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Serum- and glucocorticoid-inducible kinase SGK2 regulates human organic anion transporters 4 via ubiquitin ligase Nedd4-2

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ABSTRACT

Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters that play critical roles in the body disposition of clinically important drugs, including anti-viral therapeutics, anti-cancer drugs, antibiotics, antihypertensives, and anti-inflammatories. hOAT4 is abundantly expressed in the kidney and placenta. In the current study, we examined the regulation of hOAT4 by serum- and glucocorticoid-inducible kinase 2 (sgk2) in the kidney COS-7 cells. We showed that sgk2 stimulated hOAT4 transport activity. Such stimulation mainly resulted from an increased cell surface expression of the transporter, kinetically revealed as an increased maximal transport velocity V_{max} without significant change in substrate-binding affinity K_m. We further showed that regulation of hOAT4 activity by sgk2 was mediated by ubiquitin ligase Nedd4-2. Overexpression of Nedd4-2 enhanced hOAT4 ubiquitination, and inhibited hOAT4 transport activity, whereas overexpression of ubiquitin ligase-dead mutant Nedd4-2/C821A or siRNA knockdown of endogenous Nedd4-2 had opposite effects on hOAT4. Our co-immunoprecipitation experiment revealed that sgk2 weakened the association between hOAT4 and Nedd4-2. In conclusion, our study demonstrated for the first time that sgk2 stimulated hOAT4 transport activity by abrogating the inhibitory effect of Nedd4-2 on the transporter.

1. Introduction

Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters, which play critical roles in the body disposition of clinically important drugs, including anti-human immunodeficiency virus therapeutics, anti-tumor drugs, antibiotics, antihypertensives, and antiinflammatories. The activity of these transporters is subjected to the regulation at multiple levels such as transcription, mRNA stability, translation, and posttranslational modification [1–6]. hOAT4 is abundantly expressed in the kidney and placenta [7]. In the kidney, hOAT4 localizes at the apical membrane of the proximal tubule cells, and is involved in renal secretion and reabsorption of endogenous substances as well as many drugs and xenobiotics. In the placenta, hOAT4 is localized to the basolateral membrane of syncytiotrophoblasts [8]. In the placenta, estrogen biosynthesis uses dehydroepiandrosterone sulfate (DHEAS), a precursor produced by the fetal adrenals. Accumulation of excess DHEAS is associated with intrauterine growth retardation [9]. DHEAS is a hOAT4 substrate. Therefore, hOAT4 may play an important role in efficient uptake of DHEAS by the placenta for estrogens production and for the protection of fetus from the cytotoxicity of DHEAS.

Given such an important role, understanding the regulation of hOAT4 has profound clinical significance. We previously demonstrated that members of OAT family undergo constitutive internalization from and recycling back to cell surface and OAT transport activity can be regulated by altering the trafficking kinetics and stability of these transporters. For example, activation of protein kinase C (PKC) inhibits OAT transport activity through accelerating the internalization rate of the transporter [10,11]. Prolonged activation of PKC leads to the degradation of the internalized OAT [11]. We further demonstrated that modification of OAT by ubiquitin conjugation is an important step that precedes OAT internalization [12].

Recently, modification of receptors and channels by ubiquitin conjugation has emerged as the major regulatory mechanism of cell surface internalization, intracellular sorting, and turnover of these membrane proteins [13,14]. Ubiquitin is a highly conserved 76-amino-acid protein that forms an isopeptide bond between its C-terminal glycine and a lysine (K) residue on the target protein. Each ubiquitin moiety itself harbors seven lysine residues, allowing for the formation of ubiquitin chains linked through its

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internal lysine residues. Therefore, a substrate can be modified by different types of ubiquitin conjugation: monoubiquitination (conjugation of one single ubiquitin to one single lysine on the substrate), or polyubiquitination (extended polyubiquitin chain). More and more evidence indicates that ubiquitination serves as a major signal in PKC-regulated cellular trafficking of transporters, including dopamine transporter (DAT) [15], cationic amino acid transporter (CAT-1) [16], and glutamate transporter (GLT-1) [17].

The serum- and glucocorticoid-inducible kinases (sgk) are involved in controlling diverse cellular processes including sodium Na^+ homeostasis, osmoregulation, cell survival, and cell proliferation [18–23]. The sgk family of protein kinases has three isoforms: sgk1, sgk2 and sgk3. It has been shown that the expression, regulation, and role of sgk2 within the mammalian kidney are distinct from sgk1 and sgk3 [24]. Sgk1 and sgk3 are expressed in every tissue, whereas sgk2 seems to be present primarily in the liver, kidney, pancreas, and brain. Unlike sgk1 and sgk3, sgk2 expression in the kidney was not subjected to the regulation by aldosterone. Immunochemical characterization localized sgk1 protein to distal convoluted tubule, cortical and medullary collecting duct, whereas sgk2 protein was highly expressed in kidney proximal tubule cells, where it modulates the function of membrane proteins such as Na^+/H^+ exchanger [24]. Based on the distinct characteristics of sgk2, we investigated whether hOAT4, also highly expressed in proximal tubule cells, is regulated by sgk2. We demonstrated a new regulatory mechanism that sgk2 modulates hOAT4 expression and function through an ubiquitin ligase Nedd4-2.

2. Materials and methods

2.1. Materials

COS-7 cells were purchased from American Type Culture Collection (Manassas, VA). [^{3}H]-labeled estrone sulfate was purchased from PerkinElmer (Waltham, MA). Membrane-impermeable biotinylation reagent NHS-SS-biotin, streptavidin-agarose beads and protein G-agarose beads were purchased from Pierce (Rockford, IL). cDNAs for mouse sgk2 (wild-type sgk2 and constitutive active sgk2 (CA-sgk2)) were generously provided by Dr. Alan C. Pao from Department of Medicine, Stanford University (Stanford, CA). cDNA for human Nedd4-2 was generously provided by Dr. Peter M. Snyder of the College of Medicine, University of Iowa (Iowa City, IA). Mouse anti-myc antibody (9E10) was purchased from Roche (Indianapolis, IN). Rabbit anti-sgk2 antibody was purchased from Cell signaling (Danvers, MA). Rabbit anti-Nedd4-2 antibody was purchased from Abcam (Cambridge, MA). Mouse anti-ubiquitin antibody P4D1 and anti-β-actin were purchased 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 purchased from Ambion (Grand Island, NY). All other reagents were from Sigma-Aldrich (St. Louis, MO).

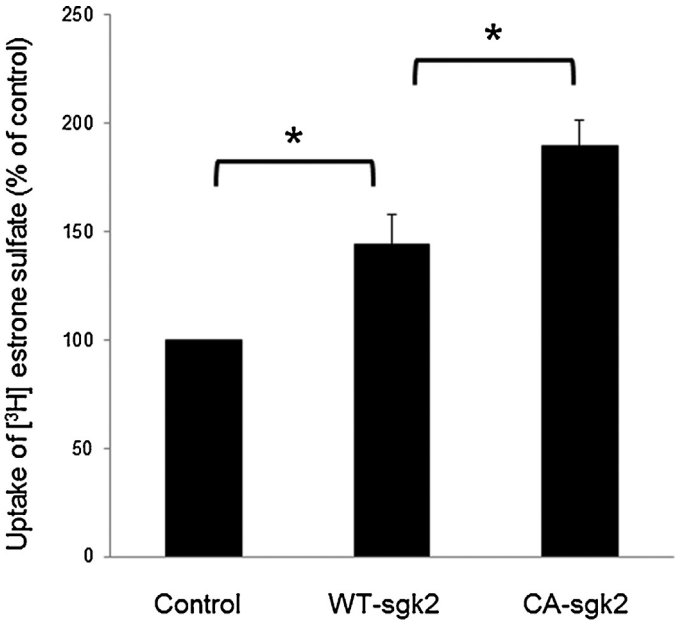

Fig. 1. Effect of sgk2 on hOAT4 transport activity.
COS-7 cells were co-transfected with hOAT4 and control vector, or with hOAT4 and wild type sgk2 (WT-sgk2), or hOAT4 and the constitutive active form of sgk2 (CA-sgk2). 3-min uptake of [^{3}H]-estrone sulfate (0.1 μM) was then measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. The data represent uptake into hOAT4-transfected cells minus uptake into mock cells (parental COS-7 cells). Values are mean ± S.E. ($n = 3$). *$P < 0.05$.

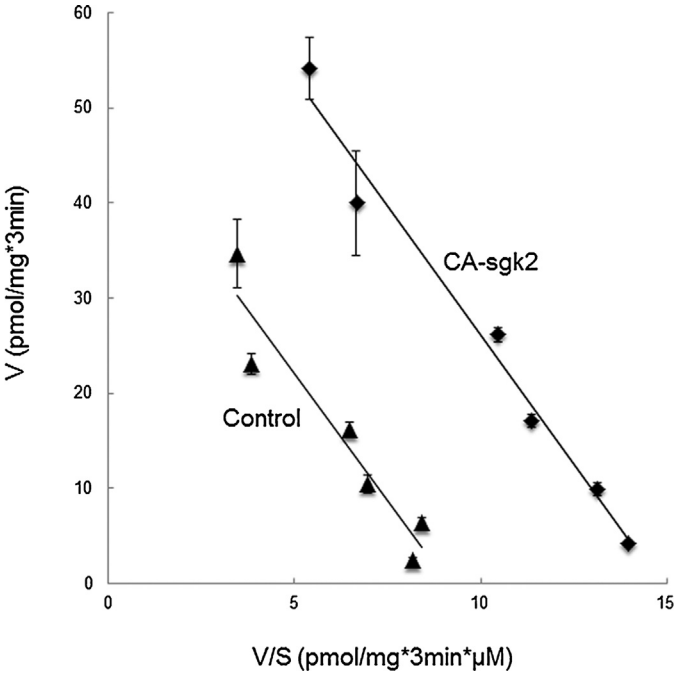

Fig. 2. Effect of sgk2 on the kinetics of hOAT4-mediated estrone sulfate transport. COS-7 cells were co-transfected with hOAT4 and the constitutive active form of sgk2 (CA-sgk2), or with hOAT4 and control vector. Initial uptake (3 min) of [^{3}H] estrone sulfate was measured at the concentration of 0.1–10 μM. The data represent uptake into hOAT4-transfected cells minus uptake into mock cells (parental COS-7 cells). Values are means ± S.E. ($n = 3$). *V*: velocity; S: substrate concentration.

2.2. Cell culture and transfection

Parental COS-7 cells were cultured 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 with plasmids was carried out for 48 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transfected cells were starved with serum free medium for 2–4 h for further experiments. Cells stably expressing hOAT4 were maintained in DMEM medium supplemented with 0.2 mg/ml G418 (Invitrogen, Carlsbad, CA), 10% fetal bovine serum.

2.3. Transport measurements

Cells were plated in 48-well plates. For each well, uptake solution was added. The uptake solution consisted of phosphate-