 transport function and phosphorylation. (A) OAT3-expressing cells were treated with Bt2-cAMP (5 nmol/L) for 2 h. 4-min uptake of [^{3}H]-estrone sulfate (ES, 300 nmol/L) was then measured. Transport activity was expressed as percentage of the uptake in control cells. The data corresponded to the uptake of OAT3-expressing cells minus uptake of untreated parental cells. Values are mean ± SD ($n = 3$); $^{*}P < 0.05$. (B) Phosphorylation of OAT3. Top panel: OAT3-expressing cells were treated with Bt2-cAMP (5 nmol/L) for 2 h. After treatment, cells were lysed, pre-cleared and immunoprecipitated with anti-myc agarose affinity gel, followed by immunoblotting (IB) with anti-phospho-Ser/Thr antibody. Bottom panel: The identical blot of the top panel was re-probed with anti-myc antibody to determine the amount of OAT3 immunoprecipitated. (C) Densitometry analysis of blot results from Fig. 5B top panel as well as from other experiments. The values are mean ± SD ($n = 3$); $^{*}P < 0.05$. Statistical analysis was performed using Student's paired t-tests.

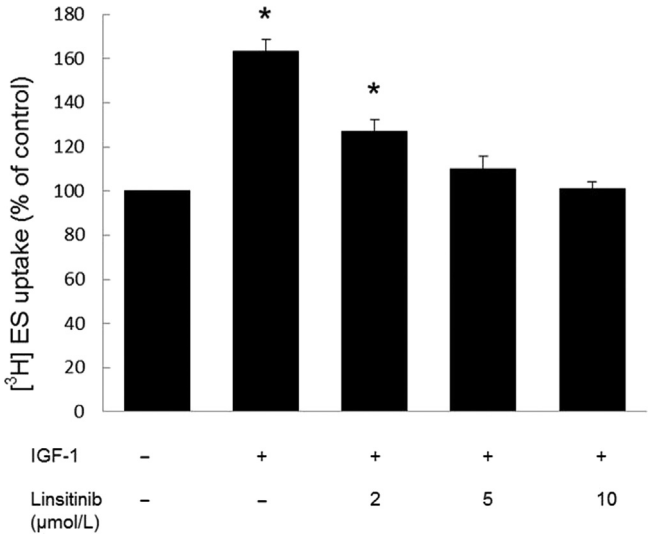

Figure 6 Linsitinib blocked IGF-1-stimulated OAT3 transport activity. OAT3-expressing cells were treated with IGF-1 (100 nmol/L) and with various concentrations of linsitinib for 4 h. 4-min uptake of [^{3}H]-estrone sulfate (ES, 300 nmol/L) was then measured. Transport activity was expressed as percentage of the uptake in control cells. The data corresponded to the uptake of OAT3-expressing cells minus uptake of untreated parental cells. Values are mean ± SD ($n = 3$); $^*P < 0.05$. Statistical analysis was performed using one-way ANOVA by using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

turn, suppresses OAT3 ubiquitination. As a result, the ubiquitination-dependent degradation is decreased and the stability of OAT3 is enhanced. Furthermore, the phosphorylation of OAT3 may not only increase its protein level but may also change its three-dimensional conformation, which leads to the change of the binding affinity of the transporter to its substrates.

Most protein substrates for PKA bear the consensus motif, R/K-R/K-X-S/T-ψ (where R is arginine, K is lysine, X is any amino acid, S is serine, T is threonine and ψ is a hydrophobic residue[53]. Using program NetPhos3.1 to predict phosphorylation sites, we identified several intracellular locations that bear the sequences for PKA phosphorylation. On the other hand, PKA phosphorylation can also occur at residues outside conventional motifs/sequences and the presence of conventional motifs does not guarantee the phosphorylation. In addition, it could not be ruled out that PKA may activate some other protein kinases that possibly phosphorylate OAT3. For example, PKB can be activated by PKA through a PI3-kinase-independent pathway[54,55] and AMP-activated protein kinase (AMPK) also can be a substrate activated by PKA[56]. The mapping of PKA-dependent phosphorylation sites on OAT3 is currently underway in our laboratory.

IGF-1 binds to its cell surface receptor to initiate the cell signaling. The inabilities to produce or to respond to IGF-1 cause several diseases and symptoms. For example, patients with Laron dwarfism showed severely low levels of IGF-1 caused by the lack of IGF-1 synthesis. The homozygous mutation of the IGF1 receptor is related with intrauterine growth retardation, insulin resistance and dysmorphism[57]. And the heterozygous mutation of IGF-1 receptor is also associated with intrauterine and postnatal growth retardation[58]. In addition, the heterozygous mutation of IGF-1 receptor was indicated to increase resistance to oxidative stress and lifespan in mice[59].

IGF-1 signaling pathway is essential for cell growth, proliferation and survival and also plays an important role in development and sustainability of malignant tumors including breast cancer, sarcoma and lung cancer[60–64]. Thus manipulation of IGF-1 signaling system is a very promising strategy for novel anticancer therapeutics and IGF-1 receptor blockade has been identified as the target for cancer treatment[65–68]. Numerous IGF-1 receptor inhibitors are at different stages of clinic trials and they can be classified into three groups including monoclonal antibody, ligand inhibitors and tyrosine kinase inhibitors. Linsitinib is a selective and potent IGF-1 receptor inhibitor and has been investigated in a phase III clinical trial for adrenocortical carcinoma[69]. Furthermore, the linsitinib clinical trials in different phases are initiated for multiple cancers including ovarian cancer, myeloma and prostate cancer[70–72]. In our studies, linsitinib significantly blocked stimulatory effect of IGF-1 on OAT3 transport activity in a dose dependent manner. Therefore, OAT transport activity should be taken into consideration in cancer patients treated with linsitinib.

Our discovery about the IGF-1 modulation of OAT3 *via* PKA signaling supports a remote sensing and signaling model for

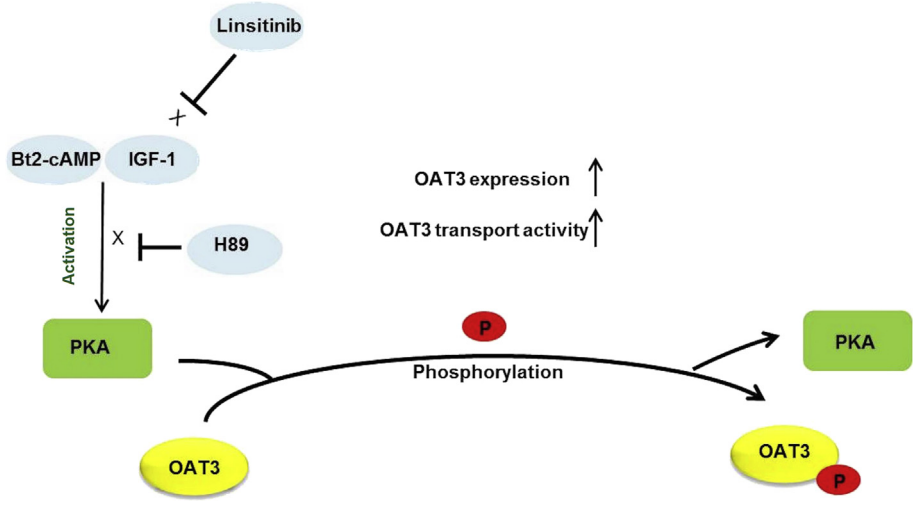

Figure 7 IGF-1/PKA signaling pathway regulates OAT3 phosphorylation, expression and transport activity. P: phosphate group.

transporters[5,9,73]. In such model, transporters in different tissues form networks and are regulated by hormones and growth factors, thereby efficiently talk among one another. In doing so, these transporters coordinately keep the balance among multiple organs and thus system homeostasis. Hormones/growth factors, produced from one organ under the influence of stimuli/environmental alterations, get into the blood stream, and then arrive at the target organs and apply their regulatory roles on transporters *via* cell signaling. Aligning with this model, our results support that IGF-1, which is formed mostly by the liver under stimuli, arrives at the kidney *via* blood stream, and then binds to its receptors and stimulates OAT3 expression and activity through PKA signaling (Fig. 7).

5. Conclusions

Our study demonstrated that IGF-1 regulates OAT3 expression and transport activity through PKA signaling pathway, possibly by phosphorylating the transporter.

Acknowledgments

This work was supported by grants (to Dr. Guofeng You) from National Institute of General Medical Sciences (R01-GM079123 and R01-GM097000, USA).

Author Contributions

Jinghui Zhang: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. Zhou Yu: Validation, Formal analysis, Investigation, Writing - Review & Editing, Visualization. Guofeng You: Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Funding acquisition.

Conflicts of Interest

The authors declare no conflicts of interest.

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