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Human Organic Anion Transporter 3 Gene Is Regulated Constitutively and Inducibly via a cAMP-Response Element

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ABSTRACT

Human organic anion transporter (OAT) 3 (SLC22A8) is localized to the basolateral membranes of renal tubular epithelial cells and plays a critical role in the excretion of anionic compounds. We previously reported that interindividual variation in the OAT3 mRNA level corresponded to interindividual differences in the rate of renal excretion of cefazolin. However, there is little information available on the molecular mechanisms regulating the gene expression of OAT3. Therefore, in the present study, we examined the transcriptional regulation of human OAT3. A deletion analysis of the OAT3 promoter suggested that the region spanning −214 to −77 base pairs was essential for basal transcriptional activity. This region contained a perfectly conserved cAMP-response element (CRE), and a mutation here led to a reduction in promoter activity. Electrophoretic mobility shift assays showed that CRE-binding protein (CREB)-1 and activating transcription factor (ATF)-1 bound to CRE. The activity of the OAT3 promoter was increased through the phosphorylation of CREB-1 and ATF-1 by treatment with 8-bromo-cAMP. This paper reports the first characterization of the human OAT3 promoter and shows that CREB-1 and ATF-1 function as constitutive and inducible transcriptional regulators of the human OAT3 gene via CRE.

The kidney plays important roles in the excretion of various drugs, toxins, and endogenous metabolites. The excretion process consists of three steps: glomerular filtration, tubular secretion, and reabsorption. Transporters expressed in renal tubular epithelial cells are mainly involved in the tubular secretion and reabsorption (Pritchard and Miller, 1996; Inui and Okuda, 1998; Wright and Dantzler, 2004). The organic anion transporter (OAT) family (OAT1–4), which is predominantly expressed in the kidney (Russel et al., 2002; Sekine et al., 2006), mediates the transport of many anionic compounds, such as β-lactam antibiotics, nonsteroidal anti-inflammatory drugs, antiviral drugs, and antitumor drug, and it regulates their excretion (Inui et al., 2000; Burckhardt and Burckhardt, 2003; Sweet, 2005).

We previously found that the mRNA level of OAT3 was higher than that of any other member of the organic ion transporter (SLC22A) family in the human kidney, and OAT3 was localized to the basolateral membranes of proximal tubules (Motohashi et al., 2002). OAT3 possessed greater activity to transport cephalosporin antibiotics, including cefazolin than OAT1 in vitro experiment (Ueo et al., 2005). Furthermore, clinical pharmacokinetic and gene expression analyses showed that only the mRNA level of OAT3 among OAT1–4 significantly correlated with the apparent elimination rate constant of the free fraction of cefazolin in patients with mesangial proliferative glomerulonephritis ($r = 0.757$; $p < 0.01$) (Sakurai et al., 2004, 2005).

The OAT3 mRNA level is assumed to be mainly under the control of transcriptional regulation. However, little is known about the functional characteristics of the promoter region of OAT3. Based on this background, we cloned the human OAT3 promoter region and examined its promoter activity using opossum kidney (OK) cells. This is the first report to identify the *cis*-element and *trans*-factors for the regulation of the human OAT family in the kidney.

Materials and Methods

Materials. [γ-^{32}P]ATP was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Restriction enzymes were from New England Biolabs (Beverly, MA). Antibodies used for supershift

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ABBREVIATIONS: OAT, organic anion transporter; OK, opossum kidney; 8-Br-cAMP, 8-bromo-cAMP; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; PKA, protein kinase A; EMSA, electrophoretic mobility shift assay; CRE, cAMP-response element; mut, mutation/mutated; ATF, activating transcription factor; CREB, CRE-binding protein; SNP, single-nucleotide polymorphism; cSNP, coding single-nucleotide polymorphism; rSNP, regulatory single-nucleotide polymorphism.

assays were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Those used for Western blotting were purchased from Cell Signaling Technology Inc. (Beverly, MA). 8-Bromo-cAMP sodium salt (8-Br-cAMP) was obtained from Sigma-Aldrich (St. Louis, MO).

5′-Rapid Amplification of cDNA Ends. To identify the transcription start site of human OAT3, 5′-rapid amplification of cDNA ends (RACE) was carried out using Human Kidney Marathon-Ready cDNA (Clontech, Mountain View, CA) according to the manufacturer's instructions. The primers for 5′-RACE were as follows: a gene-specific primer for OAT3 (accession no. NM_004254), 5′-CCCACTCTGTCACAATGGAGTCCTTGG-3′ (473 to 446); and a nested gene-specific primer for OAT3, 5′-CCCATGCTTCCCACACG-GTCCAGGATC-3′ (177 to 151). The PCR products were subcloned into the pGEM-T Easy Vector (Promega, Madison, WI) and sequenced using a multicapillary DNA sequencer RISA384 system (Shimadzu, Kyoto, Japan).

Cloning of the 5′-Regulatory Region of the OAT3 Gene. Based on the human genomic sequence (accession no. NT_033903), the 2488-base pair flanking region upstream of the transcription start site was cloned by PCR using the primers listed in Table 1 and human genomic DNA (Promega). The PCR product was isolated by electrophoresis and subcloned into the firefly luciferase reporter vector pGL3-Basic (Promega), at NheI and XhoI sites. This full-length reporter plasmid is hereafter referred to as −2488/+21.

Preparation of Deletion Reporter Constructs. The −1862/+21 construct was generated by digestion of the −2488/+21 construct with SacI, and the 5′-deleted constructs (−926/+21, −214/+21) were generated by digestion of the −2488/+21 construct with MluI and either SpeI or ApaI. The ends were blunted with T4 DNA polymerase (Takara Bio, Otsu, Japan) and then self-ligated. The −77/+21 and −11/+21 constructs were generated by PCR with primers containing an NheI site and XhoI site (Table 1). The site-directed mutations in the putative CCAAT box and CRE were introduced into the −214/+21 construct with a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the primers listed in Table 1. The nucleotide sequences of these deleted or mutated constructs were verified.

Cell Culture, Transfection, and Luciferase Assay. OK cells were cultured in medium 199 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen) without antibiotics, in an atmosphere of 5% CO_2, 95% air at 37°C and subcultured every 7 days using 0.02% EDTA and 0.05% trypsin. OK cells were plated into 24-well plates (4×10^6 cells/well) and transfected the following day with the reporter constructs and 25 ng of the *Renilla reniformis* vector pRL-TK (Promega), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. The firefly and *Renilla* activities were determined 48 h after the transfection using a dual-luciferase assay kit (Promega) and a LB940 luminometer (Berthold, Bad Wildbad, Germany). The firefly activity was normalized to *Renilla* activity. For the protein kinase A (PKA) stimulation experiment, the cells were treated with 1 mM 8-Br-cAMP for 12 h before the luciferase assay.

Electrophoretic Mobility Shift Assay. Nuclear extract was prepared from OK cells according to the method of Shimakura et al. (2005). The double-stranded oligonucleotides used in the EMSA are listed in Table 1. The OAT3 probe (−101/−75) was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara Bio), and the labeled probe was purified through a Sephadex G-25 column (GE Healthcare). EMSA was performed according to Alimov et al. (2003) but with some modifications. The OK nuclear extract (10 μg) was incubated in binding buffer [120 mM KCl, 20 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 2 mM dithiothreitol, 5% glycerol, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 10 mM NaF, 100 μM Na_3VO_4, and 2% protease inhibitor cocktail] for 30 min at 4°C. Thereafter, the labeled probe was added, and the mixture was incubated for a further 30 min at 4°C. For competition experiments and supershift assays, excess (50-fold) unlabeled oligonucleotide and antibodies (1 μg) were added 30 min before the addition of the labeled probe, respectively. The volume of the binding mixture was 20 μl throughout the experiment. The DNA-protein complex was then separated on a 4% polyacrylamide gel for 1.5 h at 200 V and room temperature in 0.5× Tris borate-EDTA buffer. Gels were dried and exposed to X-ray film for autoradiography.

Western Blot Analysis. OK cells were treated with phosphate-buffered saline in the presence or absence of 1 mM 8-Br-cAMP for 15 min before the preparation of nuclear extracts. Twenty micrograms of nuclear extract was separated on a 10% SDS polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA). Membrane blocking and antibody incubations were carried out using the PhosphoPlus CREB (Ser133) Antibody kit (Cell Signaling Technology Inc.) according to the manufacturer's instructions. The bound antibody was detected on X-ray film by enhanced chemiluminescence with horse-

TABLE 1
Oligonucleotide sequence of primers
NheI and XhoI sites are underlined. Mutations introduced into the oligonucleotides are shown in boldface.

Name	Sequence (5′-3′)	Position
Primers for cloning of the OAT3 promoter		
OAT3/−2488NheI-F	GGGCTAGCGTGATAGATCCCCCAATAAGG	−2488 to −2468
OAT3/+21XhoI-R	GGCTCGAGGCAGCTCAGCTCTAACAAGC	+2 to −21
Primers for the −77/+21 and −11/+21 deletion constructs		
OAT3/−77NheI-F	CTGCTAGCCCGCAAAAGAAAGTCAAACAT	−77 to −57
OAT3−11NheI-F	CTGCTAGCGGCACAAACACAGCTTGTTAG	−11 to +10
OAT3/+21XhoI-R	GGCTCGAGGCAGCTCAGCTCTAACAAGC	+2 to −21
Primers for the site-directed mutagenesis		
mCCAAT-F	CACAGCACTCTCCCTG**TCTG**TGACGTTAATCCGC	−107 to −74
mCCAAT-R	GCGGATTAACGTCAC**AGA**CAGGGAGAGTGCTGTG	−74 to −107
mCRE-F	CACAGCACTCTCCCTGCCAGTGA**TA**TTAATCCGCAAAAG	−107 to −69
mCRE-R	CTTTTGCGGATTAA**TA**TCACTGGCAGGGAGAGTGCTGTG	−69 to −107
Oligonucleotides for EMSA		
OAT3 (−101−75)-F	ACTCTCCCTGCCAGTGACGTTAATCCG	−101 to −75
OAT3 (−101−75)-R	CGGATTAACGTCACTGGCAGGGAGAGT	−75 to −101
mutCCAAT-F	ACTCTCCCTG**TTT**GTGACGTTAATCCG	−101 to −75
mutCCAAT-R	CGGATTAACGTCAC**AAA**CAGGGAGAGT	−75 to −101
mutCRE-F	ACTCTCCCTGCCAGTGA**GCC**TAATCCG	−101 to −75
mutCRE-R	CGGATTA**GGC**TCACTGGCAGGGAGAGT	−75 to −101
Double mut-F	ACTCTCCCTG**TTT**GTGA**GCC**TAATCCG	−101 to −75
Double mut-R	CGGATTA**GGC**TCAC**AAA**CAGGGAGAGT	−75 to −101
Sp1 consensus-F	ATTCGATCGGGGCGGGGCGAGC	
Sp1 consensus-R	GCTCGCCCCGCCCCGATCGAAT	

radish peroxidase-conjugated anti-rabbit IgG antibody (GE Healthcare).

Data Analysis. The results were expressed relative to pGL3-Basic and represent the mean ± S.D. of three replicates. Two or three experiments were conducted, and representative results are shown. In the mutational and PKA stimulation experiments, the statistical analysis was performed with the one-way analysis of variance followed by Scheffé F-post hoc testing.

Results

Determination of the Transcription Start Site of OAT3. Sequencing of the longest RACE product showed that the terminal position of OAT3 cDNA was located 126 nucleotides above the start codon, which was 10 base pairs downstream of the 5′ end of OAT3 cDNA registered in the National Center for Biotechnology Information database (accession no. NM_004254). Therefore, the 5′ end of OAT3 cDNA was numbered with +1 as the transcription start site in this study.

Determination of Minimal OAT3 Promoter. To determine the minimal region required for basal activity of the promoter, a series of deletion constructs were transfected into OK cells, and luciferase activity was measured (Fig. 1A).

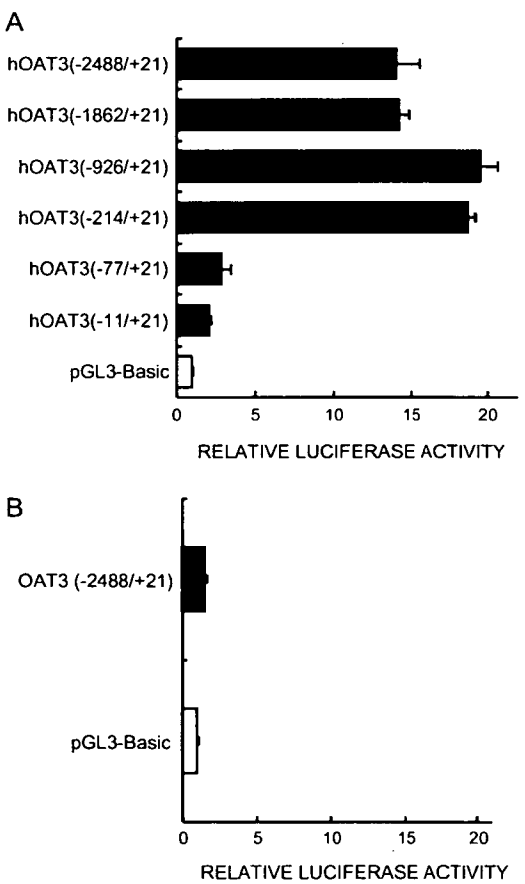

Fig. 1. Identification of the transcriptional activity of the human OAT3 promoter in OK cells. A, deletion analysis of the human OAT3 promoter in OK cells. A series of deleted promoter constructs [equimolar amounts of the −2488/+21 construct (500 ng)] were transfected into OK cells for luciferase assays. B, transcriptional activity of the human OAT3 promoter in human embryonic kidney 293 cells. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are reported as the relative -fold increase compared with pGL3-Basic and represent the mean ± S.D. of three replicates.

OK cells were used in the luciferase assay because they have an organic anion transport system (Hori et al., 1993), and the transcription factors and/or cofactors required for the expression exist intrinsically in these cells. The longest reporter constructs (−2488/+21) showed an approximately 14-fold increase in luciferase activity compared with pGL3-Basic in OK cells, but they had little promoter activity in human embryonic kidney 293 cells, which lack an organic anion transport system (Fig. 1B). The 5′-deleted constructs (−1862/+21, −926/+21, −214/+21) had the same level of activity as the longest construct, −2488/+21. In contrast, the −77/+21 construct had one-sixth of the activity of −214/+21. These results suggested that the elements important for the basal promoter activity were located between −214 and −77.

Figure 2 shows the results of a computational analysis of the −214/−77 region of the OAT3 promoter, using TRANSFAC 6.0 at www.gene-regulation.com/. This analysis revealed that there is one putative CCAAT box and one perfectly conserved cAMP-response element (CRE) in this region, suggesting that these sites contribute to the transcriptional regulation of OAT3.

Mutagenesis of CCAAT Box and CRE. To determine whether these sites were important for the promoter activity of OAT3, mutations at these sites (designated as mCCAAT and mCRE, respectively) were introduced in the −214/+21 construct and transfected into OK cells. As shown in Fig. 3, mCCAAT and mCRE reduced the luciferase activity to two-third and one-third of the wild-type level, respectively. These results suggest that the CCAAT box and CRE are responsible for the basal promoter activity of OAT3.

Electrophoretic Mobility Shift Assay. To confirm which transcription factors bind to these elements, EMSA was performed using an OAT3 probe (−101/−75) containing both the CCAAT box and CRE and nuclear extract from OK cells. The probe (−101/−75) formed a DNA-protein complex (Fig. 4A, lane 2). The formation of the complex was prevented by the addition of an excess amount of unlabeled oligonucleotide (−101/−75) but not by unrelated oligonucleotide (Fig. 4A, lanes 3 and 7), suggesting that transcription factors bind to the probe (−101/−75). Next, we prepared three oligonucleotides, mutCCAAT lacking a CCAAT box, mutCRE lacking a CRE, and double mut lacking both elements to determine which sites the protein recognized. mutCCAAT impaired the formation of the complex, but mutCRE and double mut did not (Fig. 4A, lanes 4−6), suggesting that the

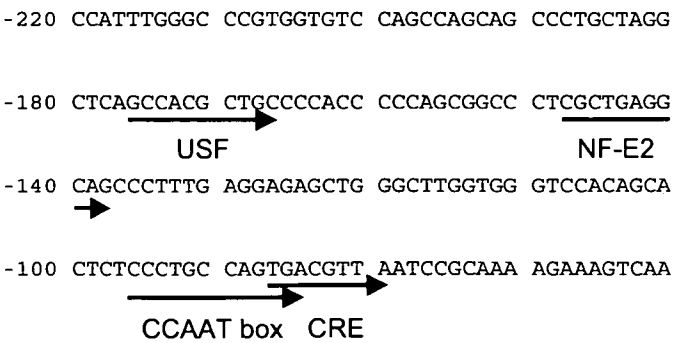

Fig. 2. Nucleotides sequence of the promoter region (−220 to −1) of human OAT3. Numbering is relative to the transcription start site. The putative binding sites for the transcription factors are indicated (the arrows indicate the direction).

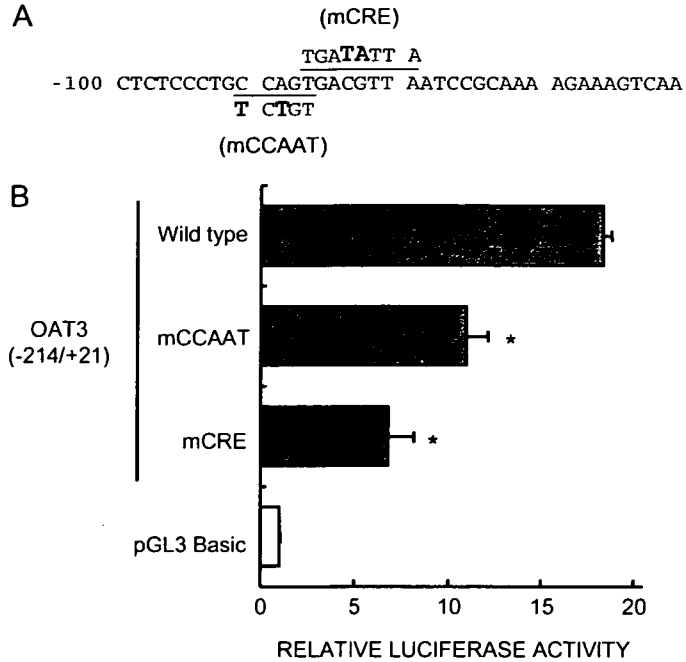

Fig. 3. Mutational analysis of the putative CCAAT box and CRE of the human OAT3 promoter. A, schematic of the mutated OAT3 (−214/+21) reporter constructs. B, these constructs (500 ng) were transfected into OK cells for luciferase assays. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are reported as the relative -fold increase compared with pGL3-Basic and represent the mean ± S.D. of three replicates. *, significantly different from wild type; $p < 0.05$.

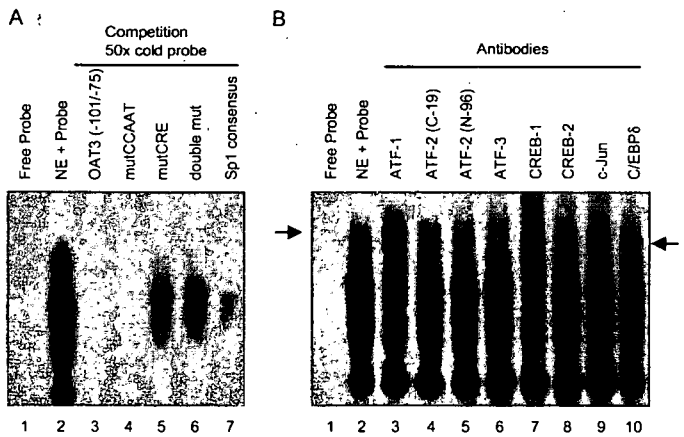

Fig. 4. EMSA using nuclear extract from OK cells and a human OAT3 probe (−101/−75). A, nuclear extract from OK cells was incubated with the ^{32}P-labeled OAT3 oligonucleotide probe (−101/−75) alone (lane 2) or in the presence of excess unlabeled oligonucleotide (−101/−75) (lane 3), excess mutated oligonucleotide (lane 4–6), and excess Sp1 oligonucleotide (lane 7). In lane 1, nuclear extract was not added. B, nuclear extract from OK cells was incubated with the ^{32}P-labeled OAT3 oligonucleotide probe (−101/−75) alone (lane 2) or in the presence of antibody against ATF-1 (lane 3), ATF-2 (C-19) (lane 4), ATF-2 (*N*-96) (lane 5), ATF-3 (lane 6), CREB-1 (lane 7), CREB-2 (lane 8), c-Jun (lane 9), and CCAAT/enhancer-binding protein δ (lane 10). In lane 1, nuclear extract was not added. Arrows indicate the supershifted complexes.

proteins that bind to the probe (−101/−75) recognize CRE, but not the CCAAT box.

It has been demonstrated that various transcription factors bind to CRE (Hai and Hartman, 2001). Figure 4B shows results of supershift assays using antibodies against transcription factors that bind to CRE. Antibodies against activating transcription factor (ATF)-1 and CRE-binding protein (CREB)-1 were able to supershift the DNA-protein complex (Fig. 4B, lanes 3 and 7). In contrast, antibodies against ATF-2, ATF-3, CREB-2 (ATF-4), c-Jun, and CCAAT/enhancer-binding protein δ did not result in a supershift (Fig. 4B, lanes 4–6 and 8–10). These results indicate that the transcription factor, which binds to CRE, consists entirely of a homodimer or heterodimer made up of ATF-1 and/or CREB-1.

Effect of PKA Activation. Both ATF-1 and CREB-1 are phosphorylated by PKA and activate the transcription of target genes. The effect of the PKA activator 8-Br-cAMP on the activity of the OAT3 promoter was investigated with the −214/+21, mCRE, and −77/+21 constructs in OK cells. Treatment with 8-Br-cAMP increased luciferase activity 2.5-fold in the −214/+21 construct. In contrast, the response to 8-Br-cAMP was diminished in the mCRE and −77/+21 constructs (Fig. 5). As shown in Fig. 6, levels of phosphorylated CREB-1 and ATF-1 increased with 8-Br-cAMP treatment. These results suggested that PKA stimulated the OAT3 promoter through phosphorylation of both CREB-1 and ATF-1.

Discussion

In the present study, we performed a functional promoter assay of human OAT3 and found that CRE is responsible for the basal and inducible promoter activity of OAT3 and that CREB-1 and ATF-1 bind to CRE. CREB-1 was first described as a transcription factor mediating induction by extracellular signals activating adenylate cyclase and PKA (Gonzalez and Montminy, 1989). Thereafter, it was demonstrated that this transcription factor is responsible for the constitutive transcriptional regulation without stimuli (Quinn, 1993). ATF-1 is capable of dimerizing with CREB-1 (Hurst et al., 1991), but it cannot stimulate gene expression unless it is combined with PKA (Rehfuss et al., 1991). It is, therefore, suggested that CREB-1 is involved in the constitutive expression of OAT3. This is the first paper to identify the *cis*-element and *trans*-factor for the regulation of the human OAT family in the kidney.

Soodvilai et al. (2004) demonstrated that the transport

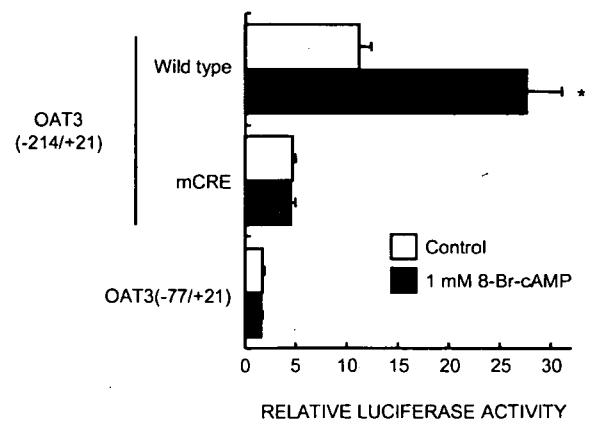

Fig. 5. Effect of 8-Br-cAMP on the promoter activity of various reporter constructs. These constructs (500 ng) were transfected into OK cells for luciferase assays. 8-Br-cAMP (1 mM) was added to the medium 36 h after transfection, and luciferase assays were carried out after 12 h. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Open columns and closed columns indicate control and 8-Br-cAMP treatment, respectively. Data are reported as the relative -fold increase compared with pGL3-Basic and represent the mean ± S.D. of three replicates. *, significantly different from control; $p < 0.05$.

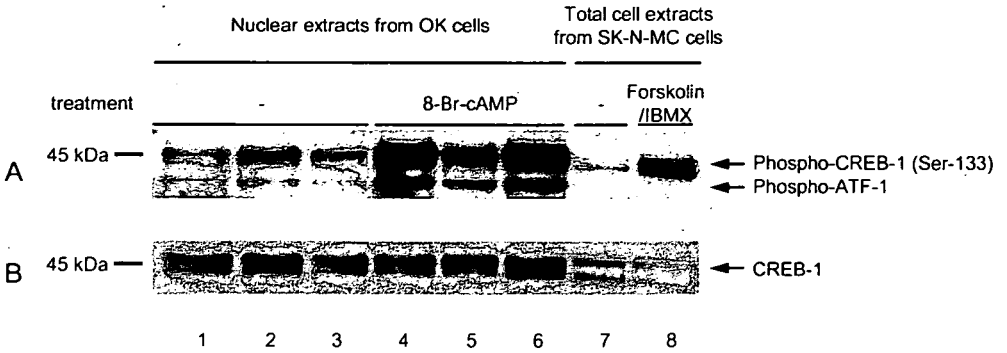

Fig. 6. Western blot analyses of nuclear extracts from OK cells treated without (lanes 1–3) or with 1 mM 8-Br-cAMP for 15 min (lanes 4–6), and total cell extracts from SK-N-MC cells, prepared without or with forskolin/3-isobutyl-1-methylxanthine (IBMX) treatment, to serve as negative and positive controls. Nuclear extracts or total cell extract (20 μg) was separated on a 10% SDS polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane. Phospho-CREB (Ser133) antibody (A) and CREB antibody (B) (1:1000 dilution) were used as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit IgG antibody was used for detection of bound antibodies. The arrowheads indicate the positions of each transcription factor.

activity of rabbit OAT3 is up-regulated by PKA (10-min stimulation) and speculated that this effect is due to the transfer of additional OAT3 transporters from an intracellular compartment to the basolateral cell membrane. Conversely, we showed that phosphorylation of CREB-1 and ATF-1 by PKA through the stimulation of 8-Br-cAMP (12 h) increased the promoter activity of OAT3. The present study revealed another possible molecular mechanism for the activation of PKA to stimulate the transport activity of OAT3, although it is unclear whether the rabbit OAT3 promoter has a CRE. It is therefore suggested that the short-term as well as long-term regulation of OAT3 is mediated by the activation of PKA. Further studies are needed to clarify the physiological and pharmacological implications of PKA signaling for the transport activity of OAT3.

In this study, a mutation in the CCAAT box reduced the luciferase activity, although no proteins bound to the CCAAT box in the EMSA experiments. Such a mutation may affect the function of CREB-1 and reduce the luciferase activity because the CCAAT box is in the vicinity of the CRE. It was reported that mutations that disrupted sequences located 5′ and 3′ of the CRE (TTACGTCA) in the promoter of the phosphoenolpyruvate carboxylkinase gene caused less severe reductions in basal promoter activity (Quinn et al., 1988), suggesting that not only the CRE but also the regions around the CRE are important for the constitutive transcriptional regulation of OAT3.

To clarify the interindividual variation in the pharmacokinetics of drugs, single-nucleotide polymorphisms (SNPs) in the coding region (cSNP) of drug transporters have been investigated (Ishikawa et al., 2004). Erdman et al. (2006) examined the allele frequency of several cSNPs in the OAT3 gene and transport characteristics and found that allele frequencies of cSNPs, which resulted in a completed loss of function, were very low (Xu et al., 2005; Erdman et al., 2006). In addition, the cSNP of OAT3 was considered to be unlikely to influence the pharmacokinetics of drugs (Nishizato et al., 2003; Sakurai et al., 2005). Recent studies have demonstrated that SNP in the promoter region (regulatory SNP; rSNP) is a candidate for the cause of the variation in the pharmacokinetics among individuals. Analyses of rSNP in the *multidrug resistance 1* gene suggested that several SNPs comprise a haplotype, influencing multidrug resistance 1 mRNA expression (Taniguchi et al., 2003; Takane et al., 2004). Screening of the rSNP of OAT3 is needed to identify

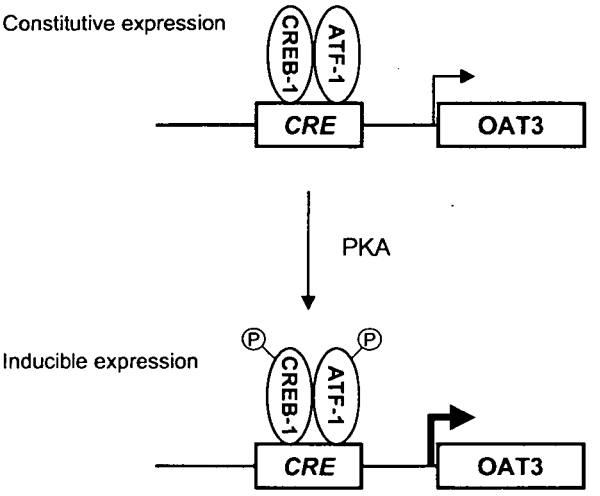

Fig. 7. Schematic model of transcriptional regulation of the human OAT3 gene. CREB-1 and ATF-1 bind to CRE and activate the transcription of the OAT3 gene (constitutive expression). PKA further activates the transcription of the OAT3 gene through phosphorylation of both CREB-1 and ATF-1 (inducible expression).

the genomic information affecting the mRNA level of OAT3. Alternatively, the regulation or modulation of CREB-1 may be involved in the interindividual difference in OAT3 mRNA levels.

In conclusion, the present study indicates that CRE is involved in the constitutive and inducible transcriptional regulation of the human OAT3 gene (Fig. 7). This is the first report to identify the *cis*-element for the regulation of the OAT family that is predominantly expressed in the kidney. It is possible that the regulation and modulation of CREB-1 affect OAT3 mRNA levels. To elucidate the interindividual variation in OAT3 mRNA expression, these factors should be taken into consideration.

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Short Communication

Interactions of Fluoroquinolone Antibacterials, DX-619 and Levofloxacin, with Creatinine Transport by Renal Organic Cation Transporter hOCT2

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: Interactions of DX-619, a novel fluoroquinolone antibacterial, and levofloxacin (LVFX) with the human renal organic cation transporter hOCT2 were studied. The intracellular accumulation of [^{14}C]creatinine in stable transfectants of HEK293 cells expressing hOCT2 (hOCT2-HEK293) as well as vector-transfected HEK293 cells (VEC-HEK293) was evaluated in the presence of DX-619 and LVFX at various concentrations. When added extracellularly, both DX-619 and LVFX inhibited the uptake of [^{14}C]creatinine (5 μM) by hOCT2-HEK293 cells in a dose-dependent manner. Unlike in hOCT2-HEK293 cells, the uptake in VEC-HEK293 cells was not inhibited by either fluoroquinolone suggesting that hOCT2 was specifically involved in the inhibition. The apparent IC_{50} value for the inhibition of [^{14}C]creatinine uptake in hOCT2-HEK293 cells was 1.29 ± 0.23 μM for DX-619 and 127 ± 27 μM for LVFX, indicating DX-619 to be ~100-fold more potent than LVFX at inhibiting the transport of [^{14}C]creatinine by hOCT2. A Dixon plot revealed that the inhibition by DX-619 of the hOCT2-mediated transport of [^{14}C]creatinine was competitive. Fluoroquinolone antibacterials have the ability to inhibit the transport of creatinine by hOCT2, with DX-619 being much more effective than LVFX.

Key words: DX-619; levofloxacin; hOCT2; creatinine; fluoroquinolone; organic cation transporter

Introduction

The level of creatinine in serum is the most commonly used clinical index of renal function. It is well recognized that the serum creatinine level is influenced by factors such as the patient's age and sex as well as the method of its determination. Because creatinine is mostly eliminated through urine *via* glomerular filtration, its renal clearance is regarded to be proportional to renal function. However, significant secretion of creatinine occurs at renal tubules, and may cause an overestimation of the glomerular filtration rate (GFR) especially in patients with decreased renal function.[1-3] Shemesh *et al.*[3] reported that the tubular secretion of creatinine is relatively constant regardless of a decreased glomerular filtration rate. Attempting to obtain a better estimation of the glomerular filtration rate by using creatinine clearance, Berglund *et al.*,[4] Burgess *et al.*[5] and van Acker *et al.*[6] administered trimethoprim[4] and cimetidine[5,6] to patients in which the tubular secretion of creatinine was blocked. Similarly, the application of cationic drugs to patients could lead to incorrect estimations of renal function because of decreased tubular secretion of creatinine.

In the renal proximal tubules, organic ion transporters mediate the tubular secretion of ionic drugs, thus contributing to the efficient extrusion of harmful substances from the body.[7-11] Human organic cation transporter 2, hOCT2, is the most abundant organic cation transporter so far reported in the human kidney.[12] hOCT2 should play significant roles in the basolateral translocation of some H_2-receptor antagonists[13,14] and biguanides[15] into epithelial cells in the renal proximal tubules. hOCT2 also accepts endogenous monoamines such as norepinephrine, serotonin, histamine and dopamine.[16] We found that creatinine is a specific substrate for the organic cation transporter hOCT2 expressed at the basolateral membranes of the human

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Abbreviations used are: hOCT2, human organic cation transporter 2; hOCT1, human organic cation transporter 1; GFR, glomerular filtration rate; LVFX, levofloxacin; MPP, 1-methyl-4-phenylpyridinium.

kidney, but not by the hepatic organic cation transporter hOCT1. In addition, the transport of creatinine by hOCT2 was inhibited by cationic drugs at around their clinical concentrations.[17] We also clarified that the transepithelial transport of creatinine across LLC-PK$_1$ cell monolayers was directional from the basolateral to apical side, and the characteristics of creatinine's uptake across basolateral membranes was comparable to that demonstrated in hOCT2-expressing HEK293 cells.[18]

DX-619 is a novel des-fluoro(6) quinolone highly active against gram positive bacteria.[19-24] DX-619 has two dissociation constants, pKa1 = 6.4 and pKa2 = 8.3, showing that the drug is a zwitterion in the physiological environment. Fukuda *et al.*[24] reported that the area under the concentration-time curve (AUC)/MIC ratio in the lungs for DX-619 was significantly higher than that for sitafloxacin and ciprofloxacin when tested in mice. However, a phase I clinical trial of DX-619 has not been completed yet, so very little is known about the pharmacokinetic properties of DX-619. We have previously clarified that levofloxacin (LVFX) is excreted into urine *via* tubular secretion in addition to glomerular filtration *via* specific transport system.[25-27] In addition, the tubular secretion of LVFX should be partly mediated by P-glycoprotein.[28] Furthermore, we clarified in cultured epithelial cells derived from pig kidney that LVFX interacts with the organic cation transport system at apical membranes but not at basolateral membranes at its therapeutic concentrations.[29-31] However, no report has been made so far regarding the interactions of fluoroquinolone antibacterials with hOCT2-mediated transport of creatinine. In the present study, we investigated interactions of DX-619 and LVFX with the hOCT2-mediated transport of creatinine, and then referred to its clinical significance.

Materials and Methods

Materials: DX-619, (−)7-[(3R)-3-(1-aminocyclopropyl)pyrrolidin-1-yl]-1-[(1R,2S)-2-fluoro-1-cyclopropyl]1,4-dihydro-8-methoxy-4-oxoquinoline-3-carboxylic acid, and LVFX (**Fig. 1**) were provided by Daiichi pharmaceuticals Co. Ltd. (Tokyo, Japan). [2-^{14}C]Creatinine hydrochloride (55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Creatinine was obtained from Nacalai Tesque (Kyoto, Japan). 1-Methyl-4-phenylpyridinium (MPP) iodide was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other compounds used were of the highest purity available.

Cell culture: hOCT2-expressing HEK293 cells (hOCT2-HEK293) and mock-transfectants established by the transfection of the plasmid vector pCMV6-XL4 into HEK293 cells (VEC-HEK293) in our previous study[17] were cultured in complete medium consisting of

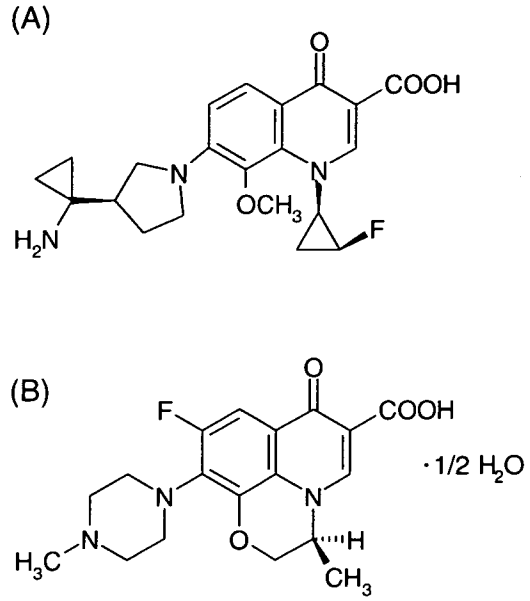

Fig. 1. Chemical structures of DX-619 (A) and LVFX (B).

Dulbecco's modified Eagle's medium with 10% fetal bovine serum in an atmosphere of 5% CO_2/95% air at 37°C. For uptake experiments, the cells were seeded onto poly-D-lysine-coated 24-well plates at a density of 2.0×10^5 cells per well. The cell monolayers were used at day 3 of culture for uptake experiments. In the present study, cells were used between the 73rd and 78th passages.

Uptake experiments using HEK293 transfectants: The uptake of [^{14}C]creatinine into cells was measured with monolayer cultures of hOCT2-HEK293 and VEC-HEK293 cells grown on poly-D-lysine-coated 24-well plates. The cells were preincubated with 0.2 mL of incubation medium for 10 min at 37°C. The medium was removed, and 0.2 mL of incubation medium containing 5 μM [^{14}C]creatinine was added. The composition of the incubation medium was as follows (in mM): 145 NaCl, 3 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, and 5 HEPES (pH 7.4). The medium was aspirated off at the end of the incubation, and the monolayers were rapidly rinsed twice with ice-cold incubation medium. The cells were solubilized in 0.5 mL of 0.5 N NaOH, and then the radioactivity in aliquots was determined by liquid scintillation counting using a Packard TRI-CARB 1900CA (PerkinElmer, Wellesley, MA, USA). The protein content of the solubilized cells was determined by the Bradford method using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine γ-globulin as a standard.[32] The uptake of [^{14}C]creatinine was determined in the presence of various concentrations of DX-619 and LVFX. The apparent IC_{50} values were calculated from inhibition plots based on the equation, $V = V_0/[1 + (I/IC_{50})^n]$, by a

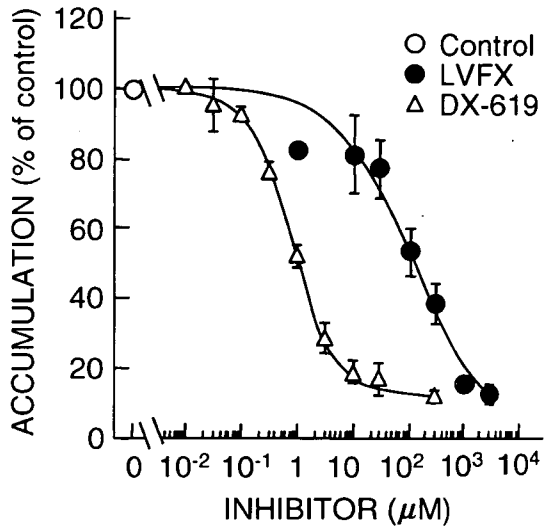

Fig. 2. Inhibition of hOCT2-mediated uptake of [^{14}C]creatinine by various concentrations of DX-619 and LVFX. HEK293 cells transfected with hOCT2 were incubated at 37°C for 2 min with 5 μM [^{14}C]creatinine (pH7.4) in the presence of DX-619 (in μM: 0.1, 0.3, 1, 3, 10, 30) or LVFX (in mM: 0.01, 0.03, 0.1, 0.3, 1, 3). Each point represents the mean ± SE of three separate experiments using three monolayers. When not shown, SE is included within the symbols.

nonlinear least squares regression analysis with Kaleidagraph Version 3.5 (Synergy Software, Reading, PA, USA).[17] V and V_0 are the uptake of [^{14}C]creatinine in the presence and absence of inhibitor, respectively. I is the concentration of inhibitor, and n is the Hill coefficient. For a Dixon plot, the uptake of [^{14}C]creatinine (5, 10 and 15 μM) was analyzed in the absence and presence of DX-619 (1 μM and 3 μM).

Statistical analyses: Data was analyzed statistically with Dunnett's test. P values of less than 0.05 were considered to be significant.

Results

Inhibition of hOCT2-mediated [^{14}C]creatinine uptake by various concentrations of DX-619 and LVFX: In order to assess the interaction of DX-619, a novel fluoroquinolone antibacterial, and LVFX with the hOCT2-mediated transport of creatinine, we measured the uptake of [^{14}C]creatinine by hOCT2-HEK293 and VEC-HEK293 cells in the absence and presence of various concentrations of DX-619 and LVFX. The hOCT2-HEK293 cells were confirmed to be able to transport [^{14}C]tetraethylammonium, a typical substrate for hOCT2, as well as [^{14}C]creatinine (data not shown). As demonstrated in our previous study,[17] the initial uptake rate of 5 μM [^{14}C]creatinine was obtained over a 2-min period with hOCT2-HEK293 cells. In the present study, both DX-619 and LVFX inhibited the uptake of 5 μM [^{14}C]creatinine for 2 min in a dose-dependent manner (**Fig. 2**). The IC_{50} values for the inhibition were 1.29 ± 0.23 μM and 127 ± 27 μM, respectively. **Table 1** gives a summary of the uptake in the absence (control) and presence of DX-619 (300 μM), LVFX (3 mM) and MPP (1 mM). Unlike in hOCT2-HEK293 cells, the uptake of 5 μM [^{14}C]creatinine in VEC-HEK293 cells was not inhibited by either of these fluoroquinolones.

Table 1. Effect of DX-619, LVFX and MPP on the uptake of [^{14}C]creatinine by VEC-HEK293 and hOCT2-HEK293 cells. The uptake of 5 μM [^{14}C]creatinine by VEC-HEK293 and hOCT2-HEK293 cells was evaluated for 2 min in the absence (control) and presence of DX-619 (300 μM), LVFX (3 mM) or MPP (1 mM). Data are expressed as the mean ± SE from three separate experiments. **, $p < 0.01$ compared to the control by Dunnet's test.

	VEC-HEK293 (nmol/mg protein/min) (% of control) n = 3	hOCT2-HEK293 (nmol/mg protein/min) (% of control) n = 3
Control	2.11 ± 0.37 (100.0)	16.58 ± 4.50 (100.0)
DX-619 (300 μM)	1.78 ± 0.18 (84.7)	1.93 ± 0.29** (11.7)
LVFX (3 mM)	1.61 ± 0.09 (76.4)	1.95 ± 0.16** (11.7)
MPP (1 mM)	1.60 ± 0.22 (76.0)	1.92 ± 0.24** (11.5)

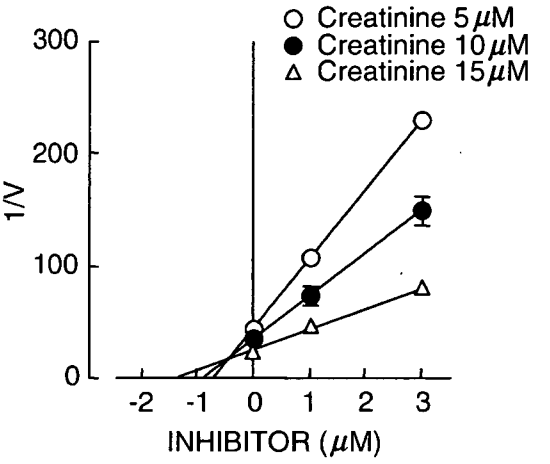

Fig. 3. Dixon plot of the inhibition of DX-619 of hOCT2-mediated transport of [^{14}C]creatinine. HEK293 cells transfected with hOCT2 were incubated at 37°C for 2 min with [^{14}C]creatinine (5 μM, open circle; 10 μM, closed circle; 15 μM, open triangle) in the absence and presence of DX-619 (1 μM or 3 μM). Each point represents the mean ± SE for three monolayers. When not shown, SE is included within the symbols.

Dixon plot of the interaction of DX-619 with hOCT2-mediated [^{14}C]creatinine transport: Next, we made a Dixon plot to clarify the type of interaction of the fluoroquinolones with the hOCT2-mediated transport of [^{14}C]creatinine. Cellular uptake of [^{14}C]creatinine (5 μM, 10 μM and 15 μM) was measured for 2 min in the absence and presence of DX-619 (1 μM and 3 μM). The Dixon plot clearly indicated that the inhibition by DX-619 of the hOCT2-mediated transport of [^{14}C]creatinine was competitive (**Fig. 3**).

Discussion

In the present study, both DX-619 and LVFX inhibited the transport of [^{14}C]creatinine in hOCT2-expressing HEK293 cells. Neither fluoroquinolone had an effect in mock-transfected HEK293 cells, suggesting that DX-619 interrupted the hOCT2-mediated transport of [^{14}C]creatinine specifically.

Serum creatinine is the most commonly used clinical marker of kidney function, because its clearance is mostly mediated by glomerular filtration. It is generally regarded that the serum creatinine level can be raised by reducing the glomerular filtration rate in patients with decreased renal functions. However, the usefulness of serum creatinine as a marker is limited by factors such as the age, sex, and amount of muscle of the patients as well as the method by which the level of creatinine is determined. Blood urea nitrogen (BUN) is also used as a clinical marker of renal function, although its value can be affected by the amount of protein ingested, protein catabolism, bleeding in the digestive tract, and the urea synthesis rate. Verho *et al.*[33] reported that the peak plasma concentration of LVFX was 6.4 μM after the oral administration of a 200-mg tablet in 6 healthy volunteers. In the present study, the peak plasma concentration was much lower than the IC_{50} of LVFX to inhibit the transport of [^{14}C]creatinine (127 $\pm$ 27 μM), suggesting that LVFX should not inhibit creatinine transport *via* hOCT2 at therapeutic concentrations. Given that similar peak plasma concentrations are obtained by administration of DX-619, the drug should markedly inhibit the transport of creatinine by hOCT2, because the IC_{50} value of DX-619 to inhibit the transport of [^{14}C]creatinine (1.29 $\pm$ 0.23 μM) was lower than the expected peak plasma concentration of DX-619. Actually, when DX-619 was administered to the healthy volunteers, an elevated serum creatinine level was observed with no apparent change in the level of BUN. In addition, the elevated serum creatinine level returned to normal with the systemic elimination of DX-619, suggesting that the tubular secretion of creatinine may be inhibited by DX-619 (unpublished observation in the clinical study).

In conclusion, DX-619 and LVFX inhibited the hOCT2-mediated transport of [^{14}C]creatinine in a competitive manner. These results suggest that the elevated serum creatinine levels after the administration of cationic drugs may be caused by the inhibition of tubular secretion of creatinine mediated in part by hOCT2.

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Induction of intestinal peptide transporter 1 expression during fasting is mediated via peroxisome proliferator-activated receptor α

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Shimakura, Jin, Tomohiro Terada, Hirofumi Saito, Toshiya Katsura, and Ken-ichi Inui. Induction of intestinal peptide transporter 1 expression during fasting is mediated via peroxisome proliferator-activated receptor α. *Am J Physiol Gastrointest Liver Physiol* 291: G851–G856, 2006. First published June 1, 2006; doi:10.1152/ajpgi.00171.2006.—We previously demonstrated that starvation markedly increased the amount of mRNA and protein levels of the intestinal H^+/peptide cotransporter (PEPT1) in rats, leading to altered pharmacokinetics of the PEPT1 substrates. In the present study, the mechanism underlying this augmentation was investigated. We focused on peroxisome proliferator-activated receptor α (PPARα), which plays a pivotal role in the adaptive response to fasting in the liver and other tissues. In 48-h fasted rats, the expression level of PPARα mRNA in the small intestine markedly increased, accompanied by the elevation of serum free fatty acids, which are endogenous PPARα ligands. Oral administration of the synthetic PPARα ligand WY-14643 to fed rats increased the mRNA level of intestinal PEPT1. Furthermore, treatment of the human intestinal model, Caco-2 cells, with WY-14643 resulted in enhanced PEPT1 mRNA expression and uptake activity of glycylsarcosine. In the small intestine of PPARα-null mice, augmentation of PEPT1 mRNA during fasting was completely abolished. In the kidney, fasting did not induce PEPT1 expression in either PPARα-null or wild-type mice. Together, these results indicate that PPARα plays critical roles in fasting-induced intestinal PEPT1 expression. In addition to the well-established roles of PPARα, we propose a novel function of PPARα in the small intestine, that is, the regulation of nitrogen absorption through PEPT1 during fasting.

Caco-2; SLC15A1; starvation; glycylsarcosine; small intestine

DIETARY PROTEINS ARE DEGRADED into a mixture of free amino acids and small peptides. A large number of studies have provided evidence that the absorption of protein digestion products in the small intestine occurs primarily in the form of small peptides rather than amino acids (22). Thus intestinal peptide transport is of major nutritional significance for the effective absorption of dietary amino nitrogen. Cellular uptake of di- and tripeptides is mediated via H^+-coupled peptide cotransporter 1 (PEPT1, SLC15A1), which is primarily expressed in the small intestine and slightly in the kidney (5). Because of its broad substrate specificity, PEPT1 can accept several peptidelike drugs such as oral β-lactam antibiotics (39) and plays important roles not only as a nutrient transporter but also as a drug transporter. A large number of functional studies using heterologous expression systems have demonstrated the molecular nature of its transport characteristics (6, 15, 20, 24). Furthermore, many studies have also been directed toward the regulation of PEPT1. For example, it has been reported that intestinal PEPT1 is regulated by various factors (1), including dietary conditions (28, 35, 42), hormones [insulin (10), thyroid hormone (2)], epidermal growth factor (27), some pharmacological agents (3, 9), and diurnal rhythm (29).

Among these factors, the dietary regulation of intestinal PEPT1 has been extensively investigated (14, 26, 28, 30, 31, 35, 42). It has been reported that short-term starvation markedly increased the amount of PEPT1 mRNA and protein expression (14, 30, 42) and uptake activity of dipeptides (42). The induction of PEPT1 expression might be an adaptive response against fasting for efficient absorption immediately after food is given again. This fasting-induced expression of PEPT1 altered the pharmacokinetic profiles of some drugs. Fasting increased the transport of the β-lactam antibiotic cefadroxil in an in situ loop experiment (26) and also increased pharmacokinetic parameters such as maximum plasma concentration and area under the plasma concentration-time curve of the β-lactam antibiotic ceftibuten in vivo (30).

Although these studies demonstrated the functional aspects of PEPT1 augmentation, the regulatory mechanisms remain to be clarified. In the present study, we assume that some metabolic signals direct this regulation. In the liver, an adaptive response to fasting has been well characterized (43). During fasting, lipolysis of stored triglycerides in adipose tissue is strongly activated, resulting in marked increase in plasma free fatty acid level. The released fatty acids are delivered to the liver, where they undergo β-oxidation for energy production. The peroxisome proliferator-activated receptors (PPARα, -β/δ, and -γ) are a family of nuclear receptors activated by fatty acid ligands (7, 12). PPARα acts as a nutritional state sensor and plays a pivotal role in the control of this adaptive response (16, 21) by inducing the transcription of genes such as the peroxisomal and mitochondrial β-oxidation pathways. Accordingly, PPARα is principally expressed in organs with a high capacity for fatty acid oxidation, such as heart, skeletal muscle, liver, and kidney. However, PPARα is also expressed in the small intestine (7) and is increased by fasting (8). This raises the possibility that PPARα might be responsible for the augmentation of PEPT1 during fasting. In the present study, we have investigated this possibility by examining PPARα activation through synthetic ligand and expression profiles of intestinal PEPT1 in fed and fasted PPARα-null mice.

MATERIALS AND METHODS

Materials. WY-14643 was purchased from Cayman Chemical (Ann Arbor, MI). Pioglitazone and GW-501516 were purchased from

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Alexis Biochemicals (Lausen, Switzerland) and Calbiochem (Darmstadt, Germany), respectively. [^{3}H]glycylsarcosine ([^{3}H]Gly-Sar; 18.5 GBq/mmol) was obtained from Moravek Biochemicals (Brea, CA). All other chemicals used were of the highest purity available.

Cell culture, treatment with PPAR ligands, and uptake study. Caco-2 cells were obtained from the American Type Culture Collection (ATCC CRL-1392) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% nonessential amino acids. Caco-2 cells were plated into 24-well plates (*day 1*) and, 2 or 9 days later, treated with the PPAR ligand WY-14643, pioglitazone, GW-501516, or DMSO (0.1%, as a control) for 24 h. The uptake experiment using [^{3}H]Gly-Sar with Caco-2 cells in a 24-well plate after WY-14643 treatment was performed according to our previous studies (38, 40).

Animal studies. Animal studies were performed in accordance with the Guidelines for Animal Experiments of Kyoto University. All protocols were previously approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (MedKyo 05189). Male Wistar rats (8 wk old) were obtained from Japan SLC. Male PPARα-null mice (B6.129S4-Ppara^{tm1Gonz} N12) and wild-type mice (C57BL/6) (8 wk old) were purchased from Taconic (Germantown, NY). The animals were fed a normal chow ad libitum except for the fasting experiments. In all experiments, the animals had free access to water. For determination of the effects of fasting, food was removed from cages 48 h before the animals were killed. For determination of the effect of PPARα ligand, fed rats were treated with WY-14643 (50 mg·5 ml^{-1}·kg^{-1}·day^{-1}, suspended in 0.5% methyl cellulose solution) or vehicle (5 ml/kg) by oral gavage for 5 days and killed after an additional 24 h. The small intestine (duodenum and upper part of the jejunum) was removed from the rats or mice under anesthesia. The kidney cortex was also removed from mice. The scraped intestinal mucosa and kidney cortex were rapidly frozen in liquid nitrogen for later preparation of total RNA.

Measurement of level of blood glucose and serum free fatty acids. Blood glucose level was quantified with the FreeStyle blood glucose monitoring system (Nipro, Osaka, Japan). Serum free fatty acid level was quantified with an enzymatic colorimetric assay (free fatty acid, Half-micro test; Roche Diagnostics, Penzberg, Germany).

Real-time PCR. Total RNA was isolated from Caco-2 cells, intestinal mucosa of rats and mice, and mice kidney cortex with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Isolated total RNA (250 or 500 ng) was reverse transcribed, and the reaction mixtures were used for real-time PCR. Real-time PCR was performed with an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) in a total volume of 20 μl containing a 2-μl aliquot of cDNA, 0.5 μM forward and reverse primers, 0.1 μM TaqMan probe, and 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems) under the following conditions: 50 cycles of 95°C for 15 s and 60°C for 60 s. The forward and reverse primers for mouse PEPT1 were 5′-CGTGCACGTAGCACTGTCCAT-3′ (positions 388–408) and 5′-GGCTTGATTCCTCCTGTACCA-3′(positions 433–453), respectively. The forward and reverse primers for rat PEPT1 were 5′-TGCACGTAGCACTGTCCATGA-3′ (positions 359–379) and 5′-CAGGGCTTGATTCCTCCTGTAC-3′ (positions 425–404), respectively. The sequence of the TaqMan probe was 5′-(6-FAM)TTGGCCTGGCCCTGATAGCCC(TAMRA)-3′, corresponding to positions 411–432 for mice, and 5′-(6-FAM)CGGCCTGGCCCTGATAGCCCT(TAMRA)-3′, corresponding to positions 381–404 for rats. The primer probe sets used for human PEPT1 (41) and rat Na$^+$-glucose cotransporter 1 (SGLT1) (13) were previously designed. The primer probe sets used for mouse and rat PPARα, mouse acyl-CoA oxidase (ACOX), rat Sp1, and rat Cdx2 were predeveloped TaqMan Assay Reagents (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also measured as an internal control with GAPDH Control Reagent (Applied Biosystems).

Data analysis. Data are expressed as means ± SE. The statistical significance of differences between the groups was analyzed with one-way ANOVA followed by Scheffé *F* post hoc testing in the experiment using Caco-2 cells and multiple concentrations of WY-14643 as a ligand. In other experiments, the nonpaired *t*-test was used. Two or three experiments were conducted, and representative results are shown.

RESULTS

Fasting-induced expression of intestinal PEPT1 is accompanied by increased mRNA level of PPARα in rats. In general, energy depletion by fasting causes a shift in whole body fuel utilization from glucose and fat in the fed state to almost exclusively fat. Under our experimental conditions, 48-h fasting led to reductions in body weight and blood glucose level and dramatic increment in serum free fatty acid level, reflecting the metabolic switching mentioned above (Fig. 1). Under this condition, we determined mRNA levels of intestinal PEPT1 and PPARα (Fig. 2). The transcription factor Sp1 and caudal-related homeobox protein Cdx2 were also measured because we recently demonstrated that PEPT1 is transcriptionally regulated by Sp1 and Cdx2 (33, 34). As expected, fasting significantly induced mRNA levels of PEPT1, consistent with our previous result (30). The expression level of PPARα was increased to twofold by fasting, whereas the increments in Sp1 and Cdx2 were smaller than that in PPARα.

Expression of PEPT1 mRNA is induced by PPARα ligand WY-14643 in Caco-2 cells. To assess the potential involvement of PPARα in PEPT1 expression, we treated an intestinal model system, Caco-2 cells just before and after confluence, for 24 h with the PPARα ligand WY-14643 and measured the expression levels of PEPT1 mRNA and the activity of [^{3}H]Gly-Sar uptake (Fig. 3). PEPT1 mRNA expression levels and the activity of [^{3}H]Gly-Sar uptake were significantly increased in response to WY-14643 in the cells of both stages. Thus it was suggested that the induction of PEPT1 mRNA by WY-14643 led to a concomitant increase of the transport function. Next,

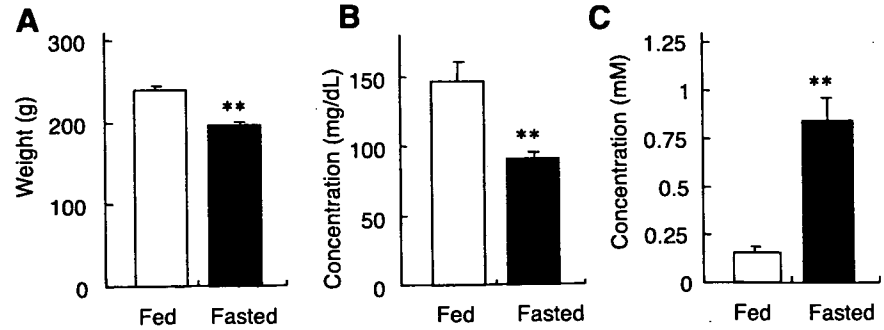

Fig. 1. Effects of fasting on body weight (*A*), blood glucose (*B*), and serum free fatty acids (*C*) in rats. Blood glucose and serum free fatty acid levels in 48-h fasted or fed rats were determined as described in MATERIALS AND METHODS. Data are means ± SE for 5 rats. **Significantly different from fed rats ($P < 0.01$).

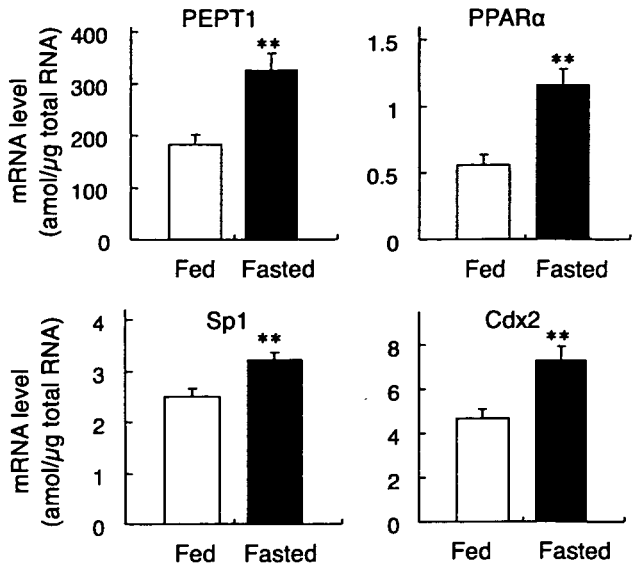

Fig. 2. Effects of fasting on mRNA levels of peptide cotransporter 1 (PEPT1), peroxisome proliferator-activated receptor (PPAR)α, Sp1, and Cdx2 in rat small intestine. Total RNA was isolated from the small intestine of 48-h fasted or fed rats, transcribed to cDNA, and subjected to real-time PCR analysis. The results corrected by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels are means ± SE for 5 rats. **Significantly different from fed rats ($P < 0.01$).

Caco-2 cells were treated with the PPARα ligand WY-14643 and, increasing concentrations of the PPARγ ligand pioglitazone and the PPARβ/δ ligand GW-501516 (Fig. 4). The activity of [^{3}H]Gly-Sar uptake increased only when PPARα ligand WY-14643 was administered.

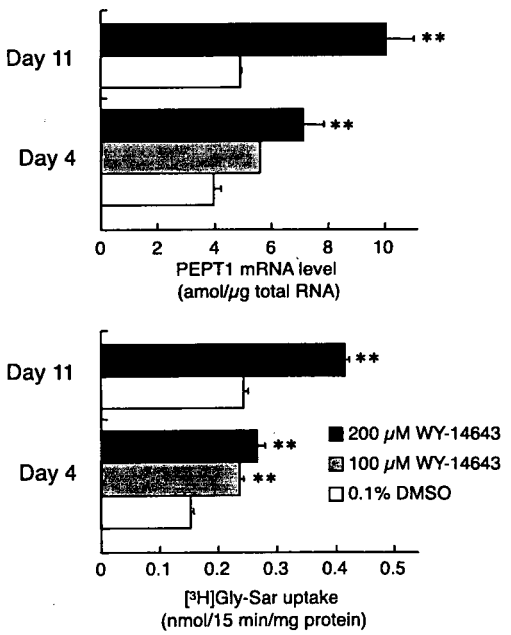

Fig. 3. Expression of PEPT1 mRNA and [^{3}H]glycylsarcosine ([^{3}H]Gly-Sar) uptake in Caco-2 cells treated with WY-14643. Twenty-four hours after WY-14643 treatment, Caco-2 cells were subjected to total RNA isolation or [^{3}H]Gly-Sar uptake experiment on *day 4* and *day 11*. The concentrations of WY-14643 were set to 100 and 200 μM on *day 4* and 200 μM on *day 11*. Total RNA was transcribed to cDNA and subjected to real-time PCR analysis. For the uptake experiment, Caco-2 cells were incubated with 25 μM [^{3}H]Gly-Sar for 15 min at 37°C. Data are means ± SE for 3 monolayers. Data of mRNA analysis are corrected by GAPDH levels. **Significantly different from control (0.1% DMSO) ($P < 0.01$).

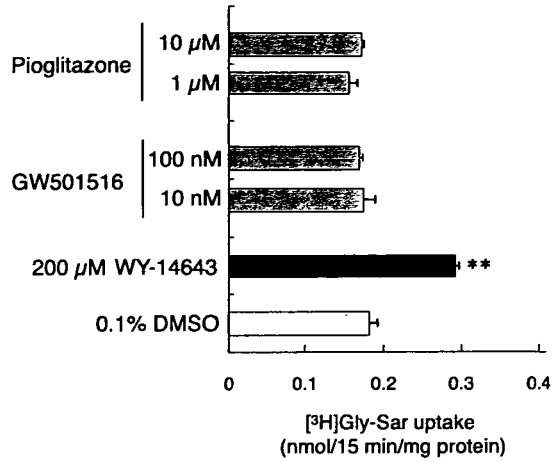

Fig. 4. [^{3}H]Gly-Sar uptake in Caco-2 cells treated with PPAR ligands for 24 h. WY-14643, pioglitazone, and GW-501516 were used as the ligands for PPARα, -γ, and -β/δ, respectively. The day after treatment (*day 4*), Caco-2 cells were incubated with 25 μM [^{3}H]Gly-Sar for 15 min at 37°C. Data are means ± SE for 3 monolayers. **Significantly different from control (0.1% DMSO) ($P < 0.01$).

Effect of WY-14643 on expression levels of intestinal PEPT1 mRNA in rats. We subsequently administered WY-14643 (50 mg/kg) orally to fed rats for 5 days to examine the effect of PPARα ligand on PEPT1 expression in vivo. For comparison, the mRNA levels of SGLT1 were also determined. SGLT1 is expressed at the brush-border membranes of intestinal epithelial cells (11) as PEPT1 but is reported to show no significant change in its expression level during fasting (14). As expected, mRNA levels of PEPT1 were significantly increased by WY-14643, whereas those of SGLT1 were not changed (Fig. 5).

Effect of fasting on expression levels of intestinal PEPT1 in PPARα-null mice. To confirm the contribution of PPARα to fasting-induced PEPT1 expression, we measured mRNA levels of PEPT1 in the small intestine of wild-type and PPARα-null mice (Fig. 6). Fasting induced the expression of PEPT1 and PPARα in wild-type mice as in rats, although the induction of PPARα was not statistically significant. In contrast, this fasting response of PEPT1 was completely abolished in PPARα-null mice, indicating the critical role of PPARα in this response.

Effect of fasting on expression levels of renal PEPT1 in PPARα-null mice. PEPT1 is also expressed in the kidney, although its expression level is much lower than that in the small intestine. Thus we investigated the effect of fasting on expression levels of PEPT1 and PPARα in the kidney (Fig. 7). For comparison, mRNA levels of ACOX, which is a peroxisomal β-oxidation enzyme and a representative PPARα target gene, were simultaneously determined. In contrast to the intestine, renal PEPT1 was not induced by fasting in either wild-type or PPARα-null mice, which is in agreement with our previous result in rats (30). ACOX expression was markedly enhanced, although the PPARα level did not show significant elevation. These results suggested that the lack of fasting-induced response of renal PEPT1 was not due to insufficient levels of endogenous ligands or PPARα.

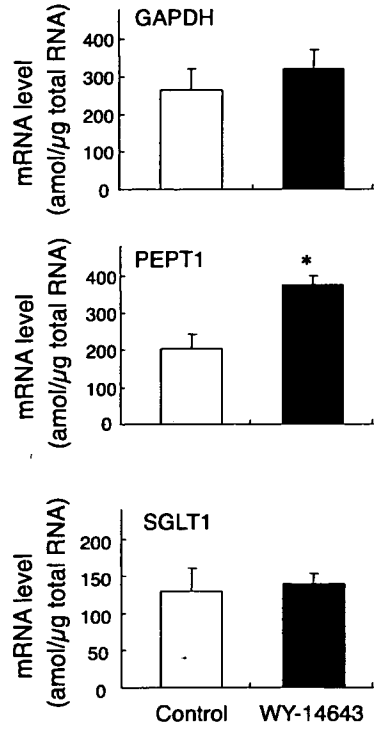

Fig. 5. mRNA levels of PEPT1, Na^+-glucose cotransporter (SGLT)1, and GAPDH in the small intestine of rats dosed with WY-14643 (50 mg/kg) for 5 days. Total RNA was isolated from the small intestine, transcribed to cDNA, and subjected to real-time PCR analysis. Data are means ± SE for 3 rats. *Significantly different from control ($P < 0.05$).

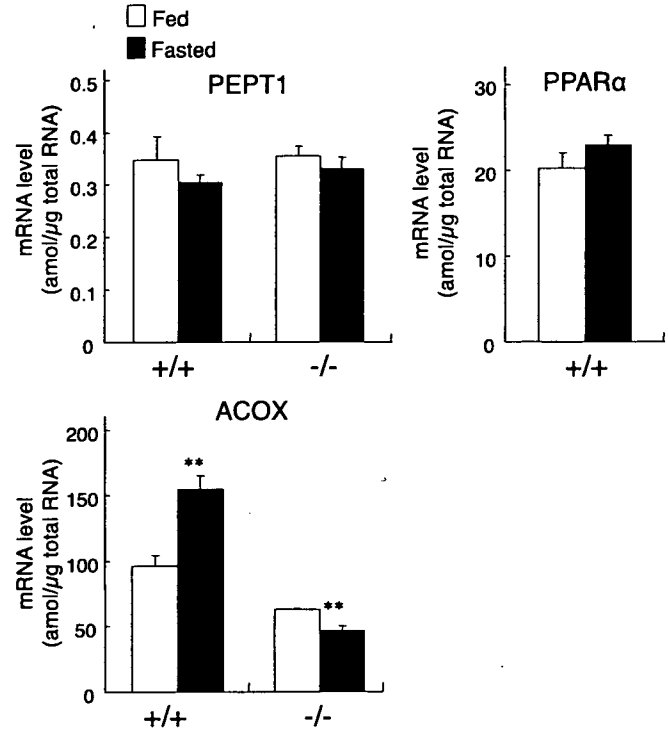

Fig. 7. mRNA levels of PEPT1, PPARα, and PPARα target gene acyl-CoA oxidase (ACOX) in the kidney of fasted or fed wild-type and PPARα-null mice. Total RNA was isolated from the kidney cortex of 48-h fasted or fed mice, transcribed to cDNA, and subjected to real-time PCR analysis. Data corrected by GAPDH levels are means ± SE for 6 mice. **Significantly different from fed mice of the same genotype ($P < 0.01$).

DISCUSSION

In the present study, we investigated the regulatory mechanism underlying the augmentation of intestinal PEPT1 in fasting. In agreement with previous results (4, 8), it was confirmed that the serum concentration of free fatty acids and the expression level of intestinal PPARα mRNA were increased under our experimental condition. We showed that PPARα played a critical role in the augmentation of intestinal PEPT1 as a fasting response mainly through PPARα-null mice. In support of the knockout mouse data, we used Caco-2 cells to show that the PPARα ligand WY-14643 upregulated PEPT1, because Caco-2 cells expressed endogenous PPARα (data not shown). Several studies have used Caco-2 cells as an intestinal model for activating PPAR target genes by synthetic PPARα ligands (19, 32, 37). Furthermore, the effect of WY-14643 on PEPT1 expression was also observed in vivo in rats. These findings led us to speculate that elevated free fatty acids serve as a metabolic signal and activate intestinal PPARα, resulting in the augmentation of PEPT1 in an adaptive response to fasting.

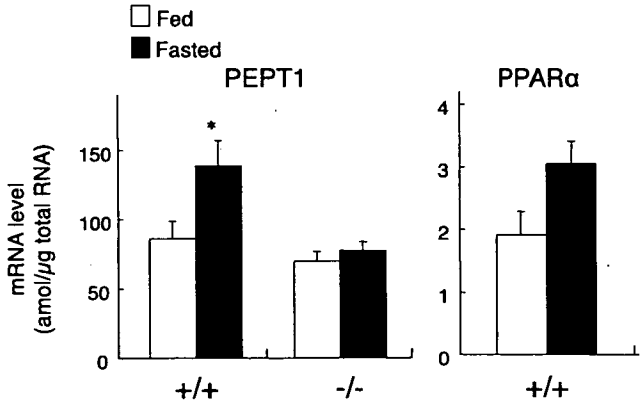

Fig. 6. mRNA levels of PEPT1 and PPARα in the small intestine of fasted or fed wild-type (+/+) and PPARα-null (−/−) mice. Total RNA was isolated from the small intestine of 48-h fasted or fed mice, transcribed to cDNA, and subjected to real-time PCR analysis. Data corrected by GAPDH levels are means ± SE for 6 mice. *Significantly different from fed mice of the same genotype ($P < 0.05$).

With regard to the roles of PPARα in the small intestine, there are several studies. PPARα is reported to be involved in lipid absorption through regulation of fatty acid binding protein (7). In addition, PPARα influences cholesterol absorption through modulation of ATP binding cassette transporter A1 activity in the intestine (18). PPARα also regulates other intestinal genes such as CYP1A1 (32), bile acid binding protein (19), retinol-binding protein (37), and 17β-hydroxysterol dehydrogenase type 11 (25). The presented function of PPARα, i.e., augmentation of PEPT1, is quite different from those previously demonstrated from the point of view that peptide absorption was implicated as a target. In fasting, sloughing of mucosal cells into the intestinal lumen is observed (23), resulting in atrophy of mucosa and decreased mucosal weight. It may be possible to speculate that increased PEPT1 minimizes the loss of nitrogen by efficient absorption of small peptides derived from sloughing cells or secreted hormones. Furthermore, induced PEPT1 will lead to efficient absorption of peptides immediately after food is given again. It has also

been reported that PPARα downregulates the hepatic genes involved in amino acid metabolism, leading to an overall decrease of amino acid degradation (17). It seems reasonable to suppose that PPARα functions to minimize the loss of body amino acids both by suppressing amino acid degradation and by increasing peptide absorption during fasting.

In contrast to the small intestine, no PEPT1 induction was observed in the kidney, suggesting different regulatory mechanisms for PEPT1 between them. This cannot be explained solely by the lack of activation of renal PPARα, because the expression of the representative PPARα target gene, ACOX, was markedly increased. PPARα and free fatty acids may be necessary but not sufficient for the induction of renal PEPT1.

In our previous and present study, the fasting response of PEPT1 was discussed mainly from the findings obtained in rats or mice. In humans, WY-14643 induced the expression of PEPT1 in a human intestinal cell line, Caco-2. This result, together with the fact that plasma free fatty acids are also increased during fasting in humans (36), suggested that a similar regulation of PEPT1 by PPARα might exist also in humans. To test the possibility that PPARα directly regulates the human PEPT1 promoter, we searched for the potential PPAR response element (PPRE) on the promoter as far as 10 kb upstream of the transcription start site and found several proposed PPREs. However, none of these sites, which were subcloned upstream of the PEPT1 proximal promoter, enhanced basal promoter activity in response to WY-14643 treatment in Caco-2 cells (data not shown). Alternatively, it is possible that PPARα stimulates the expression of other transcription factors that regulate PEPT1. mRNA levels of Sp1 and Cdx2 were increased in fasted rats. However, these factors may not be responsible for the augmentation of PEPT1 by PPARα because the PEPT1 promoter activity responsive to Sp1 or Cdx2 was not altered by WY-14643 treatment (data not shown). The functional PPRE and/or other regulatory region related to PPARα may be located in more distal regions or intronic regions.

The observation that PPARα regulates PEPT1 expression raises the question of whether the administration of PPARα agonist increases PEPT1 function in the clinical situation. This issue is important from the viewpoint of clinical drug-drug interaction because PPARα ligands belonging to the fibrate class have been used widely as hypolipidemic drugs. Oral dosing of WY-14643 in rats resulted in increase of PEPT1 expression. It is unclear whether clinically used PPARα ligands affect the expression of human intestinal PEPT1 in vivo. Further studies will be needed to estimate the change in the clinical pharmacokinetics of PEPT1 substrates and the impact on its therapeutic effects.

In conclusion, we demonstrated that PPARα plays a critical role in the augmentation of PEPT1 during fasting. We propose a novel function of PPARα in the small intestine, that is, the regulation of nitrogen absorption through PEPT1 as an adaptive response to fasting.

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Cisplatin and Oxaliplatin, but Not Carboplatin and Nedaplatin, Are Substrates for Human Organic Cation Transporters (SLC22A1–3 and Multidrug and Toxin Extrusion Family)

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ABSTRACT

We have examined the role of the human organic cation transporters [hOCTs and human novel organic cation transporter (hOCTN); SLC22A1–5] and apical multidrug and toxin extrusion (hMATE) in the cellular accumulation and cytotoxicity of platinum agents using the human embryonic kidney (HEK) 293 cells transiently transfected with the transporter cDNAs. Both the cytotoxicity and accumulation of cisplatin were enhanced by the expression of hOCT2 and weakly by hOCT1, and those of oxaliplatin were also enhanced by the expression of hOCT2 and weakly by hOCT3. The hOCT-mediated uptake of tetraethylammonium (TEA) was markedly decreased in the presence of cisplatin in a concentration-dependent manner. However, oxaliplatin showed almost no influence on the TEA uptakes in the HEK293 cells expressing hOCT1, hOCT2, and hOCT3. The hMATE1 and hMATE2-K, but not hOCTN1 and OCTN2, mediated the cellular accumulation of cisplatin and oxaliplatin without a marked release of lactate dehydrogenase. Oxaliplatin, but not cisplatin, markedly decreased the hMATE2-K-mediated TEA uptake. However, the inhibitory effect of cisplatin and oxaliplatin against the hMATE1-mediated TEA uptake was similar. The release of lactate dehydrogenase and the cellular accumulation of carboplatin and nedaplatin were not found in the HEK293 cells transiently expressing these seven organic cation transporters. These results indicate that cisplatin is a relatively good substrate of hOCT1, hOCT2, and hMATE1, and oxaliplatin is of hOCT2, hOCT3, hMATE1, and hMATE2-K. These transporters could play predominant roles in the tissue distribution and anticancer effects and/or adverse effects of platinum agent-based chemotherapy.

Four platinum-based anticancer drugs are currently registered for clinical use. *cis*-Diamminedichloroplatinum II (cisplatin) has been clinically used for over 30 years and continues to play an essential role in cancer chemotherapy against solid tumors of the prostate, bladder, colon, lung, testis, liver, and brain (Ho et al., 2003). However, severe nephrotoxicity limits its clinical application because it was reported that an increase in the serum creatinine concentration was observed in 41% of patients treated with a high dose of cisplatin (de Jongh et al., 2003). *cis*-Diammine(1,1-cyclobutanedicarboxylato)platinum II (carboplatin), *trans*-L-1,2-diaminocyclohexaneoxalatoplatinum II (oxaliplatin), and *cis*-diammineglycolatoplatinum (nedaplatin) are analogs of cisplatin and show a lowered nephrotoxicity compared with cisplatin. However, it is not clear why the nephrotoxicity of these analogs is low, although cisplatin induces severe nephrotoxicity. The chemical structures of platinum agents are shown in Fig. 1.

The mechanisms of cellular uptake and efflux of platinum agents are not fully understood. In our laboratory, the cisplatin uptake was reported to be mediated by specific transporter(s) in the renal tubular cells (Okuda et al., 1999). Recently, we reported that rat kidney-specific organic cation transporter 2 (rOCT2/Slc22a2) transported cisplatin and was responsible for cisplatin-induced nephrotoxicity (Yonezawa et al., 2005). Just after our report, hOCT2 was reported to transport cisplatin (Ciarimboli et al., 2005). However, it is not clear whether these analogs were transported by OCT2.

The organic ion transporter family (SLC22) consists of the organic anion transporter (OAT), OCT, and novel organic cation transporter (OCTN), which transport drugs,

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ABBREVIATIONS: r, rat; OCT, organic cation transporter; h, human; OAT, organic anion transporter; OCTN, novel organic cation transporter; MATE, multidrug and toxin extrusion; HEK, human embryonic kidney; TEA, tetraethylammonium; MPP, 1-methyl-4-phenylpyridium; PAH, *p*-aminohippurate; ES, [6,7-(*N*)]esterone-3-sulfate; ICP-MS, inductively coupled plasma-mass spectrometry; LDH, lactate dehydrogenase.

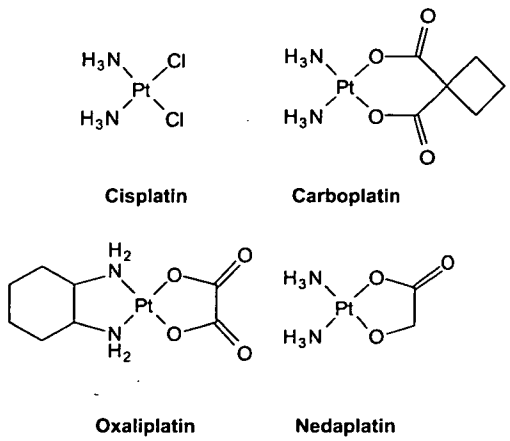

Fig. 1. Chemical structures of four platinum agents.

toxins and endogenous metabolites (Inui et al., 2000; Jonker and Schinkel, 2004). hOCT1 is expressed mainly in the liver, hOCT2 in the kidney (Gorboulev et al., 1997), and hOCT3 is predominantly expressed in the placenta and weakly in the intestine, heart, brain, and kidney (Kekuda et al., 1998). The substrate specificities of hOCT1, hOCT2, and hOCT3 have been considered to not be the same. hOCTN1 and hOCTN2 were expressed not only in the kidney but also in other tissues. They were expressed in the apical membrane of renal proximal tubules. Human multidrug and toxin extrusion 1 (hMATE1) was recently cloned and characterized (Otsuka et al., 2005). It was expressed in the liver, kidney, and skeletal muscle. Moreover, we cloned hMATE2-K, which is specifically expressed in the apical membrane of renal epithelial cells and transports cationic drugs (Masuda et al., 2006).

Based on these backgrounds, we hypothesized that organic cation transporters could transport four platinum drugs and play an important role in the cellular accumulation and toxicity of platinum agents. In the present study, we examined whether the basolateral cation transporters hOCT1, hOCT2, and hOCT3, and the apical cation transporters, hMATE1, hMATE2-K, hOCTN1, and hOCTN2, transported platinum agents and affected the cytotoxicity induced by these drugs.

Materials and Methods

Cell Culture and Transfection. HEK293 cells (CRL-1573; American Type Culture Collection, Manassas, VA) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (Whittaker Bioproducts Inc., St. Louis, MO) in an atmosphere of 5% CO_2-95% air at 37°C.

For a transient expression system, pCMV6-XL4 plasmid vector DNA (OriGene Technologies, Rockville, MO), pcDNA3.1(+) plasmid vector DNA (Invitrogen, Carlsbad, CA), or pBK-CMV plasmid vector DNA (Stratagene, La Jolla, CA) containing hOCT1, hOCT2, hOCT3, hMATE1, hMATE2-K, hOCTN1, hOCTN2, hOAT1, and hOAT3 cDNA was purified using Midi-V100 Ultrapure Plasmid Extraction Systems (Viogene, Sunnyvale, CA). The day before transfection, HEK293 cells were seeded onto poly-D-lysine-coated 24-well plates at a density of 2.0×10^5 cells per well. The cells were transfected with 800 ng of plasmid DNA per well in a combination of empty vector and transporter cDNA using 2 μl of Lipofectamine 2000 (Invitrogen) per well according to the manufacturer's instructions. The amount of transporter cDNA was 100 ng except in the experiment examining the transporter cDNA-dependence. Forty-eight hours after the transfection, the cells were used for the experiments.

Uptake Experiment. Cellular uptake of tetraethylammonium (TEA; 88.8 MBq/mmol; PerkinElmer Life Analytical Sciences, Boston, MA), [^{3}H]1-methyl-4-phenylpyridium acetate ([^{3}H]MPP; 2.7 TBq/mmol; PerkinElmer), *p*-[^{14}C]aminohippurate ([^{14}C]PAH; 1.9 GBq/mmol; PerkinElmer), and [6,7-^{3}H(N)]esterone-3-sulfate, ammonium salt ([^{3}H]ES; 2.1 TBq/mmol, PerkinElmer) was measured with HEK293 cells grown on poly-D-lysine-coated 24-well plates. The composition of the incubation buffer was as follows: 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, and 5 mM HEPES, pH 7.4 or pH 8.4 adjusted with NaOH. Experimental procedures were performed as described previously (Urakami et al., 2004).

To measure the cellular accumulation of the platinum agents, HEK293 cells were seeded on poly-D-lysine-coated 24-well plates. Cells were incubated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum containing cisplatin (Sigma), carboplatin (Sigma), oxaliplatin (a gift from Yakult Co. Ltd., Tokyo, Japan), and nedaplatin (LKT Laboratories, Inc., St. Paul, MN). After the incubation, the monolayers were rapidly washed twice with ice-cold incubation buffer containing 3% bovine serum albumin (Nacalai Tesque Inc., Kyoto, Japan) and then washed three times with ice-cold incubation buffer. The cells were solubilized in 0.5 N NaOH, and the amount of platinum was determined using inductively coupled plasma-mass spectrometry (ICP-MS) by the Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories, Ltd. (Wakayama, Japan). The protein content of the cell monolayers solubilized in 0.5 N NaOH was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Measurement of Released Lactate Dehydrogenase Activity. The cytotoxicity of the platinum agents was measured with HEK293 cells seeded on poly-D-lysine-coated 24-well plates. Cells were incubated with the medium containing platinum agent with or without cimetidine (Nacalai) for 2 h. After the removal of the medium, a drug-free medium was added. After incubation for 24 h, the medium was collected, and the lactate dehydrogenase (LDH) activity in the medium was measured using an LDH cytotoxicity detection kit (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions. Cytotoxicity was evaluated by measuring the LDH activity in the medium. The total LDH activity was defined as the LDH activity in the medium containing 1% Triton X-100. LDH release (percentage) represents (LDH activity − LDH activity of control)/(total LDH activity − LDH activity of control) × 100.

Statistical Analysis. Data are expressed as means ± S.E.M. Data were analyzed statistically using the unpaired Student's *t* test. Multiple comparisons were performed with Dunnett's two-tailed test after a one-way ANOVA. Probability values of less than 0.05 were considered statistically significant.

Results

Cytotoxicity of Cisplatin in HEK293 Cells Transiently Expressing hOCT2. We examined the expression level-dependent effect of hOCT2 on the cisplatin-induced cytotoxicity. When HEK293 cells transfected with 50 to 800 ng of hOCT2 cDNA per well were treated with 500 μM cisplatin for 2 h and subsequently cultured in normal medium for 24 h, the release of LDH into the culture medium was increased in a hOCT2 expression-dependent manner (Fig. 2A). Based on these results, we decided that cells were transfected with 100 ng of transporter cDNA. Moreover, cimetidine inhibited the cytotoxicity induced by 500 μM cisplatin in a concentration-dependent manner with an IC_{50} value of 109.3 ± 4.4 μM (mean ± S.E.M. of three separate experiments) in hOCT2-expressing cells (Fig. 2B).