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Original Article

Short-term and long-term effects of protein kinase C on the trafficking and stability of human organic anion transporter 3

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Abstract: Human organic anion transporter 3 (hOAT3) belongs to a family of organic anion transporters that play critical roles in the body disposition of numerous clinically important drugs. Therefore, understanding the regulation of this transporter has profound clinical significance. In the current study, we investigated the short-term and long-term regulation of hOAT3 by protein kinase C (PKC). We showed that short-term activation of PKC by phobol 12-Myristate 13-Acetate (PMA) inhibited hOAT3 activity through accelerating its internalization from cell surface to intracellular recycling endosomes. The colocalization of hOAT3 with EEA1-positive recycling endosomes was demonstrated by immunolocalization with confocal microscopy. Furthermore, we showed that long-term activation of PKC resulted in the enhanced degradation of cell surface hOAT3. The pathways for hOAT3 degradation were further examined using proteasomal and lysosomal inhibitors. Our results showed that both proteasomal inhibitors and the lysosomal inhibitors significantly blocked hOAT3 degradation. These results demonstrate that PKC plays critical roles in the trafficking and the stability of hOAT3.

Keywords: Drug transporter, regulation, protein Kinase C, internalization, degradation

Introduction

The organic anion transporter (OAT) family mediates the body disposition of a diverse array of environmental toxins, and clinically important drugs, including anti-HIV therapeutics, antitumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories [1-5]. Therefore, understanding the regulation of these transporters has profound clinical significance.

Several OAT members have been cloned. These OATs are expressed in distinct tissues and cell membranes [1-5]. In the kidney, OAT1 and OAT3 utilize a tertiary transport mechanism to move organic anions across the basolateral membrane into the proximal tubule cells for subsequent exit across the apical membrane into the urine for elimination. Through this tertiary transport mechanism, Na^+/K^+-ATPase maintains an inwardly directed (blood-to-cell) Na^+ gradient. The Na^+ gradient then drives a sodium dicarboxylate cotransporter, sustaining an outwardly directed dicarboxylate gradient that is utilized by a dicarboxylate/organic anion exchanger, namely OAT, to move the organic anion substrate into the cell. This cascade of events indirectly links organic anion transport to metabolic energy and the Na^+ gradient, allowing the entry of a negatively charged substrate against both its chemical concentration gradient and the electrical potential of the cell. Generation of OAT1 and OAT3 null mice confirmed an essential role for these transporters in the renal transport [6, 7].

hOAT1 and hOAT3 have both shared and distinct properties. We previously showed that short-term activation of PKC inhibits OAT1 transport activity by reducing its surface expression

through enhancing the rate of OAT1 internalization from cell surface to intracellular compartments [8]. However, whether OAT3 follows similar regulatory pathway is unknown. Moreover, the effects of long-term PKC activation on these transporters have never been investigated. These investigations are described in the current studies.

Materials and methods

Materials

[^{3}H] estrone sulfate was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA). NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce Chemical (Rockford, IL, USA). Mouse anti-myc antibody was purchased from Roche. (Indianapolis, IN, USA). Mouse anti-EEA1 antibody was purchased from BD Biosciences (San Jose, CA, USA). Alexa Fluor® 488 goat anti-mouse IgG (H+L) and Alexa Fluor® 555 goat anti-rabbit IgG (H+L) were purchased from Molecular Probes (Eugene, OR, USA). Goat anti-mouse IgG conjugated to horseradish peroxidase and SuperSignal West Dura extended duration substrate kit were purchased from Thermo Scientific (Waltham, MA, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Cell cultures

Cells stably expressing hOAT3 [9] were maintained in DMEM medium supplemented with 0.2 mg/ml G418, 10 % fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml).

Transport measurement

Cells were plated in 48-well plates. For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/Ca^{2+}/Mg^{2+} (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4) and [^{3}H] estrone sulfate. At the times indicated, the uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquotted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values are means ± S.D. (n = 3).

Cell surface biotinylation

Cell surface expression levels of hOAT3 were examined using the membrane-impermeant biotinylation reagent NHS-SS-biotin. The cells were seeded onto six-well plates. After 24 hrs, the medium was removed and the cells were washed twice with 3 ml of ice-cold PBS, pH 8.0. The plates were kept on ice, and all solutions were kept ice-cold for the rest of the procedure. Each well of cells was incubated with 1 ml of freshly made NHS-SS-biotin (0.5 mg/ml in PBS, pH 8.0) in two successive 20 min incubations on ice with very gentle shaking. Biotinylation was quenched by first briefly washing each well with 3 ml of 100 mM glycine and followed by incubation with 100 mM glycine on ice for 20 min. The cells were then dissolved on ice for 40 min in 400 µl of lysis buffer [10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 1 % Triton X-100, and protease inhibitors (200 µg/ml phenylmethylsulfonyl fluoride and 3 µg/ml leupeptin), pH 7.4]. The unlysed cells were removed by centrifugation at 16,000 X g at 4 °C. Streptavidin-agarose beads were then added to the supernatant to isolate cell membrane protein. hOAT3 was detected in the pool of surface proteins by polyacrylamide gel electrophoresis and immunoblotting using an anti-myc antibody (1:100). Myc was tagged at the carboxyl terminus of hOAT3 for its immunodetection [9].

Internalization assay

We followed the procedure described previously by our laboratory [8]. hOAT3-expressing cells underwent biotinylation with 0.5 mg/ml sulfo-NHS-SS-biotin as described above. Following biotinylation, one set of cells was washed with PBS and kept at 4 °C to determine the total initial surface hOAT3 and stripping efficiency. To initiate internalization, cells in the duplicate plate were washed repeatedly with pre-warmed (37 °C) PBS containing either 1 µM of PMA or PBS only and incubated with the same solutions at 37 °C for indicated time periods. Residual cell surface biotin was stripped by incubating cells three times for 20 min with freshly prepared 50 mM MesNa in NT buffer (150 mM NaCl, 1 mM EDTA, 0.2 % bovine serum albumin, 20 mM Tris, pH 8.6). Stripping efficiency was determined for each experiment on biotinylated cells kept in parallel at 4 °C. Cells were lysed in lysis buffer with protease inhibitor cocktail. Biotinylated proteins were separated from non-

biotinylated proteins by streptavidin-agarose resin similarly as we described above. Samples were then eluted from the beads by adding sample buffer and resolved by SDS-PAGE and analyzed by western blotting with anti-myc antibody. Relative hOAT3 internalized was calculated as % of the total initial cell surface hOAT3 pool.

Immunofluorescence analysis

hOAT3-expressing cells were grown on coverslips (22 mm) for 48 hrs, washed three times in PBS, and then fixed with 3% paraformaldehyde for 20 min at room temperature, permeabilized with 0.01% Triton x-100 for 5 min three times, and incubated with 5% dry milk at room temperature for 1 h. Afterwards, the cells were incubated with rabbit anti-c-Myc antibody (Sigma, 1:300) to label hOAT3-tagged with c-myc and with mouse anti-EEA1 antibody (1:250) at 4 °C overnight. The coverslips were then incubated with Alexa Fluor® 488 goat anti-mouse IgG (H+L) (1:500) or Alexa Fluor® 555 goat anti-rabbit IgG (H+L) (1:1000) at room temperature for 2 hrs. After washing, the coverslips were mounted on slides for image acquisition and analysis. Samples were visualized with a Zeiss LSM-510 laser-scanning microscope.

Degradation of cell surface hOAT3

hOAT3 expressing cells were plated in 35mm dishes. Each dish of cells was incubated with 1ml of cell membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin (1 mg/ml in PBS pH8.0, supplemented with 1mM Ca^{2+} and 1mM Mg^{2+} 1mM) in two successive 20 minutes incubations under trafficking impermissive condition (4°C) with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each dish was rinsed with 2ml PBS containing 100mM glycine and then incubated with the same solution for 20 minutes on ice, to ensure complete quenching of the unreacted NHS-SS-biotin. The biotin-labeled cells were incubated in DMEM containing 1 μM DMSO or 1 μM PMA at 37°C. Treated cells were collected at 2, 4, and 6 hours and lysed in lysis buffer with protease inhibitor cocktail. The cell lysates were cleared by centrifugation at 16,000 × g at 4 °C. 50μl of streptavidin-agarose beads were then added to the supernatant to isolate cell membrane proteins. Samples were loaded on 7.5% SDS-PAGE minigels and analyzed by immunoblotting with anti-myc antibody.

Inhibition of Proteases

Degradation of cell surface hOAT3 (described as above) was determined in the presence and absence of proteasome inhibitor MG132 (5 μM), and lactacystin (5 μM) or lysosomal inhibitors leupeptin/pepstatin A (50, 2 μg/ml), and chloroquine (100μM) individually for specific time point as indicated in the figure legends.

Electrophoresis and immunoblotting

Protein samples (100 μg) were resolved on 7.5 % SDS-PAGE mini-gels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5 % nonfat dry milk in PBS-0.05 % Tween, and incubated overnight at 4 °C with anti-myc antibody (1:100). The membranes were washed and then incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (1:5,000), and signals were detected using a SuperSignal West Dura extended duration substrate kit. Images were captured by Fluorchem ®8800 system (Alpha Innotech, San Leandro, CA, USA). Density of bands was analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

Data analysis

Each experiment was repeated a minimum of three times. The statistical analysis given was from multiple experiments. Statistical analysis was performed using Student's paired *t*-tests. A P value < 0.05 was considered significant.

Results

Constitutive and PKC-regulated trafficking of hOAT3

We previously showed that hOAT1 constitutively internalizes from and recycles back to the plasma membrane and that inhibition of hOAT1 activity by short-term (30 min) activation of PKC results from a reduced surface expression of hOAT1 through accelerating internalization of the transporter [8]. hOAT1 and hOAT3 have shared and distinct properties. hOAT3 activity was also inhibited by short-term activation of PKC with PMA and such inhibition was reversed in the presence of PKC inhibitor staurosporin

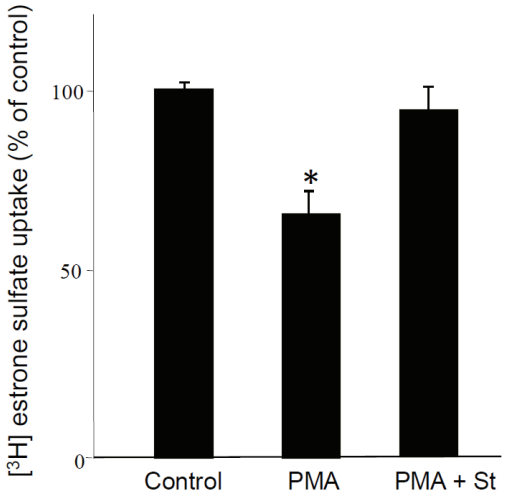

Figure 1. Effect of PKC on hOAT3 activity. COS-7 cells stably expressing hOAT3 were incubated for 30 min with 1 μM PKC activator PMA in the presence and absence of PKC inhibitor staurosporin (St, 2 μM) added directly to the culture media. After washing the cells, 3-min uptake of [^{3}H] estrone sulfate (0.1 μM) was measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. Values are mean ± S.D. (*n* = 3). *Asterisks* indicate values significantly different ($p < 0.05$) from that of control.

(**Figure 1**). However whether the mechanism underlying PKC regulation of hOAT3 is similar to that hOAT1 is unknown. To address this issue, we took a biotinylation-based strategy. hOAT3-expressing cells were biotinylated with cell-impermeable biotinylation reagent sulfo-NHS-SS -biotin under trafficking-impermissive condition (4 °C). The labeled cells were then rewarmed back to trafficking-permissive condition (37 °C) to allow internalization to occur in the presence and the absence of PKC activator PMA. At indicated time points after initiation of internalization, biotin from biotinylated proteins remaining on the surface was removed by treatment with MesNa, a nonpermeant reducing agent that cleaves disulfide bond and liberates biotin from biotinylated proteins at the cell surface. The amount of biotinylated proteins resistant (inaccessible) to MesNa treatment was defined as "the amount of protein internalized." Our result (**Figure 2**) showed that under the basal condition, the amount of the surface-labeled hOAT3 internalized increasingly with time. Approximately 5%, 15%, or 33% of surface-labeled hOAT3 were detectable in the intracellular compartments after 5-, 15-, or 30-min initiation of internalization. Therefore, hOAT3 undergoes constitutive internalization in COS-7 cells. Furthermore, our result (**Figure 3**) showed that the amount of surface-labeled hOAT3 internalized in the presence of PMA was much greater than that in the absence of PMA, suggesting that short-term activation of PKC by PMA inhibits hOAT3 transport activity through accelerating hOAT3 internalization into the intracellular compartments. Total expression of hOAT3 was not affected under such condition (not shown).

Immuno-localization of hOAT3 and EEA1

The cellular distribution of hOAT3 was examined by immunofluorescence microscopy. We previously showed that hOAT1 constitutively traffics between plasma membrane and recycling endosomes [8]. To determine whether hOAT3 traffics through the same route, we immunolocalized hOAT3 and EEA1, a recycling endosome marker. The fluorescence images (**Figure 4**) showed that hOAT3 indeed partially colocalized with EEA1-positive recycling endosomes (shown

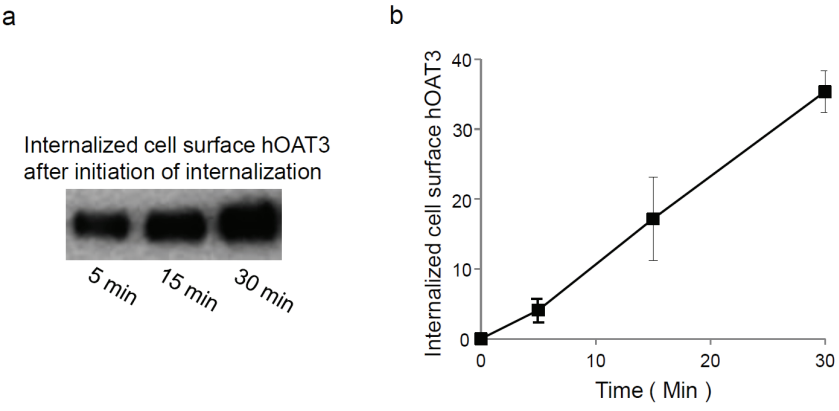

Figure 2. Biotinylation analysis of constitutive hOAT3 internalization in COS-7 cells. a. hOAT3 internalization was analyzed as described in "Materials and Methods" section followed by immunoblotting using anti-myc antibody (1:100). b. Densitometry plot of results from Figure 2a as well as from other experiments. Internalized hOAT3 was expressed as % of total initial cell surface hOAT3 pool. Values are mean ± S.E. (*n* = 3).

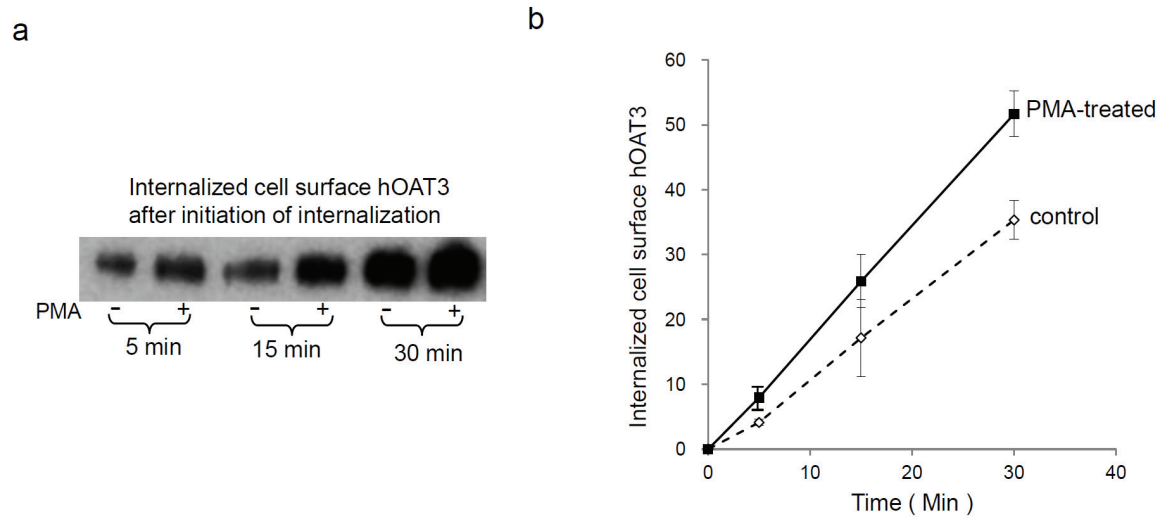

Figure 3. Biotinylation analysis of PKC-modulated hOAT3 internalization in COS-7 cells. a. hOAT3 internalization was analyzed in the presence and absence of PKC activator PMA (1 μM) as described in "Materials and Methods" section followed by immunoblotting using anti-myc antibody (1:100). b. Densitometry plot of results from Figure 3a as well as from other experiments. Internalized hOAT3 was expressed as % of total initial cell surface hOAT3 pool. Values are mean ± S.E. (*n* = 3).

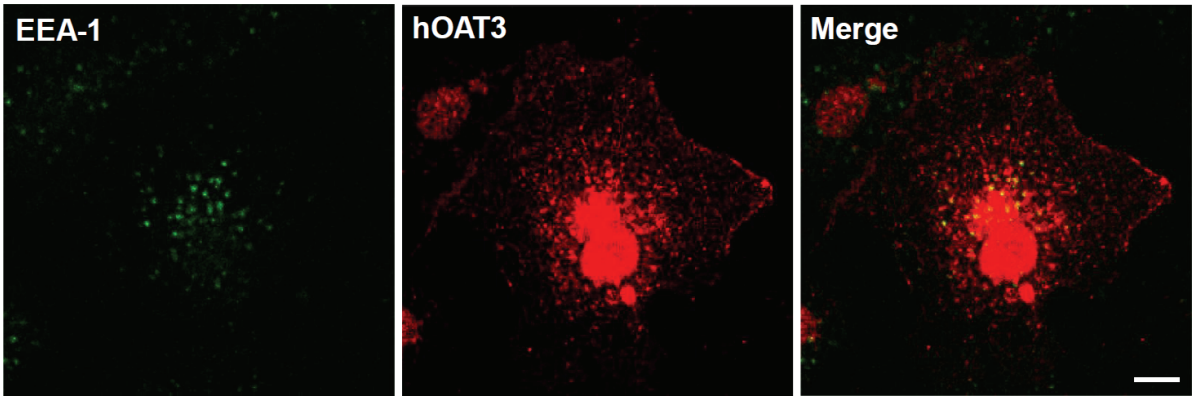

Figure 4. Immuno-localization of hOAT3 and EEA1. The cells were immunostained for hOAT3, and early endosome marker EEA1. Fluorescence images were taken for hOAT3 (red), and EEA1 (green). The merged image of hOAT3 and EEA1 was shown as orange/yellow. Bar = ~ 10 μm.

as orange/yellow color).

Constitutive and PKC-regulated degradation of hOAT3

Our results above examined the effect of short-term activation of PKC (≤ 30 min) on cell surface hOAT3. The effect of long-term activation of PKC on any member of OAT family has never been investigated. We therefore examined the effect of long-term activation of PKC on cell surface hOAT3. hOAT3-expressing cells were biotinylated with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then treated with or without PMA at 37°C for 2, 4, and 6 hrs. Treated cells were lysed and cell surface proteins were isolated using streptavidin-agarose beads, followed by immunoblotting with anti-myc antibody. Our results (**Figure 5**) showed that the rate of cell surface hOAT3 degradation increased significantly after 2 hrs of treatment with PMA as compared to that of

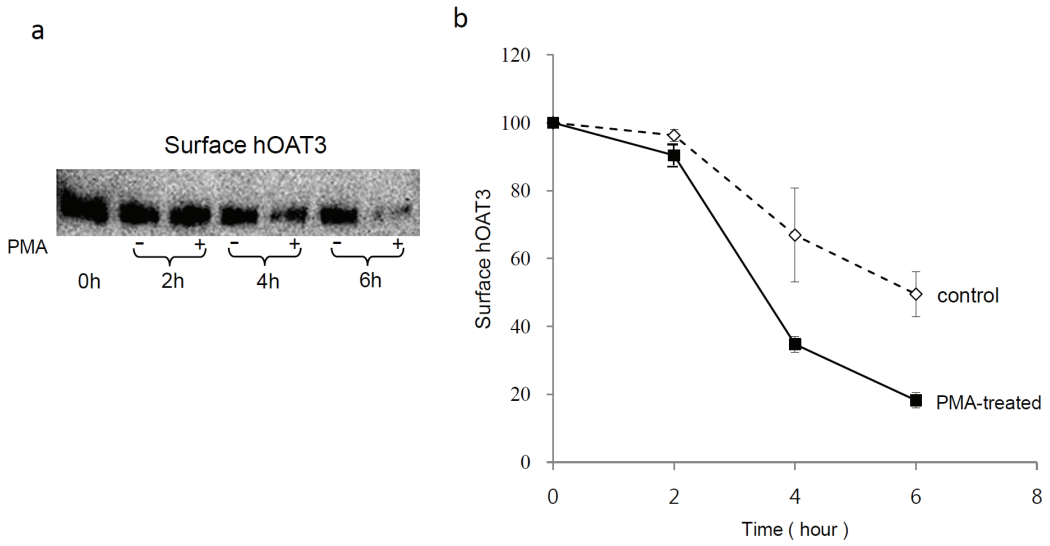

Figure 5. Biotinylation analysis of constitutive and PKC-modulated degradation of surface hOAT3. a. Cell surface hOAT3 degradation was analyzed in the presence and absence of PKC activator PMA (1 μM) as described in "Materials and Methods" section followed by immunoblotting using anti-myc antibody (1:100). b. Densitometry plot of results from Figure 5a as well as from other experiments. The amount undegraded cell surface hOAT3 was expressed as % of total initial cell surface hOAT3 pool. Values are mean ± S.E. (*n* = 3).

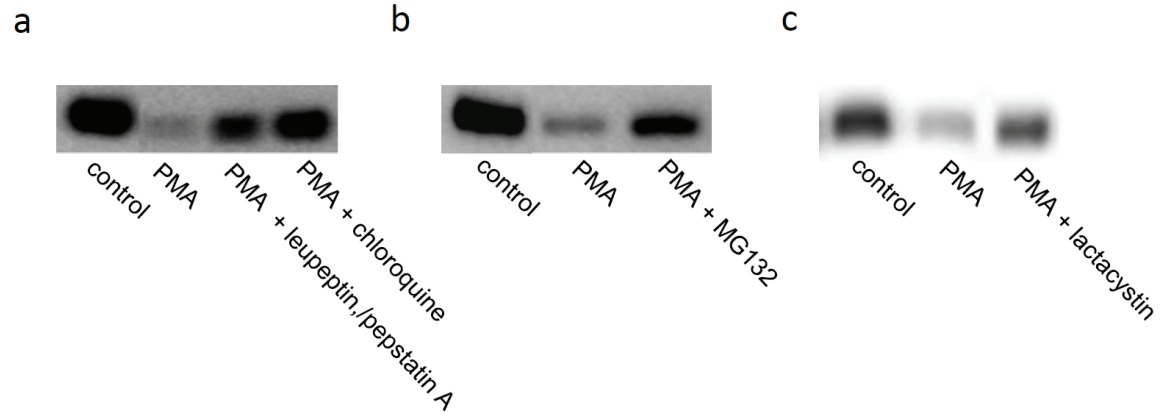

Figure 6. Effects of protease inhibitors on the degradation of cell surface hOAT3. Cell surface hOAT3 degradation was determined as described in "Materials and Methods" section in the presence of various protease inhibitors (6 hrs): a. lysosomal inhibitors leupeptin (50 μg/ml), pepstatin A (2 μg/ml) and chloroquine (100 μM). b. proteasomal inhibitor MG132 (5 μM). c. proteasomal inhibitor lactacystin (5 μM).

control.

Degradation pathways of hOAT3

We next examined the degradation pathway of cell surface hOAT3 using a battery of different inhibitory reagents. Cells degrade proteins through two major systems, the proteasome and the lysosome. These different pathways of proteolysis can be determined by their sensitivity to different inhibitors. Degradation of polypeptides by the proteasome can be inhibited by MG132 and lactacystin, whereas lysosomal proteolysis can be inhibited by leupeptin, pepstatin A and chloroquine. For our experiment, hOAT3-expressing cells were biotinylated with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then treated

with PMA at 37°C for 6 hrs in the presence of various protease inhibitors. Treated cells were lysed and cell surface proteins were isolated using streptavidin-agarose beads, followed by immunoblotting with anti-myc antibody. Our results (**Figure 6**) showed that both proteasome inhibitors MG132 and lactacystin and lysosomal inhibitors leupeptin, pepstatin A and chloroquine significantly blocked hOAT3 degradation.

Discussion

The organic anion transporter (OAT) family mediates the body disposition of a diverse array of environmental toxins, and clinically important drugs. Therefore, understanding the regulation of these transporters has profound clinical significance. In the current study, we investigated both the short-term and the long-term effects of PKC activation on cell surface hOAT3. Short-term regulation usually occurs when body has to deal with rapidly changing amounts of substances as a consequence of variable intake of drug, fluid, and meal as well as metabolic activity, whereas long-term regulation usually happens in situations in which the body undergoes massive changes, for example during development or in diseases.

We choose COS-7 cells for our study because these cells offer several useful advantages for study of the cloned organic anion transporter. (i) These cells were directly derived from the kidney and have been very useful in understanding other renal transport processes and cellular functions, including organic cation transport [10, 11]. (ii) This cell line does not express endogenous OATs. Therefore, expression of OAT3 in COS-7 cells will allow us to dissect the transport characteristics of OAT3 in a relevant mammalian system without the possibly confounding effects of other organic anion transporters. (iii) They possess endogenous PKC and PKA signaling pathways and provide a good experimental model system for studying the regulatory mechanisms underlying many transport processes [12, 13]. (iv) The transport characteristics of OAT3 in COS-7 cells were in a good agreement with that observed in other systems [14-16].

The amount of OATs at the cell surface is critical for their drug transport activity. We previously established that hOAT1 undergoes constitutive internalization from and recycling back to cell surface and that acute activation of PKC inhibits hOAT1 activity by reducing hOAT1 cell surface expression through accelerating its internalization from cell surface to intracellular compartments without affecting the total expression of the transporter [8]. hOAT1 and hOAT3 have both shared and distinct properties. For example, these transporters have different substrate specificities. Our current study on the effect of short-term activation of PKC on cell surface hOAT3 demonstrated that hOAT1 and hOAT3 shared similar regulatory mechanisms: acute activation of PKC inhibits hOAT3 activity through accelerating its internalization from cell surface to intracellular compartments, part of which were EEA1-positive recycling endosomes as demonstrated through our immunolocalization study (Figs. 3 and 4). The partial colocalization of hOAT3 with EEA1 could arise from the possibility that recycling endosomes are heterogeneous in their biochemical compositions, ion transport properties, and pH values [17]. Therefore, it may be possible that some of hOAT3 resides in a different subpopulation of recycling endosomes from that enriched in EEA1. It is also worth to note that hOAT3 is over-expressed in COS-7 cells. In such an over-expression system, it is not surprising to see that hOAT3 does not completely overlap with EEA1.

We then went further to investigate the effect of long-term PKC activation on cell surface hOAT3, an important issue, which has never been explored for any member of OAT family. We showed that prolonged treatment of hOAT3-expressing cells with PKC activator PMA resulted in an accelerated degradation of the transporter in both proteasome and lysosome (Figs. 5.and 6). Our unpublished result indicates that PKC regulates OAT trafficking through ubiquitination of the transporters. A protein can be modified by different types of ubiquitin conjugation such as monoubiquitination and polyubiquitination. In addition, a polyubiquitin chain can bear different linkages. It has been shown that different types and linkages of ubiquitination may direct the ubiquitinated protein to degrade in lysosome or proteasome [18]. We are currently investigating the specific type and linkage of hOAT3 ubiquitination.

In conclusion, the major finding from the current study is that i) hOAT3 undergoes constitutive internalization, ii) short-term activation of PKC inhibits hOAT3 activity by accelerating hOAT3

internalization from cell surface to recycling endosomes without affecting the total expression of the transporter, and iii) long-term activation of PKC resulted in hOAT3 degradation in both lysosome and proteasome.

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