Regulation of Human Organic Anion Transporter 3 by Peptide Hormone Bradykinin

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ABSTRACT

Human organic anion transporter (hOAT) 3 belongs to a family of organic anion transporters that play critical roles in the body disposition of numerous clinically important drugs. In the current study, we examined the regulation of hOAT3 by peptide hormone bradykinin (BK) in COS-7 cells. BK (≤ 500 nM) induced a concentration- and time-dependent stimulation of hOAT3 activity, kinetically revealed as an increased $V_{\max}$. Such an increase in $V_{\max}$ resulted from an increased cell surface expression without a change in total cell expression of the transporter. BK-induced stimulation of hOAT3 activity could be prevented by treating hOAT3-expressing cells with staurosporine, a general inhibitor for protein kinase C (PKC). To obtain further infor-

mation on which PKC isoform mediates BK regulation of hOAT3 activity, cellular distribution of various PKC isoforms was examined in cells treated with BK. We showed that BK treatment resulted in a significant translocation of PKC δ , PKC ϵ , and PKC ζ from cytosol to membrane. We further showed that BK treatment enhanced association of hOAT3 with PKC δ , PKC ϵ , and PKC ζ and that isoform-specific inhibitor for PKC δ , PKC ϵ , and PKC ζ reversed BK effect on hOAT3 activity. We therefore concluded that BK stimulated hOAT3 activity through activation of PKC δ , PKC ϵ , and PKC ζ , which then led to the redistribution of hOAT3 from the intracellular compartments to the cell surface and to the up-regulation of hOAT3 activity.

Organic anion transporters (OATs) play essential roles in the body disposition of a diverse array of environmental toxins and clinically important drugs, including anti-human immunodeficiency virus therapeutics, antitumor drugs, antibiotics, antihypertensives, and anti-inflammatories (You, 2002, 2004a,b). Therefore, understanding the regulation of OATs will have significant impact on the future design of strategies aimed at maximizing therapeutic efficacy and minimizing toxicity.

Multiple OAT isoforms have been cloned and their expressions identified in distinct tissues and cell membranes. Human OAT (hOAT) 1 and hOAT3 are predominantly expressed in the kidney and localized at the basolateral membranes of renal proximal tubules (Hosoyamada et al., 1999; Cha et al., 2001). Moreover, it has been reported that the mRNA levels of hOAT1 and hOAT3 were much higher than those of other organic ion transporters in the human kidney cortex

(Motohashi et al., 2002). The membrane localization and function of these transporters position them for an active role in the tubular secretion of organic anions, including various clinically used drugs, as well as endogenous compounds, from the circulation and in determining therapeutic efficacy and toxicity of the drugs.

Bradykinin (BK), a nine amino-acid peptide hormone, has been shown to regulate the function of numerous kidney membrane transport processes, such as chloride conductance, Na^+/H^+ exchange, and organic anion and organic cation transport (Slotki et al., 1990; Hohage et al., 1998; Gekle et al., 1999; Tiwari et al., 2007). Multiple signaling pathways, including the protein kinase A pathway and protein kinase C (PKC) pathway, are involved in bradykinin-induced regulation (Tippmer et al., 1994; Sena et al., 1996; Ross and Joyner, 1997; Tiwari et al., 2007). BK regulation through PKC involved different PKC isoforms (Tippmer et al., 1994; Sena et al., 1996; Ross and Joyner, 1997). The PKC isoforms are divided into three categories based upon the cofactors that are required for optimal catalytic activity. Conventional PKCs (α , β , and γ) are calcium-dependent and are stimulated by the second messenger diacylglycerol. Novel PKCs (δ , ϵ , η , and θ) are also activated by diacylglycerol but are calcium-

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ABBREVIATIONS: OAT, organic anion transporter; hOAT, human organic anion transporter; BK, bradykinin; PKC, protein kinase C; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

independent. Atypical PKCs (ζ and λ) require neither calcium nor diacylglycerol for optimal activity. These isoforms have cell type-dependent expression to mediate distinct cellular response. In the current study, we investigated the role of BK in the function of hOAT3 and the protein kinases involved in this process. We chose estrone sulfate, a high-affinity substrate for OAT3 (Cha et al., 2001), for our functional assays. Although estrone sulfate can be the substrate for other transporters, the functional data from our studies were obtained by subtracting the uptake values in parental COS-7 cells from the uptake values in hOAT3-expressing cells, ensuring that the transport of estrone sulfate was indeed by hOAT3 and not by other endogenous estrone sulfate transporters.

Materials and Methods

Materials. [3 H]Estrone sulfate was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Sulfo-NHS-SS-biotin and streptavidin-agarose beads were purchased from Thermo Fisher Scientific (Waltham, MA). Anti-myc antibody 9E10 was from Mount Sinai School of Medicine (New York, NY). PKC isoform-specific antibodies were from BD Biosciences (San Jose, CA). PKC ϵ peptide inhibitor was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); PKC ζ peptide inhibitor was purchased from Tocris Bioscience (Ellisville, MO); and rottlerin and staurosporine were purchased from LC Laboratories (Woburn, MA). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Cell Culture. Parental COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml) in a 5% CO₂ atmosphere at 37°C. COS-7 cells stably expressing hOAT3-myc were maintained in the same medium containing 0.2 mg/ml Geneticin (G418; Invitrogen, Carlsbad, CA). The myc epitope was tagged to the carboxyl terminus of hOAT3 to facilitate the immunodetection of hOAT3.

Transport Measurement. Cells plated in 48-well plates were treated with each reagent at 37°C for certain time periods as indicated. For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/Ca²⁺/Mg²⁺ (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 1 mM CaCl₂, and 1 mM MgCl₂, pH 7.4) and [3 H]estrone sulfate. At the times indicated, 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 measured in aliquots for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values are means $\pm$ S.E. ($n = 3$).

Cell Surface Biotinylation. Cell surface expression levels of hOAT3 were examined using the membrane-impermeant biotinylation reagent Sulfo-NHS-SS-biotin (Thermo Fisher Scientific). The cells were seeded onto six-well plates at 8×10^5 cells per well. After 24 h, 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 Sulfo-NHS-SS-biotin (0.5 mg/ml in PBS) in two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS containing 100 mM glycine and then incubated with the same solution for 20 min on ice to ensure complete quenching of the unreacted sulfo-NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 μ l of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1/100 protease inhibitor mixture, pH 7.4). The unlysed cells were removed by centrifugation at 13,000 rpm at 4°C. Streptavidin-agarose beads (50 μ l; Thermo Fisher Scientific) were then added to the supernatant to isolate cell membrane protein.

hOAT3 was detected in the pool of surface proteins by electrophoresis and immunoblotting using an anti-myc antibody (1:500).

Subcellular Fractionation. The cells grown on a 100-mm dish were homogenized in isolation buffer (50 mM Tris-HCl, pH 7.2, 10% glycerol, and 1/100 protease inhibitor mixture). The harvested cells were sonicated twice for 10 s each, and the cell lysate was then centrifuged at 1000g for 10 min at 4°C to remove unbroken cells. Ultracentrifugation (L7-55 ultracentrifuge; Beckman Coulter, Fullerton, CA; rotor 70.1 Ti) was followed to separate the supernatant into cytosolic and crude membrane fractions at 120,000g for 45 min at 4°C. The particular fraction was resuspended in the sample buffer. The proteins were eluted with Laemmli buffer containing β -mercaptoethanol and analyzed by immunoblotting with PKC isoform-specific antibodies.

Coimmunoprecipitation. The cells were lysed in immunoprecipitation buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 10% glycerol, and 1/100 protease inhibitor mixture). Cell lysates were precleaned with protein A-agarose beads to reduce nonspecific binding at 4°C for 1.5 h. Anti-myc antibody (1:100) was incubated with appropriate volume of protein A-agarose beads at 4°C for 1.5 h. The precleaned protein sample was then mixed with antibody-bound protein A-agarose beads and underwent end-over-end rotating at 4°C overnight. Proteins bound to the protein A-agarose beads were eluted with Laemmli buffer containing β -mercaptoethanol and analyzed by immunoblotting with PKC isoform-specific antibodies.

Electrophoresis and Western Blotting. Protein samples (100 μ g) were resolved on 7.5% SDS-PAGE minigels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS-0.05% Tween 20, washed, and incubated overnight at 4°C with primary antibody. The membranes were washed and then incubated with appropriate secondary antibody conjugated to horseradish peroxidase (1:5000), and signals were detected using a SuperSignal West Dura extended duration substrate kit (Thermo Fisher Scientific).

Data Analysis. Statistical analysis was conducted using Student's paired *t* test for comparing two treatments. A one-way analysis of variance followed by a Dunnett's post hoc test was used for comparing among more than two treatments. A *P* value <0.05 was considered significant.

Results

Effects of BK on hOAT3 Activity. We examined whether treatment with BK could affect hOAT3 transport activity in COS-7 cells. Because the hOAT3 expression vector used in the current study does not contain the promoter region of hOAT3, the long-term regulation at the transcriptional level cannot be investigated. We only focused on the short-term regulation of the transporter (within a time frame of 2 h). BK induced a concentration-dependent regulation of [3 H]estrone sulfate uptake with the concentration of maximum stimulation at 500 nM (Fig. 1a). At this concentration, we observed a time-dependent stimulation of estrone sulfate uptake (Fig. 1b), with maximal stimulation after 30-min treatment. To examine the mechanism of BK-induced stimulation of hOAT3 activity, we determined [3 H]estrone sulfate uptake at different substrate concentrations. An Eadie-Hofstee analysis of the derived data (Fig. 2) showed that pretreatment with BK resulted in an increased $V_{\max}$ (0.015 ± 0.001 pmol $\cdot$ μ g⁻¹ $\cdot$ min⁻¹ with untreated cells and 0.020 ± 0.001 pmol $\cdot$ μ g⁻¹ $\cdot$ min⁻¹ in the presence of BK) with no significant change in the affinity (K_m) for estrone sulfate (5.09 ± 0.27 μ M with untreated cells and 4.81 ± 0.31 μ M in the presence of BK). The K_m of hOAT3 for estrone sulfate determined in our system is comparable with that obtained

Fig. 1. Effect of BK on hOAT3. hOAT3-expressing cells were treated with BK at various concentrations (a), or at various times (b), followed by [³H]estrone sulfate uptake (100 nM for 4 min). Uptake activity was expressed as a percentage of the uptake measured in untreated cells. The results represent data from three experiments. The uptake values in mock cells (parental COS-7 cells) were subtracted. Values are means $\pm$ S.E. ($n = 3$).

Fig. 2. Effect of BK on the kinetics of estrone sulfate transport. COS-7 cells expressing hOAT3 were pretreated with or without BK (500 nM) for 30 min, and initial uptake (4 min) of [³H]estrone sulfate was measured at 0.1 to 10 μ M estrone sulfate. The data represent uptake into hOAT3-transfected cells minus uptake into mock cells (parental COS-7 cells). Values are means $\pm$ S.E. ($n = 3$). V, velocity; S, substrate concentration.

Fig. 3. Effect of BK on cell surface and total cell expression of hOAT3. a, Western blot analysis of total cell expression and cell surface expression of hOAT3. COS-7 cells stably expressing hOAT3 were treated with or without BK (500 nM for 30 min). The lysed cell proteins, or the biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads and separated by SDS-PAGE, followed by Western blotting with anti-myc antibody (1:500). b, densitometry analyses of the transporter expression from the experiment shown in a and other experiments. *, $P < 0.05$.

from other systems (Cha et al., 2001). Determination of the protein concentrations in control cells confirmed that BK treatment did not change the total protein content of the cultures (data not shown).

Effect of BK on hOAT3 Expression. An increased $V_{\max}$ could be affected by either an increased number of the transporter at the cell surface or an increased transporter turnover number. Experiments that differentiate between these possibilities were conducted by measuring transporter expression both at the cell surface and in the total cell lysates.

We showed that BK treatment resulted in an increased cell surface expression of hOAT3 without affecting the total cell expression of the transporter (Fig. 3).

BK Regulation of hOAT3 Activity through PKC. hOAT3-expressing COS-7 cells were treated with BK in the presence of staurosporine, a general inhibitor for PKC. As shown in Fig. 4, staurosporine efficiently reversed the stimulating effect of BK on hOAT3 activity.

Identification of PKC Isoforms Involved in BK Effect. COS-7 cells have been shown to express several PKC

Fig. 4. Effect of inhibitor for PKC on BK-induced stimulation of hOAT3 activity. hOAT3-expressing cells were pretreated with staurosporine, a PKC inhibitor (St; 2 μ M for 5 min) followed by incubation with BK (500 nM for 30 min) in the presence or absence of St (2 μ M). The uptake of [³H]estrone sulfate (100 nM for 4 min) was then performed. The results represent data from three experiments. The uptake values in mock cells (parental COS-7 cells) were subtracted. Values are mean $\pm$ S.E. ($n = 3$).

isoforms, including PKC α , PKC ϵ , PKC δ , PKC ζ , and PKC ι (Chen and Exton, 2004). Our results showed that, among all these PKC isoforms, treatment of cells with BK resulted in a significant translocation of PKC δ , PKC ζ , and PKC ϵ from cytosol to membrane fraction (Fig. 5). We further showed, through coimmunoprecipitation experiments, that BK enhanced the association of hOAT3 with PKC δ , PKC ζ , and PKC ϵ (Fig. 6). Finally, BK-induced stimulation of hOAT3 activity was prevented by pretreating the cells with each of the PKC isoform-specific inhibitors (Fig. 7). These results confirm the involvement of PKC δ , PKC ζ , and PKC ϵ in the action of BK on hOAT3.

Discussion

In the current study, we investigated the effect of BK on the function of hOAT3 as well as the signaling molecules involved in this process. We carried out studies in COS-7 cells because of their many advantages. 1) They were derived directly from kidney and have been very useful in understanding other renal epithelial transport processes and cellular functions, including organic cation transport (Zhang et al., 2002; Nagai et al., 2006). 2) Our Western blot demonstrated that these cells do not express endogenous OATs (data not shown). Therefore, expression of hOAT3 in such cells will allow us to dissect the transport characteristics of hOAT3 in a relevant mammalian system without the possibly confounding effects of other organic anion transporters. 3) These cells possess endogenous PKC and protein kinase A signaling pathways and provide a good experimental model system for studying the regulatory mechanisms underlying many transport process (Kazanietz et al., 2001; Cobb et al., 2002).

BK induced a biphasic effect on the uptake of estrone sulfate mediated by hOAT3 (Fig. 1, a and b), with the max-

imal stimulation at a BK concentration of 500 nM. With the concentration of BK higher than 500 nM, the stimulation became less significant. BK has been reported to exhibit opposite effects on many biological systems (Crocker and Willavoys, 1975; Riethmüller et al., 2006), dependent on the experimental conditions as well as the signaling pathways involved. Therefore, we speculate that at high concentration (>500 nM), BK began to affect multiple processes, which led to the overall diminished stimulation of estrone sulfate uptake. We chose a concentration of BK at 500 nM for our subsequent studies, because this concentration has been widely used to investigate BK effect in both in vitro and in vivo conditions (Bagaté et al., 2000; Faussner et al., 2003). Our kinetic analysis of hOAT3 activity (Fig. 2) showed that the enhanced transport activity was contributed by an increased maximal transport velocity (V_{max}) without affecting the binding affinity (K_m) for the substrates. Furthermore, our cell surface biotinylation experiments demonstrated that the increased V_{max} was a result of an increased cell surface expression of hOAT3 without change in the total expression of the transporter (Fig. 3). These results suggest that a redistribution of hOAT3 from intracellular compartments to cell surface occurred in response to BK treatment.

The only moderate stimulation of hOAT3 activity (~30%) by BK is consistent with the dynamic nature of the transporter. We demonstrated previously (Zhang et al., 2008; Duan et al., 2010) that both hOAT1 and hOAT3 undergo constitutive internalization and recycling between cell surface and intracellular compartments and that regulation of these transporters can be achieved by altering its already existent trafficking kinetics. We therefore speculate that BK may stimulate hOAT3 activity by either attenuating its internalization, or accelerating its recycling or a combination of both. Due to such dynamic nature of hOAT3, at any given time, there is always certain amount of hOAT3 leaving the cell surface due to the component of constitutive internalization even with the maximal stimulation of BK, which explains why we only observed a moderate stimulation of approximately 30% by BK.

We showed that BK-induced stimulation of hOAT3 activity could be prevented by treating hOAT3-expressing cells with staurosporine, a general inhibitor for PKC (Fig. 4). Staurosporine exerts its inhibition effect on PKC by binding to the ATP binding site on the catalytic domain of the kinase (Rüegg and Burgess, 1989). Unfortunately, the ATP binding site of PKC shares great homology with the ATP binding sites of other protein kinases, making it difficult for staurosporine to achieve high selectivity for PKC (Rüegg and Burgess, 1989). However, our further studies provided strong evidence that the PKC mediated the effect of BK. This conclusion was based on the following points. First, BK induced significant translocation of three PKC isoforms PKC δ , PKC ζ , and PKC ϵ from cytosol to plasma membrane (Fig. 5). Second, BK enhanced the association of hOAT3 with PKC δ , PKC ζ , and PKC ϵ (Fig. 6). Third, BK-induced stimulation of hOAT3 activity could be reversed by treating hOAT3-expressing cells with PKC isoform-specific inhibitors (Fig. 7). Therefore this is the first study to demonstrate that three PKC isoforms are involved in BK effect on hOAT3 activity. The exact contribution of each PKC isoform in the process of BK effect on hOAT3 is however difficult to evaluate notably because PKC isoforms do not act independently but rather cross-react in a

Fig. 5. Western blot analyses of subcellular fraction expression of PKC isoforms in cells treated with or without BK. COS-7 cells stably expressing hOAT3 treated with or without BK (500 nM for 30 min) were lysed and fractionated into crude membrane and cytosol, and the proteins were separated by SDS-PAGE, followed by Western blotting with PKC isoform-specific antibodies. Total cell lysate was also prepared. Only the PKC isoforms that showed translocations are shown here.

Fig. 6. Effect of BK on the association of hOAT3 with PKC δ , PKC ζ , and PKC ϵ . **a**, coimmunoprecipitation of hOAT3 with PKC isoforms. COS-7 cells expressing hOAT3 were lysed in 0.5 to 1% Triton X-100 and subjected to immunoprecipitation with anti-myc antibody, followed by immunoblotting with PKC isoform-specific antibodies. **b**, densitometry analyses of results in **a** as well as other experiments. *, $P < 0.05$.

complex manner. For example, PKC α is able to elevate PKC δ amounts at both transcriptional and post-transcriptional levels (Romanova et al., 1998), whereas PKC ϵ has the propensity to activate novel PKCs by phosphorylation (Rybin et al., 2003). In addition, a previous study clearly demonstrated that there exists a spatiotemporally coordinated cascade in which PKC isoforms are tightly linked (Collazos et al., 2006). This would largely explain why we could not evidence, in the present study, any additive effects of PKC isoform-specific inhibitor in reversing BK activation of hOAT3.

It is important to note that BK was shown previously to inhibit organic anion transport activity in isolated rabbit proximal tubules (Gekle et al., 1999), an observation opposite to what we observed in the current study. Again, as explained above, BK has been shown to exert opposite effects on many biological systems (Crocker and Willavoys, 1975; Riethmüller et al., 2006), dependent on the experimental conditions as well as the signaling pathways involved. The discrepancy between our and Gekle's studies could be explained as follows. First, rabbit organic anion transporter(s) with fluorescein as a substrate was investigated in their study,

Fig. 7. Effect of PKC isoform-specific inhibitor on BK-mediated hOAT3 activity. hOAT3-expressing cells were pretreated with rottlerin (specific for PKC δ ; 2 μ M for 20 min), with PKC ζ peptide inhibitor (specific for PKC ζ ; 1 μ M for 20 min), or with PKC ϵ peptide inhibitor (specific for PKC ϵ ; 1 μ M for 20 min) followed by incubation with BK (500 nM for 30 min). The uptake of [³H]estrone sulfate (20 μ M for 4 min) was then performed. The results represent data from three experiments. The uptake values in mock cells (parental COS-7 cells) were subtracted.

whereas single transporter human OAT3 was investigated in our current study. Second, their study was carried out in isolated rabbit proximal tubule, whereas our study was carried out in COS-7 cells. Due to these differences, the signaling pathway involved in both studies may well be different. The inhibitory effect of BK on rabbit organic anion transport activity in their study may involve the activation of PKC α , whereas the stimulatory effect of BK on human OAT3 activity in our current study involves the activation of PKC δ , PKC ζ , and PKC ϵ . Indeed, we have recently shown (Li et al., 2009; Duan et al., 2010) that activation of PKC α by angiotensin II exhibits an inhibitory effect on the activities of both hOAT1 and hOAT3, whereas Barros et al. (2009) showed that activation of PKC ζ by insulin exhibits an stimulatory effect on the activities of both rat OAT1 and rat OAT3.

Active transport of endogenous metabolites and xenobiotics from blood to urine across the cells of the renal proximal tubule is an important protective mechanism. In addition, the rate of drug elimination from the body critically determines the therapeutic efficacy of the drug and its toxicity. hOAT3 plays an important role in these processes.

To the best of our knowledge, our findings are the first to demonstrate that hOAT3 activity is under control of physiological stimuli BK through specific PKC isoforms. Although BK has been shown to regulate numerous renal transport processes, its *in vivo* role on OAT expression and function is largely unexplored. Elucidating the *in vivo* relevance of our findings will be the focus of our future studies.

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