HHS Public Access

Author manuscript

J Pharmacol Sci. Author manuscript; available in PMC 2021 February 05.

Published in final edited form as:

J Pharmacol Sci. 2018 February ; 136(2): 79–85. doi:10.1016/j.jphs.2017.12.011.

The Activity of Organic Anion Transporter-3: Role of Dexamethasone

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Abstract

Human organic anion transporter-3 (hOAT3) is richly expressed in the kidney, where it plays critical roles in the secretion, from the blood to urine, of clinically important drugs, such as anti-viral therapeutics, anti-cancer drugs, antibiotics, antihypertensives, and anti-inflammatories. In the current study, we examined the role of dexamethasone in hOAT3 transport activity in the kidney HEK293 cells. Cis-inhibition study showed that dexamethasone exhibited a concentration-dependent inhibition of hOAT3-mediated uptake of estrone sulfate, a prototypical substrate for the transporter, with IC_{50} value of 49.91 μM. Dixon plot analysis revealed that inhibition by dexamethasone was competitive with a Ki = 47.08 μM. In contrast to the cis-inhibition effect of dexamethasone, prolonged incubation (6 hrs.) of hOAT3-expressing cells with dexamethasone resulted in an upregulation of hOAT3 expression and transport activity, kinetically revealed as an increase in the maximum transport velocity V_{max} without meaningful alteration in substrate-binding affinity K_m. Such upregulation was abrogated by GSK650394, a specific inhibitor for serum-and glucocorticoid-inducible kinases (sgk). Dexamethasone also enhanced sgk1 phosphorylation. Our study demonstrated that dexamethasone exhibits dual effects on hOAT3: it is a competitive inhibitor for hOAT3-mediated transport, and interestingly, when entering the cells, it stimulates hOAT3 expression and transport activity through sgk1.

Keywords

Organic Anion Transporter; Drug Transport; Regulation; Dexamethasone; Serum and Glucocorticoid-Inducible Kinase

Introduction

Organic anion transporter-3 (OAT3) belongs to a class of organic anion transporters (OATs) consisting of over 10 membrane proteins. OAT3 is expressed at the basolateral membrane of the renal proximal tubule cells and plays a critical role in the secretion of many clinical drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, antihypertension drugs, and anti-inflammatories [1–6].

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CONFLICT OF INTEREST

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

We previously established that OATs are subjected to the regulation by the serum- and glucocorticoid-inducible kinases (sgk) [7–9]. Sgk consists of three serine/threonine kinase isoforms sgk1, sgk2 and sgk3. These kinases regulate many cellular processes such as Na^+ balance, osmoregulation, cell survival, and proliferation [10–16]. They also play important roles in oncology, diabetes, and obesity [17]. Sgk1 and sgk3 are expressed ubiquitously, whereas sgk2 is restricted to tissues such as liver, kidney, pancreas, and brain. We previously demonstrated that sgk1 and sgk2 stimulate OAT activity by weakening the association of OAT with a ubiquitin ligase Nedd4–2, which leads to a deceleration of ubiquitin-dependent OAT internalization from the plasma membrane to intracellular compartments. As a result, the amount of OAT at the cell surface is enhanced and OAT transport activity is increased [7, 8].

Glucocorticoids are hormones that regulate numerous physiological activities related with metabolic, cardiovascular, and inflammatory processes [18]. Excess of glucocorticoids contribute to obesity, hyperlipidemia, hypertension, and glucose intolerance [19]. Glucocorticoids have been used for the treatment of diarrhea related to inflammatory bowel diseases and nontropical sprue [20]. Several studies have shown that one of the signaling molecule downstream glucocorticoids is sgk [21–23]. In the current study, we investigated the role of dexamethasone, a synthetic glucocorticoid, in OAT3 expression and transport activity.

Materials and Methods

Materials -

The HEK293 cells were purchased from ATCC (Manassas, VA). [^{3}H]-labeled estrone sulfate ([^{3}H]-ES) was purchased from PerkinElmer (Waltham, MA). cDNA for mouse Flag-tagged sgk1 was generously provided by Dr. Alan C. Pao from the Department of Medicine, Stanford University, (Stanford, CA). Dexamethasone and all other reagents were purchased from Sigma-Aldrich (St Louis, MO).

Cell culture and transient transfection -

Parental HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Corning, Corning, NY) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) at 37°C in 5% CO_2. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfection of cDNAs following the manufacturer's instructions. 48 h after transfection, the cells were used for further experiments. Cells stably expressing hOAT3 were maintained in DMEM medium supplemented with 0.2 mg/ml G418 (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum.

Transport measurement -

The transport activity of hOAT3 was determined by measuring [^{3}H]-ES uptake into hOAT3-expressing cells. The uptake solution consists of phosphate-buffered saline (PBS) with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4) and [^{3}H]-ES (300 nM). At the time points indicated, uptake was terminated by removing the uptake solution followed by

washing with ice-cold PBS twice. The cells were then lysed in 0.2 N NaOH, neutralized with 0.2 N HCl and transferred into scintillation vials for liquid scintillation counting.

Cell surface biotinylation -

The expression level of hOAT3 at the cell surface was examined by using a biotinylation strategy. The cells in monolayer culture were washed with ice-cold PBS and then incubated with 1 ml of NHS-SS-biotin (0.5 mg/ ml in PBS/CM) on ice for two consecutive 20 min periods under gentle shaking. Biotinylation was stopped by rinsing with 100 mM glycine in PBS/CM. Afterwards, the cell extracts were prepared in lysis buffer (10 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 with 1/100 protease inhibitor cocktail) for 30 min at 4°C and cleared by centrifugation at 16,000×g at 4°C. The supernatant was mixed with streptavidin-agarose beads (Pierce, Rockford, IL) to isolate cell surface proteins. Membrane-expressed hOAT3 was detected by SDS-PAGE and immunoblotting with an anti-myc antibody (epitope myc was tagged to hOAT3).

Electrophoresis and immunoblotting -

The protein samples were separated on 7.5% SDS-PAGE minigels (Bio-Rad, Hercules, CA) and electroblotted on to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The blots were blocked with 5% nonfat dry milk for 1–2 h in PBS-Tween 20 (PBST; 0.05% Tween-20 in PBS) at room temperature, washed and incubated overnight at 4°C with appropriate primary antibodies. The primary antibodies included mouse anti-E cadherin (Abcam, Cambridge, MA), rabbit anti-sgk1 (Cell Signaling, Danvers, MA), mouse anti-myc (Roche, Indianapolis, IN), mouse anti-β-actin, and mouse anti-P-sgk1 (S422) (Santa Cruz, Santa Cruz, CA). The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies, followed by detection with a SuperSignal West Dura Extended Duration Substrate kit (Pierce, Rockford, IL). The FluorChem 8000 imaging system (Alpha Innotech Corp., San Leandro, CA) was applied to quantify the nonsaturating, immunoreactive protein bands.

Concentration-dependent inhibition studies -

Inhibition studies were performed at varying concentrations of Dexamethasone. hOAT3 specific uptake was obtained by subtracting [^{3}H]-ES uptake into parental cells from the uptake into hOAT3-expressing cells. The IC_{50} (concentration of the drugs required to inhibit 50% of ES uptake) was determined by nonlinear regression using GraphPad Prism.

Dixon plot -

The mechanism of inhibition was determined by linear regression analysis of reciprocal saturable uptake (1/v) for different substrate concentrations (1.2 μM or 2.4 μM ES) as a function of inhibitor concentration. hOAT3 uptake was determined at 4 min in both the absence and presence of varying concentrations of dexamethasone. The specific uptake was obtained by subtracting [^{3}H]-ES uptake into parental cells from the uptake into hOAT3-expressing cells. The data were analyzed by linear regression with GraphPad Prism. Inhibition constant Ki was obtained by fitting the data with eq. 1 [24–26], where C is the concentration of substrates (μM) and K_m is the Michaelis constant (μM). An IC_{50} is a

relative value, which depends on the concentrations of the transporter, substrate and inhibitor with other experimental conditions used in the assay, while K_i is an intrinsic, thermodynamic quantity that is independent of the substrate but depends on the transporter and inhibitor. For competitive inhibitors, the relationship between IC_{50} and K_i is stated in equation below.

$$K_i = \frac{IC_{50}}{1 + {C}/{K_m}} \quad (1)$$

Data analysis -

Each experiment was repeated at least three times. Student's paired t-tests were used to perform statistical analysis. A value of $p < 0.05$ was considered significant.

Results

Cis-inhibition of hOAT3-mediated estrone sulfate (ES) uptake by dexamethasone -

Cis-inhibition studies were performed in hOAT3-expressing HEK293 cells. The 4-min uptake of [^{3}H]-ES (300 nM) in the presence of dexamethasone (100 μM) or probenecid (100 μM) were measured. Probenecid is a known competitive inhibitor for OATs. As shown in Fig. 1, dexamethasone exhibited ~80% inhibition of ES uptake, similar to the inhibitory potency of probenecid.

Dose-dependent effects of dexamethaosne on hOAT3-mediated transport -

Dose response curve was then constructed to evaluate the effectiveness of dexamethasone as an inhibitor of hOAT3 uptake. 4-min uptake of [^{3}H]-ES (300 nM) was measured in the presence of 0.1–1000 μM dexamethasone. Our result showed that dexamethasone inhibited hOAT3-mediated ES uptake in a concentration-dependent manner with IC_{50} values of 49.91 μM (Fig. 2). IC_{50} value is the concentrations at which 50% inhibition is achieved.

Dixon plot analysis -

To further characterize the mechanism of inhibition and to determine the K_i values (inhibition constant), uptake in the presence and absence of dexamethasone was analyzed via Dixon plot (Fig. 3). Dexamethasone demonstrated a competitive mechanism of inhibition of [^{3}H]-ES uptake by hOAT3 (as the lines for substrate concentrations converge above the x axis). We then determined the inhibition constant K_i by fitting the data to a competitive inhibition model, as described by eq. 1. The K_m values of OAT3 for ES was 5μM, the inhibition constant (K_i) of dexamethasone was then estimated as 47.08 μM.

Long-term treatment of hOAT3-expressing cells with dexamethasone stimulates hOAT3 transport activity –

The above studies (Figs. 1–3) were designed by measuring the 4-min uptake of [^{3}H]-ES (300 nM) in the presence of dexamethasone (100 μM). However, the long-term effect of dexamethasone on hOAT3 is not known. In the current experiment, we pretreated hOAT3-

expressing cells with dexamethasone for 0 – 6 hrs., followed by measuring [^{3}H]-ES uptake. Our results showed that long-term treatment of hOAT3-expressing cells with dexamethasone resulted in a stimulation of hOAT3-mediated [^{3}H]-ES uptake with 40% stimulation after 6 hrs. pretreatment (Fig. 4a). Dexamethasone also induced a dose-dependent stimulation of hOAT3 mediated transport with 6 hrs. pretreatment (Fig. 4b). To examine the mechanism of dexamethasone-induced stimulation of hOAT3 activity, we determined hOAT3-mediated [^{3}H]-ES uptake at different substrate (ES) concentrations. An Eadie-Hofstee analysis of the derived data (Fig. 5) showed that treatment of hOAT3-expressing cells with dexamethasone resulted in an increased maximal transport velocity V_{max} of hOAT3 (330 ± 24 pmol·mg^{-1}·4min^{-1} with control cells and 387 ± 29 pmol·mg^{-1}·4min^{-1} with cells transfected with dexamethasone) with no significant change in the substrate-binding affinity K_m of the transporter (5.07 ± 0.49 μM with control cells and 5.08 ± 0.51 μM with cells transfected with dexamethasone).

Effect of dexamethasone on hOAT3 expression –

An increased Vmax (Fig. 5) could be a result from either an increased number of the transporter at the cell surface or an increased transporter turnover number. We conducted experiments that differentiate between these possibilities by measuring transporter expression both at the cell surface and in the total cell lysates. We showed that dexamethasone treatment resulted in an increased cell surface expression of hOAT3 without affecting the total cell expression of the transporter (Fig. 6).

Sgk inhibitor GSK650394 abrogates the stimulatory effect of dexamethasone on hOAT3 transport activity –

Several studies have shown that one of the signaling molecule downstream glucocorticoids is the serum- and glucocorticoid-inducible kinases (sgk). In this experiment, we examined whether sgk mediates the effect of dexamethasone on hOAT3. We treated hOAT3-expressing cells with dexamethasone with or without a sgk-selective inhibitor GSK650394, followed by the measurement of hOAT3-mediated uptake of [^{3}H]-ES. As shown in Fig. 7, dexamethasone stimulated hOAT3-mediated transport ~40% in control cells, whereas such stimulation was blocked in the presence of GSK650394.

Dexamethsaone enhanced sgk1 phosphorylation –

Previous investigations [23, 27, 28] reported that activation of sgk1 is dependent upon the phosphorylation of this kinase, at least in part, by phosphorylating sgk1 at serine residue 422 (Ser 422). Thus, we examined the effect of dexamethasone on the phosphorylation levels of sgk1. hOAT3-expressing cells were pretreated with dexamethasone for 6 hrs. (10nM). Treated cells were then lysed, followed by immunoblotting (IB) with anti-phospho-sgk1-specific antibody. Our results showed that dexamethasone significantly enhanced the level of sgk1 phosphorylation as compared to that of the control (Fig. 8a) without change in the total expression of sgk1 (Fig. 8c). The expression level of cellular protein marker β-actin was also not changed.

Discussion

Organic anion transporters (OATs) are critical players in the therapeutic efficacy and toxicity of many drugs. Therefore, uncovering how OATs are regulated at the molecular and cellular levels is clinically and pharmacologically significant. The current investigation revealed that dexamethasone, a synthetic glucocorticoid, has a significant role in modulating hOAT3 expression and transport activity.

Glucocorticoids are known to affect a variety of renal functions. Glucocorticoid excess can lead to metabolic alkalosis and potassium depletion [29]. In addition, glucocorticoids such as dexamethsone have been shown to specifically regulate, through glucocorticoid receptors, a set of transporters/exchangers (e.g. Na^+:HCO_3^- cotransporter, Na^+/H^+ exchanger) in renal proximal tubules, where OATs are expressed [22, 30, 31].

We chose to perform our investigation in human kidney HEK293 cells, a widely-used cell model for answering mechanistic questions of many renal transport processes [32–35]. Therefore, our studies in these cells will provide insights into the future investigation in evaluating whether similar regulation is working *in vivo*.

From our investigation, we gained several pieces of valuable information. Dexamethasone has dual effects on hOAT3 transport activity. First, when co-present with hOAT3 substrate for a brief period of time (4 min), dexamethasone acts as an inhibitor for hOAT3-mediated transport (Fig. 1). Further characterization of the inhibition mechanism by Dixon plot revealed that the dexamethasone is a competitive inhibitor (Fig. 3). Such mode of inhibition is that the binding of the inhibitor to the active site on the transporter excludes the binding of the substrate and *vice versa.* The IC_{50} value of dexamethasone for hOAT3 determined in the present study is 49.91 μM (Fig. 2). This is the concentrations at which 50% inhibition was achieved. The maximum plasma concentration (C_{max}) of dexamethasone is 0.16 μM (64.4μg/L), as suggested by spoorenberg et al. [36]. Corrected by unbound fraction value of 0.272 [37], the unbound maximum plasma concentration ($C_{u,max}$) of dexamethasone is ~0.045 μM. A $C_{u,max}/IC_{50}$ value greater than 0.1 would indicate a potential for drug-drug interaction [38]. The $C_{u,max}/IC_{50}$ value of dexamethasone for hOAT3 was < 0.1. Therefore, the propensity for dexamethasone to cause drug-drug interaction through inhibition of hOAT3 is low.

Interestingly, long-term treatment of hOAT3-expressing cells at the clinically relevant concentration of dexamethasone (10nM), displayed an opposite effect of dexamethasone on hOAT3 transport activity. Instead of an inhibitory role, pretreatment of hOAT3-expressing cells with dexamethasone for 6 hrs. resulted in a 40% stimulation of hOAT3 activity (Fig. 4). The stimulation of hOAT3 transport activity by dexamethasone correlated with an enhanced hOAT3 expression at the cell surface. One of the signaling pathways downstream of dexamethasone is the serum- and glucocorticoid-inducible kinases (sgk) pathway. Our lab previously demonstrated that sgk stimulates OAT expression and activity by weakening the interaction of OAT with a ubiquitin ligase Nedd4–2 and therefore preventing Nedd4–2-dependent OAT internalization from the cell surface [7–9]. To examine the role of sgk in the effect of dexamethasone on hOAT3, we pretreated hOAT3-expressing cells with

dexamethasone in the presence or absence of sgk-specific inhibitor GSK650394. We showed that GSK650394 abrogated stimulatory effect of dexamethasone on hOAT3 transport activity, suggesting that dexamethasone exerts its effect on hOAT3 through the activation of sgk (Fig. 7). This conclusion was further reinforced by our results showing that dexamethasone enhanced sgk phosphorylation (Fig. 8). Phosphorylation of sgk was previously reported to be a prerequisite for the activation of this kinase [23, 28]. Based on previously published work, dexamethasone diffuses passively into cells [39], stimulates PI3K signaling pathway through binding to glucocorticoid receptor [22], which leads to the phosphorylation of SGK1 and enhancement of SGK1 activity.

Dexamethasone is a synthetic glucocorticoid. Because of its potent anti-inflammatory and immunosuppressant effect, it has been widely used to treat inflammatory and autoimmune conditions, such as rheumatic problems, and severe allergies [40]. What is more, it is often used with chemotherapy in cancer patient to counteract certain side effects of their antitumor treatments [41]. Our *in vitro* study showed that at a clinical relevant concentration (10nM), long-term treatment with dexamethasone stimulated hOAT3 transport activity, suggesting that hOAT3-mediated drug elimination might be affected if a hOAT3 substrate/drug is taken with dexamethasone simultaneously. The *in vivo* study aiming at identifying the effect of dexamethasone on hOAT3 activity is currently being carried out in our lab.

In summary, current study uncovered dual roles of dexamethasone in hOAT3 transport activity: dexamethasone can act as a competitive inhibitor for hOAT3-mediated transport. Interestingly, once entering the cells, dexamethasone activates sgk1, which leads to an enhanced hOAT3 expression at the cell surface and an enhanced hOAT3 transport activity.

ACKNOWLEDGMENT

This work was supported by grant (to Dr. Guofeng You) from National Institute of General Medical Sciences (R01-GM079123).

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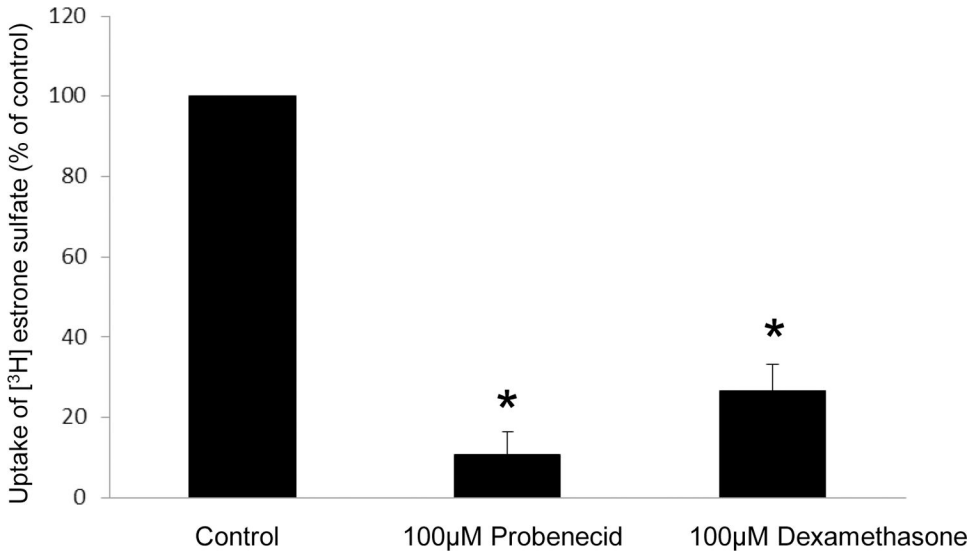

Fig. 1. Cis-inhibition of hOAT3-mediated [^{3}H]-ES uptake by dexamethasone.

4-min uptake of 300 nM [^{3}H]-ES in the presence of dexamethasone (100 μM) or probenecid (100 μM) was measured. Each data point represented only carrier-mediated transport after subtraction of values from parental cells. Uptake activity was expressed as percentage of uptake measured in control cells from three independent experiments. Results shown are means ± S.E. of three separate experiments. *P<0.05

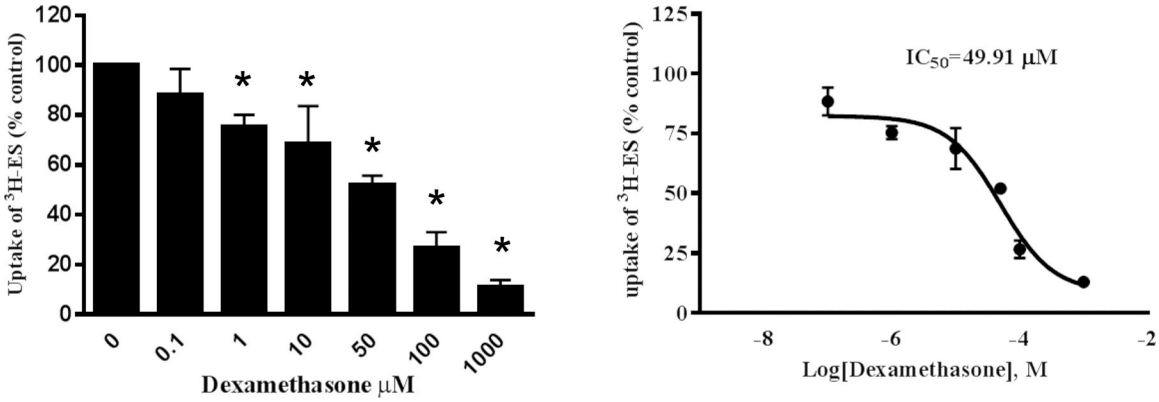

Fig. 2. Concentration dependence of dexamethasone inhibition on hOAT3-mediated uptake.

hOAT3-expressing cells were incubated for 4 min with 300 nM [^{3}H]-ES in the presence of various concentrations of dexamethasone. Each data point represented only carrier-mediated transport after subtraction of values from parental cells. Uptake activity was expressed as percentage of uptake measured in control cells from three independent experiments. Results shown are means ± S.E. of three separate experiments. *P<0.05. The line represents a best fit of data using nonlinear regression analysis.

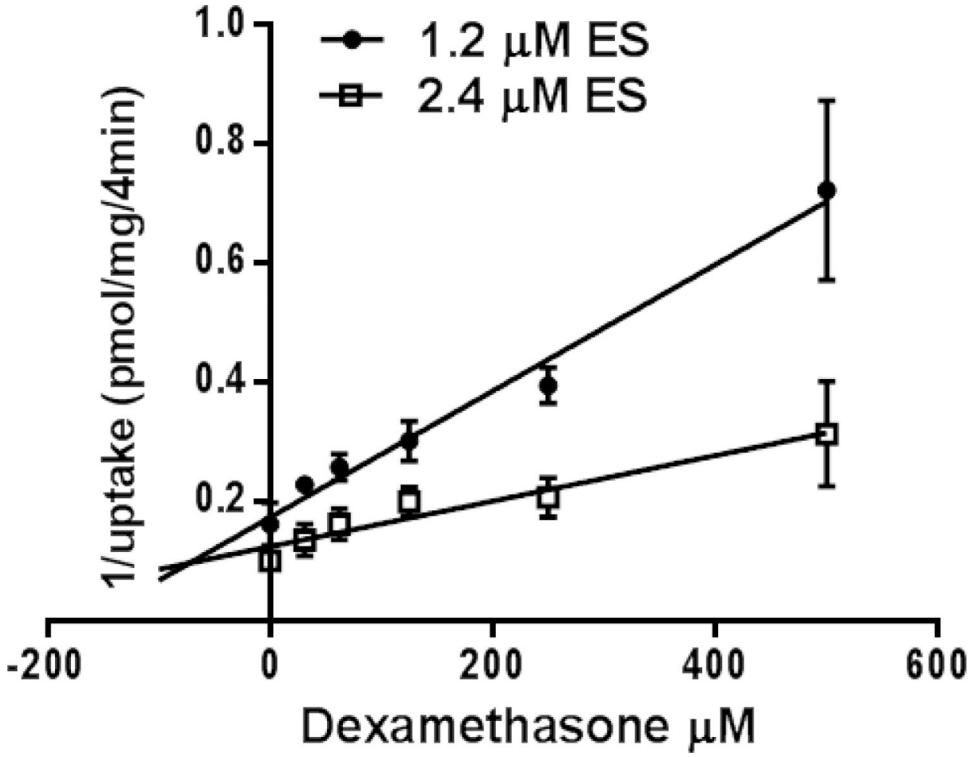

Fig. 3. Dixon plot analysis of the inhibitory effects of dexamethasone on hOAT3-mediated transport.

[^{3}H]-ES uptake (1.2 μM and 2.4 μM) was determined at 4 min in the presence of varying concentrations of dexamethasone. Each data point represented only carrier-mediated transport after subtraction of values from parental cells. Results shown are means ± SE percentage of uptake measured in control cells. The data were fitted by linear regression and K_i was calculated.

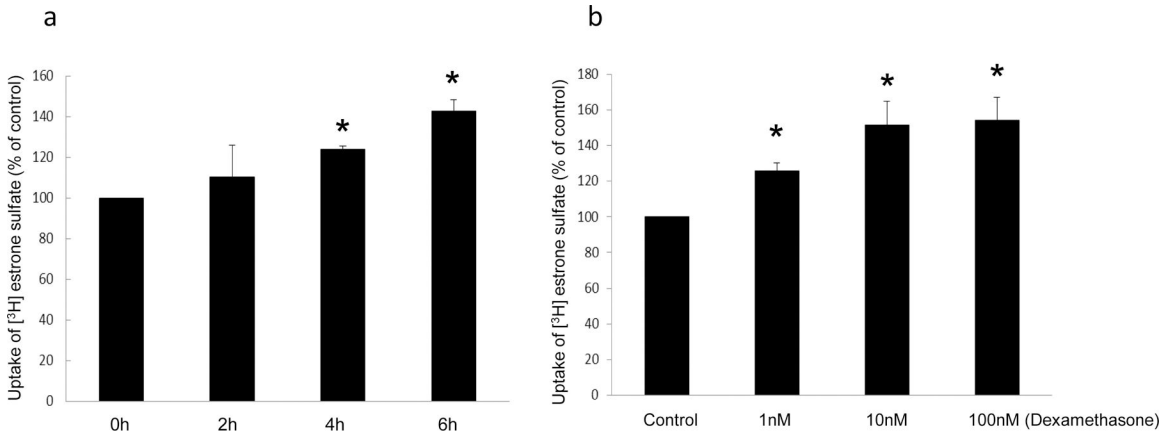

Fig. 4. Long-term treatment of hOAT3-expressing cells with dexamethasone stimulates hOAT3 transport activity.

(a). Time dependence. hOAT3-expressing cells were pretreated with dexamethasone (10nM) for 2, 4 and 6 hrs. 4-min uptake of [^{3}H]-ES (300 nM) was then measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. The data represent uptake into hOAT3-expressing cells minus uptake into mock cells (parental cells). Values are mean ± S.E. (n = 3). *P<0.05. (b). Dose dependence. hOAT3-expressing cells were pretreated for 6 hrs. with dexamethasone at varies doses. 4-min uptake of [^{3}H]-ES (300 nM) was then measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. The data represent uptake into hOAT3-expressing cells minus uptake into control cells (parental cells). Values are mean ± S.E. (n = 3). *P<0.05.

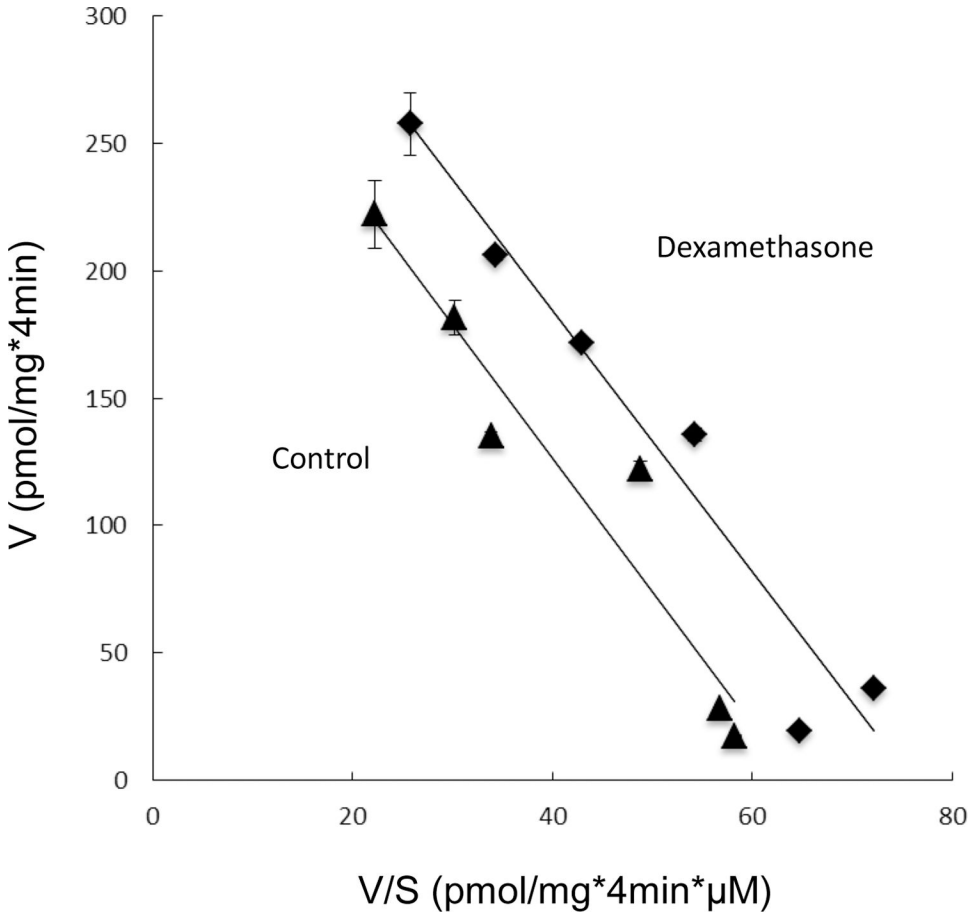

Fig. 5. Kinetic analysis of hOAT3-mediated ES transport.

Kinetic characteristics were determined at substrate concentration ranging from 0.05 to 10 μM (ES, 4-min uptake) after pretreatment of dexamethasone for 6 hrs. (10 nM). The data represent uptake into hOAT3-expressing cells minus uptake into control cells (parental cells). Values are mean ± S.E. (n = 3). Transport kinetic values were calculated using the Eadie–Hofstee transformation.

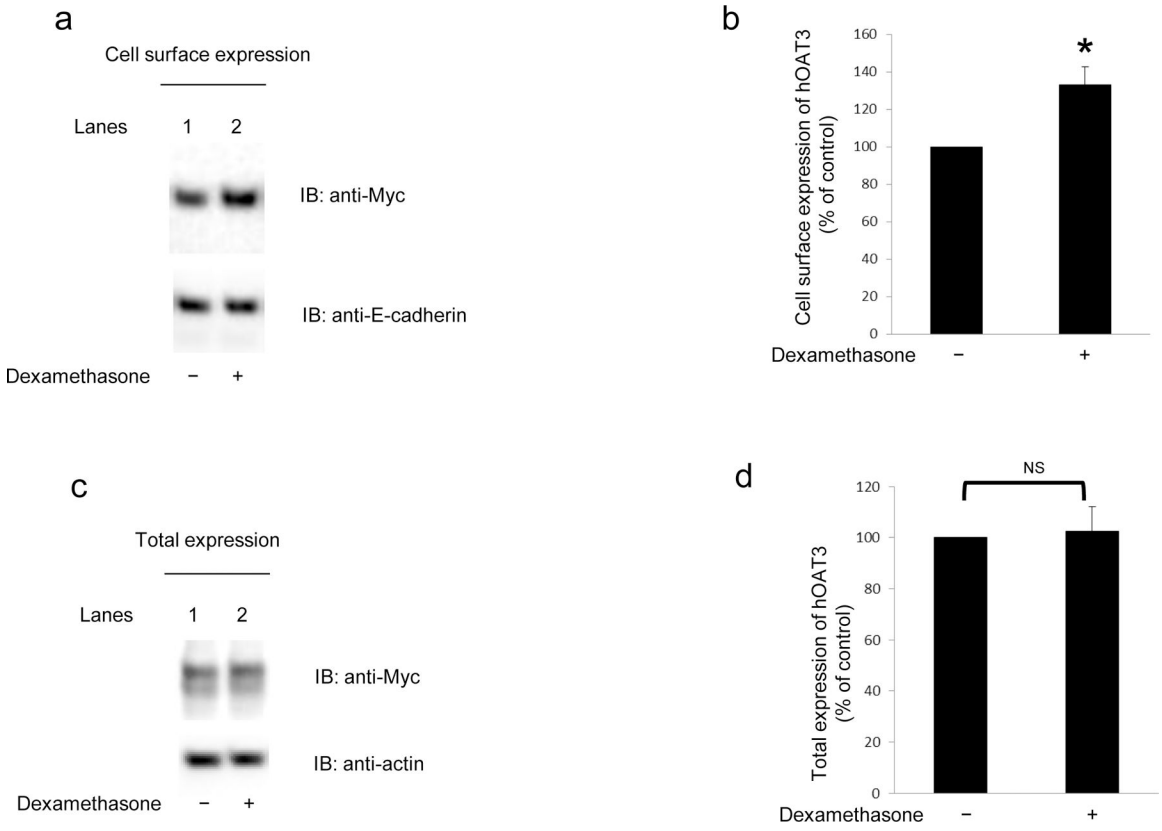

Fig. 6. Effect of dexamethasone on hOAT3 expression.

(a). *Top panel:* Cell surface expression of hOAT3. hOAT3-expressing cells were pretreated with the dexamethasone (10nM, 6 hrs.). Cells were labeled with biotin. Biotinylated cell surface proteins were separated with streptavidin beads, followed by immunoblotting (IB) with an anti-myc antibody (hOAT3 was tagged with epitope myc for immunodetection). *Bottom panel:* The expression of cell surface protein marker E-cadherin. The same blot from the top panel was re-probed with anti- E-cadherin antibody. (b). Densitometry plot of results from Fig. 6a, top panel as well as from other experiments. The values are mean ± S.E. (n = 3). *P<0.05. (c). *Top panel:* Total cell expression of hOAT3. hOAT3-expressing cells were pretreated with the Dexamethasone (10nM, 6 hrs.). Cells were lysed, followed by immunoblotting (IB) with an anti-myc antibody. *Bottom panel:* Total cell expression of cellular protein marker β-actin. The same blot from top panel was re-probed with anti-β-actin antibody. (d). Densitometry plot of results from Fig. 6c, top panel as well as from other experiments. The values are mean ± S.E. (n = 3). *P<0.05.

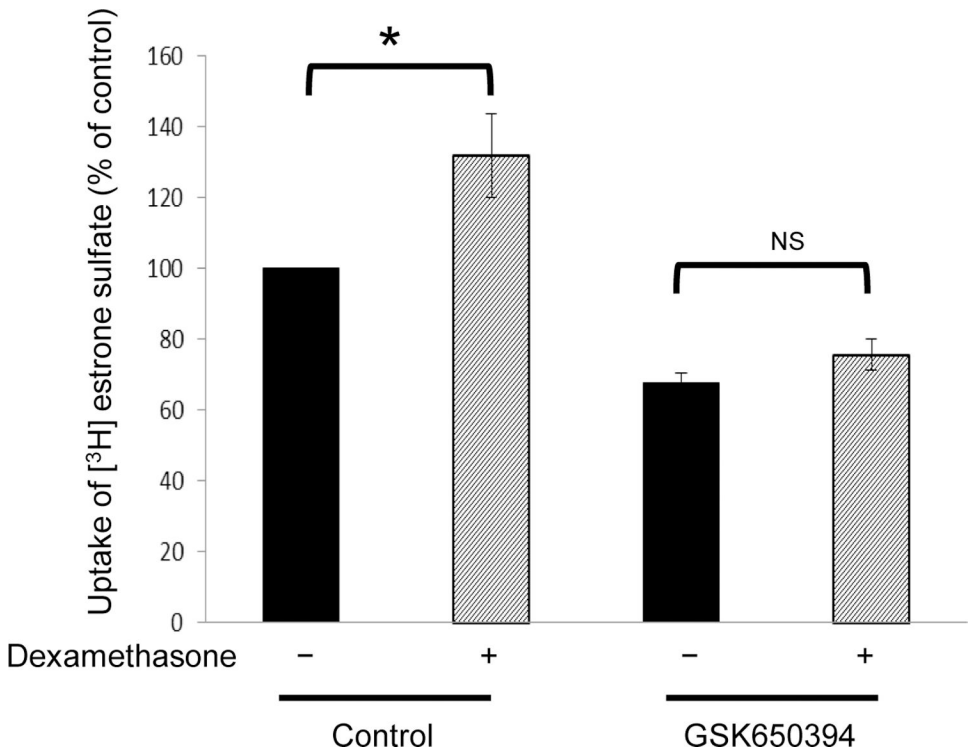

Fig. 7. Sgk inhibitor GSK650394 abrogates the stimulatory effect of dexamethasone on hOAT3 transport activity.

hOAT3-expressing cells were incubated for 6 hrs. with 10 nM dexamethasone in the presence and absence of sgk inhibitor GSK650394 (100 nM). After washing the cells, 4-min uptake of [^{3}H]-ES (300 nM) was measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. The data represent uptake into hOAT3-expressing cells minus uptake into mock cells (parental HEK293 cells). The values are mean ± S.E. (n = 3). *P<0.05.

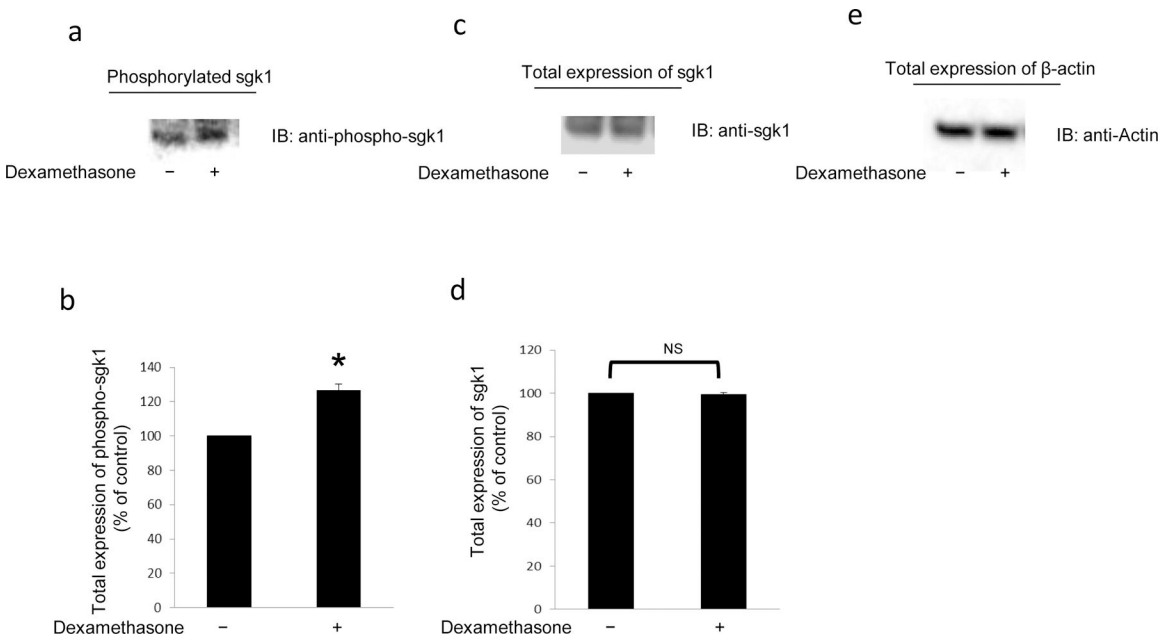

Fig. 8. Dexamethasone phosphorylates sgk1.

(a) Total cell expression of phospho-sgk1. hOAT3-expressing cells were pretreated with dexamethasone (10nM, 6 hrs.). Cells were lysed, followed by immunoblotting (IB) with anti-phospho-sgk1(Ser422)-specific antibody. (b) Densitometry plot of results from Fig. 8a, as well as from other experiments. The values are mean ± SE (n = 3). (c) The same blot from Fig. 8a was re-probed with anti-sgk1. (d) Densitometry plot of results from Fig. 8c as well as from other experiments. The values are mean ± SE (n = 3). (e) The same blot from Fig. 8a was re-probed with anti-β-actin antibody. β-actin is a cellular protein marker.