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Characterization of Monocarboxylate Transport in Human Kidney HK-2 Cells

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Abstract: The objectives of this study were to characterize the expression and function of monocarboxylate transporters (MCTs) in human kidney HK-2 cells and to compare the expression of MCTs in HK-2 cells to that found in human kidney. mRNA and protein expression of MCTs were determined by RT-PCR and Western analyses, respectively, while immunofluorescence staining was used to determine the membrane localization of MCT1. The driving force, transport kinetics, and inhibition of two MCT substrates, D-lactate and butyrate, were characterized in HK-2 cells. mRNA of MCT1, -2, -3, -4 isoforms were present in HK-2 cells and in human kidney cortex. MCT1 was present predominantly on the basal membranes of HK-2 cells. The cellular uptake of D-lactate and butyrate exhibited pH- and concentration-dependence (D-lactate, K_m of 26.5 $\pm$ 2.2 mM and V_{max} of 72.0 $\pm$ 14.5 nmol mg^{-1} min^{-1}; butyrate, K_m of 0.8 $\pm$ 0.3 mM, V_{max} of 29.3 $\pm$ 2.5 nmol mg^{-1} min^{-1}, and a diffusional clearance of 2.1 μL mg^{-1} min^{-1}). The uptake of D-lactate and butyrate by HK-2 cells was inhibited by MCT analogues and the classical MCT inhibitors α-cyano-4-hydroxycinnamate, pCMB, and phloretin. The uptake of D-lactate and butyrate by HK-2 cells significantly decreased after transfection with small-interference RNA for MCT1. In summary, MCTs were present in both HK-2 cells and human kidney cortex, and HK-2 cells exhibited polarized MCT expression and pH-dependent transport of D-lactate and butyrate. Our results also support the usefulness of HK-2 cells as an in vitro model for studying monocarboxylate transport in renal proximal tubule cells.

Keywords: Monocarboxylate transporter (MCT); HK-2 cells; RNA interference; cellular transport

Introduction

The family of monocarboxylate transporters (MCTs) consists of 14 members (MCT1−14).[1] MCT1−4 have been shown to be proton coupled monocarboxylate transporters,[1,2] with similar, but not identical, substrates, including endogenous compounds, such as lactate, pyruvate, butyrate, and ketone bodies,[2−5] and exogenous compounds, such as fos-

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carnet, nateglinide, simvastatin acid, and lovastatin acid.[6−9] Among these four MCTs, MCT1, the first member identified, has been the most extensively characterized. MCT1 transports monocarboxylates in a pH-dependent manner with a moderate K_m for D-lactate in the mM range, and a lower K_m for its L-isomer. The tissue distribution of MCT1 is ubiquitous and includes intestine, colon, muscle, heart, brain, kidney, and red blood cells.[1,2] MCT2 is a pyruvate transporter,[3] with a much higher affinity for pyruvate than other MCTs. MCT2 has been detected in liver and brain, and it is believed that MCT2 is the main MCT for neuronal cells that use lactate as an energy source.[10,11] MCT3 has a limited tissue distribution and is restricted to the basal membrane of retinal pigment epithelium.[12,13] MCT4 is believed to be the major MCT in white skeletal muscles, although other MCT isoforms are also present in this tissue.[1,14] MCT4 has a much lower affinity for monocarboxylates in general than MCT1, with K_m values about an order of magnitude higher than that of MCT1.[14,15] Recently, MCT6 has been suggested as bumetanide transporter in a pH- and membrane-potential-sensitive but not a proton-gradient-dependent manner.[16] MCT6 also transports various drugs but not L-lactate or L-tryptophan.[16] MCT8 has been identified as a thyroid hormone transporter that does not transport lactate or aromatic amino acids.[17] MCT10 (also abbreviated as TAT1) has been identified as a sodium- and proton-independent aromatic amino acid transporter,[18] which is distributed mainly in the intestine and kidney in humans.[19]

The objective of this investigation was to characterize the expression and function of MCT1−4 in HK-2 cells, an immortalized normal human kidney cell line, and to compare expression to that in human kidney. MCTs are present in the human kidney, where they are involved in the reabsorption of short-chain fatty acids and ketone bodies.[1] Among these isoforms, MCT1 and MCT2 mRNA and protein have been detected in human kidney tissues.[2] mRNA expression of MCT5 and MCT7 has also been reported in kidney tissues,[20] although their roles as transporters have not been explored. The HK-2 cell line is a human proximal tubular epithelial cell line that was immortalized with human papilloma virus E6/E7 genes.[21] This cell line retains the functional characteristics of proximal tubular epithelium of human kidney, and it has been used to study proximal tubular cell injury and repair.[22] It has been shown that these cells retain functional transport activities of glucose transporters, as have been characterized in proximal tubular cells.[21] Other transporters have also been identified in this cell line, including P-glycoprotein.[23−25] HK-2 cells have been employed to study active vectorial transport[26] and secretion of protein products.[27] It has been reported that HK-2 cells can form a monolayer under culture conditions and may polarize into apical and basal membranes.[27] In this study, we have determined the expression of MCT isoforms in HK-2 cells using RT-PCR and Western blot, characterized the membrane localization of MCT using immunofluorescence staining, and studied the cellular transport of the MCT substrates D-lactate and butyrate. Our data reveal that (1) HK-2 cells have a MCT expression pattern highly similar to that found in normal human kidney, (2) MCT1 is distributed mainly on the basal membrane of HK-2 cell membrane with little expression on the apical membrane, (3) the transport of D-lactate and butyrate is pH-dependent, concentration-dependent, and inhibited by MCT transport inhibitors and substrates, and (4) MCT1 is the main transporter that is responsible for monocarboxylate transport in HK-2 cells.

Materials and Methods

L-Lactate, D-lactate, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), α-cyano-4-hydroxycinnamate (CHC),

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Table 1. Primers Designed for MCT Isoforms

isoform	direction	sequences	prod.
MCT1	forward	5′-TGGATGGAGAGGAAGCTTTCTAAT-3′	151
(SLC16A1)	reverse	5′-CACACCAGATTTTCCAGCTTTC-3′	
MCT2	forward	5′-GCCCACTGGCACAGGACTA-3′	251
(SLC16A7)	reverse	5′-CACAATAGCCCCACAGGACAT-3′	
MCT3	forward	5′-GGATGCGTTGAAGAACTATGAGATC-3′	213
(SLC16A8)	reverse	5′-CCGGGTTCCTCTGCAACA-3′	
MCT4	forward	5′-CACGGCATCGTCACCAACT-3′	200
(SLC16A3)	reverse	5′-ACAGCCTGGATAGCAACGTACAT-3′	

Table 2. Kinetic Parameters for D-Lactate and Butyrate Uptake in HK-2 Cells ($N = 9-12$, Mean ± SE)

	D-lactate	butyrate
K_m (mM)	26.5 ± 2.2	0.8 ± 0.3
V_{max} (nmol mg^{-1} min^{-1})	72.0 ± 14.5	29.3 ± 2.5
P (μL mg^{-1} min^{-1})		2.1 ± 0.4

p-chloro-mercuribenzoic acid (pCMB) butyrate, phloretin, rifampicin, probenecid, and tetraethylammonium chloride (TEA) were purchased from Sigma (St. Louis, MO). [2,3-^{3}H]-D-Lactate (specific activity 20 Ci/mmol) and [^{14}C]-butyrate (specific activity 56 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Human Kidney-2 (HK-2) cells were purchased from the American Type Culture Collection (Manassas, VA). Keratinocyte-Serum Free Medium was from Gibco BRL (Buffalo, NY). Biodegradable counting scintillate was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell Culture. HK-2 cells were maintained in keratinocyte-serum free medium (GIBCO-BRL 17005-042) with 5 ng mL^{-1} recombinant epidermal growth factor and 0.05 mg mL^{-1} bovine pituitary extract (without antibiotics added), with fresh medium added every 2−3 days. Cells were incubated at 37.0 °C in a 5% CO_2/95% air environment. Cells reached confluence after 5−7 days in culture. They were harvested for studies between passages 13 and 30. Cells were subcultured at a ratio of 1:4 using 0.05% trypsin and 0.53 mM EDTA. Cells were seeded in 35 mm (diameter) plastic culture dishes 2 days before uptake studies.

RT-PCR. Human kidney cortex from kidneys with no suitable transplant recipient were obtained from Upstate New York Transplantation Service (Buffalo NY), following approval from the Institutional Review Board, University at Buffalo. Total RNA was isolated from HK-2 cells and human kidney cortex with the SV total RNA Isolation system (Promega Madison, WI). First strand cDNA was synthesized from total RNA by reverse transcriptase (RT) using oligo dT primers with Brilliant Two step QRT-PCR core reagent kit (Cedar Creek, TX). PCR was performed using an Eppendorf Mastercycler gradient PCR system. Primers were designed by Primer Express software program. The primers specific to human MCT1, MCT2, MCT3, and MCT4 are listed in Table 1 with their gene name below their protein name. The nucleotide sequences for these primers were designed on the basis of the Genebank database. The PCR reaction mixtures contained 200 μM of each dNTP, 0.2 μM of each forward and reverse primer, and 0.5 unit/reaction TAQ DNA polymerase in 1X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3 (at 25 °C), 15 mM magnesium) through 40 cycles of 94 °C 1 min, 60 °C 1 min, 72 °C 1 min. The PCR products were separated by electrophoresis on 2% agarose gel and then stained by ethidium bromide and visualized under UV light.

Western Blotting. The expression of γ-glutamyl transpeptidase (GGT), Na^+/K^+-ATPase, MCT1, MCT2, and MCT4 in HK-2 cells and human kidney cortex was examined by Western blotting. HK-2 cells were harvested and lysed with lysis buffer (0.15 M NaCl, 5 mM EDTA, 1% Triton X100, 10 mM Tris-Cl pH 7.4, 5 mM DTT, 100 μM phenylmethanesulfonyl fluoride (PMSF) in isopropanol, 5 mM ϵ-aminocaproic acid) on ice for 30 min and then centrifuged at 16000*g* for 20 min at 4 °C. The supernatants were harvested for Western blot. Human kidney cortex tissues were homogenized and lysed similarly to HK-2 cells. The supernatants were separated on 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS and transferred to nitrocellulose membrane. Membranes were blocked by 5% (w/v) milk (Bio-Rad, Hercules, CA) in Tris-buffered saline containing 0.1% (v/v) Tween 20 at 4 °C overnight. The membranes were incubated with primary antibodies for GGT (Lab Vision Corp., Fremont CA), Na^+/K^+-ATPase (Sigma, St. Louis MO), MCT1 (1 μg mL^{-1}), MCT2 (1 μg mL^{-1}) and MCT4 (1 μg mL^{-1}) (US Biological, Swampscott, MA) 2 h at room temperature or overnight at 4 °C. The membranes were then incubated with specific horseradish peroxidase-conjugated secondary antibodies (Chemicon, Temecula, CA) for 1 h at room temperature. Immunoblots were developed with the enhanced chemiluminescence system (ECL) (Amersham International, U.K.).

Immunofluorescence Staining. HK-2 cells were maintained on Transwell permeable supports (Corning Inc., Acton, MA) at 37.0 °C in 5% CO_2/95% air environment, similar to previous studies.[27] Cells were cultured in Dulbecco's modified Eagle medium (D-MEM/F12, Invitrogen Corp., Carlsbad, CA) with 10% fetal bovine serum (FBS), 100 units of penicillin, and 100 μg mL^{-1} streptomycin. The medium was kept fresh daily. For immunofluorescence analysis, cells on Transwell permeable supports were washed with phosphate buffered saline (PBS) twice and then fixed with 5% formalin at room temperature for 10 min. Cells were permeabilized with 0.2% Triton X100 (Sigma, St. Louis, MO) in PBS (pH 7.5) for 5 min. Cells were washed and blocked with 2% of bovine serum albumin (BSA) for 30 min at room tempera-

ture. After washing, cells were incubated with primary antibodies for MCT1 (1:50 dilution), MCT2 (1:50), GGT (1:40 dilution), Na^+/K^+-ATPase (1:100 dilution), and Zonula Occludens 1 (1:50 dilution) (ZO-1, US Biological, Swampscott, MA) in 1% of BSA for 1 h at room temperature. After washing, either fluorescein isothiocyanate (FITC) or rhodamine-conjugated secondary antibodies were added and incubated for 45 min at room temperature. After washing, cells on Transwell permeable supports were mounted with DAPI medium (Vector laboratory, Burlingame, CA) on Superfrost/plus glass microscope slides (Fisher Scientific). Confocal microscopy was performed using a Leica TCS SP2 true confocal scanner (Leica Microsystem, Bannockburn, IL) equipped with Leica confocal software.

Uptake Studies. Growth medium was removed from the cell monolayers, and cells were washed three times with uptake buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, pH7.5). One milliliter of buffer containing 0.2 μCi of [^{14}C]-butyrate or 0.5μCi [^{3}H]-D-lactate was added to the dishes. For the kinetic studies, cells were incubated at room temperature for 0.5, 1, 5, 10, 15, 30, 60, and 120 min. Concentration-dependent uptake was examined by incubating cells with different concentrations of D-lactate or butyrate for 10 or 5 min, respectively. For the pH-dependent studies, the cells were incubated with uptake buffer with different pH values (pH 5.5, 6.0, 6.5, 7.0, 7.5), while for the sodium-dependent studies, the cells were incubated with sodium buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, pH 7.5) or sodium free buffer (137 mM *N*-methyl-D-glucamine, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, pH7.5). Inhibitors used in the cell studies included CHC, DIDS, probenecid, rifampicin, TEA, phloretin, L-lactate, D-lactate, and butyrate. The uptake was stopped by aspirating the buffer and washing three times with ice-cold stop buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, pH 7.5). The cells were solubilized by 1 mL of lysis buffer containing 0.3 N NaOH and 1% SDS. Samples were collected from each dish after 1 h, and the radioactivity was determined by mixing 3 mL of scintillation liquid with 200 μL of lysed sample and counted with a liquid scintillation counter (1900 CA, Tricarb liquid scintillation analyzer, Packard Instrument Co. Downers Grove, IL). Protein concentrations were determined by the bicinchoninic acid protein assay kit (BCA, Pierce Chemicals, Rockford, IL) with bovine serum albumin as standard. The results were normalized for the protein content of the cells in each dish, and accumulation was expressed as pmol or nmol mg protein^{-1} min^{-1}.

Directional Flux Study. HK-2 cells grown on Transwell permeable support were used for directional flux study using ^{3}H-D-lactate as the substrate. The studies were performed as previously described.[28] Briefly, the radiolabeled substrate was dosed at the apical or basal chamber at pH 6.0, room temperature; and the appearance of the substrate in the opposite chamber was used to calculate the rate of transport. The calculation of apparent permeability was carried out as previously described:[28]

$$P_{app} = \frac{\Delta Q}{\Delta t}\frac{1}{AC_0}$$

where $\Delta Q/\Delta t$ is the rate of the appearance of radiolabeled substrates in the receiving chamber; C_0 is the initial concentration of the radiolabeled compound in the donor chamber; and A is the cell monolayer surface area (4.71 cm^2).

RNA Interference. Two siRNA constructs that target on exon5 or exon3 of the mRNA of MCT1 were designed and purchased from Ambion Inc. (Ambion Inc., Houston, TX). The siRNA sequences are as follows: (sense/antisense, 5′- - - 3′) #1 GCAGUAUCCUGGUGAAUAAtt, UUAUUCACCAGGAUACUGCtg, and #2 CGAAUAAAGAUAGGAUUGGtt, CCAAUCCUAUCUUUAUUCGtt. The scrambled control RNA was also purchased from Ambion Inc. Cells were seeded in 6-well plates at 20−40% confluence 1 day before the transfection. Cells were transfected with siRNA at a final concentration of 20 nM using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After transfection, the cells were cultured for 48 h and the change of MCT1 expression was examined using Western blot analysis. The uptake of ^{14}C-butyrate and ^{3}H-D-lactate in transfected cells was also examined after transfection.

Data Analysis. The data are presented as mean ± SD. Statistical significance among means of more than two groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Differences with a p value of 0.05 or less were considered as statistically significant. Data analysis was performed using GraphPad Prism (GraphPad Inc., San Diego, CA).

The transport kinetic parameters, Michaelis−Menten constant K_m and maximum uptake rate V_{max}, were determined by fitting the data using weighted nonlinear regression analysis (Winnolin 2.1, Pharsight Corp., Cary, NC) and the following equations:

$$v = \frac{V_{max}C}{K_m + C} \quad (1)$$

$$v = \frac{V_{max}C}{K_m + C} + PC \quad (2)$$

Here v is the uptake rate of D-lactate or butyrate and C is the concentration of D-lactate or butyrate. P is the nonsaturable uptake clearance. The goodness of fit was determined by the sum of the squared derivatives, the residual plot, and the Akaike information criterion (AIC). The equation that provided the smallest CV% and AIC for the data was used in obtaining K_m and V_{max} parameters for the uptake data.

Results

Expression of MCT Isoforms mRNA in HK-2 Cells and Human Kidney Cortex. The expression of mRNA for MCT1−4 in HK-2 cells and in human kidney cortex from

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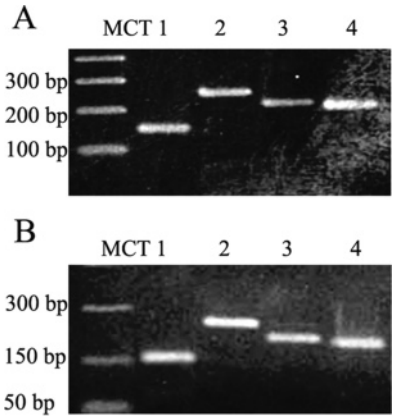

Figure 1. mRNA expression of MCT isoforms in HK-2 cells and human kidney cortex determined by RT-PCR: (A) MCT1, -2, -3, and -4 mRNA expression in HK-2 cells; (B) MCT1, -2, -3, and -4 mRNA expression in human kidney cortex.

two individuals was examined by RT-PCR with specific primers for each isoform. Our results showed that mRNA of MCT1, -2, -3, and -4 was expressed in HK-2 cells and human kidney cortex samples from two individuals (Figure 1). The PCR product of MCT3 was sequenced directly after being purified using Qiaquick PCR purification kit (Qiagen Inc, Valencia, CA). The resulting sequence was blasted against the NCBI database and corresponded to the human MCT3 gene.

Expression of MCT1, -2, and -4 Proteins in HK-2 Cells and in Human Kidney Cortex. We have found that specific kidney proximal tubule cell enzymes, GGT and Na^+/K^+-ATPase, were present in HK-2 cells (Figure 2A). The MCT1 and -2 proteins were expressed in both HK-2 cells and human kidney cortex (Figure 2B). The protein of MCT4 was also detected in HK-2 cells. The protein of MCT1 was detected as 42 kDa, which was similar to a previous determination using a similar antibody.[4] We also used red blood cells as a positive control and detected a single significant band around 42 kDa (data not shown). The protein of MCT2 was detected around 38 kDa, which was also similar to previous reported results using a similar antibody.[29] The MCT4 antibody detected a band at ~ 46 kDa, which was similar to a previously reported size of 45 kDa.[30]

MCT Distribution on HK-2 Cell Membranes. Using immunofluorescence staining, we demonstrated that kidney proximal tubule specific enzyme Na^+/K^+-ATPase was distributed mainly on basal plasma membranes (Figure 3A), while GGT was distributed mainly on apical cell membranes (Figure 3A). We also observed that ZO-1 was localized at the cell−cell junctions of HK-2 cells (Figure 3A), which suggested that tight junctions were formed among cultured cells. We observed that MCT1 was distributed exclusively on the basal membrane of HK-2 cells, with minimal

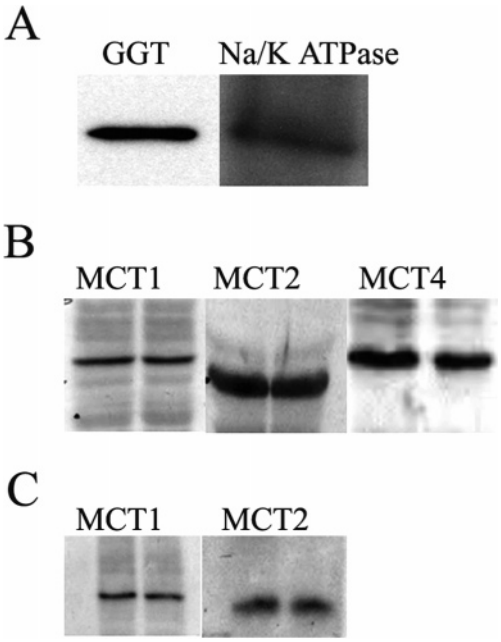

Figure 2. Western blot analysis of GGT, Na^+/K^+-ATPase, MCT1, MCT2, and MCT4 expression in HK-2 cells and human kidney cortex: (A) GGT (90 kDa) and Na^+/K^+-ATPase (105 kDa) expression in HK-2 cells; (B) MCT1 (44 kDa), MCT2 (38 kDa), and MCT4 (46 kDa) expression in HK-2 cells; (C) MCT1 and MCT2 protein expression in human kidney cortex from two individuals.

localization on the lateral and apical membranes (Figure 3A). The colocalization of MCT1 with Na^+/K^+-ATPase on the cell membranes further suggested that MCT1 was distributed on the cell basal membranes (Figure 3B). There was more nonspecific filter binding with rhodamine staining than with the FITC staining, resulting in a greater background staining. The immunofluoresecence staining of other MCT isoforms, MCT2, MCT4, and MCT6, was not successful. The MCT2 antibody produced a significant but weak stain that was greater on basal membranes than lateral and apical membranes (data not shown). The staining with the MCT4 antibody was weak or negligible. The immunofluoresecence staining for all the proteins tested was compared to their isotype controls, where 2% of bovine serum albumin (BSA) was incubated with the cells instead of the primary antibody and followed by a corresponding secondary antibody.

MCT-Mediated Cellular Uptake of D-Lactate and Butyrate. Time Course of D-Lactate and Butyrate Uptake. The uptake of 25 nM [^{3}H]-D-lactate in HK-2 cells was examined for up to 2 h at room temperature (Figure 4A) and was found to be linear up to 20 min. Therefore a 10 min incubation time was used to determine the initial uptake rate of D-lactate. The uptake of 0.1 mM [^{14}C]-butyrate in HK-2 cells was examined for up to 1 h at room temperature (Figure 4B) and was found to be linear for up to 5 min. Subsequently, a 5 min incubation time was used to determine the initial uptake rate of butyrate.

The Effect of Extracellular Sodium and pH on D-Lactate and Butyrate Uptake. The effect of extracellular

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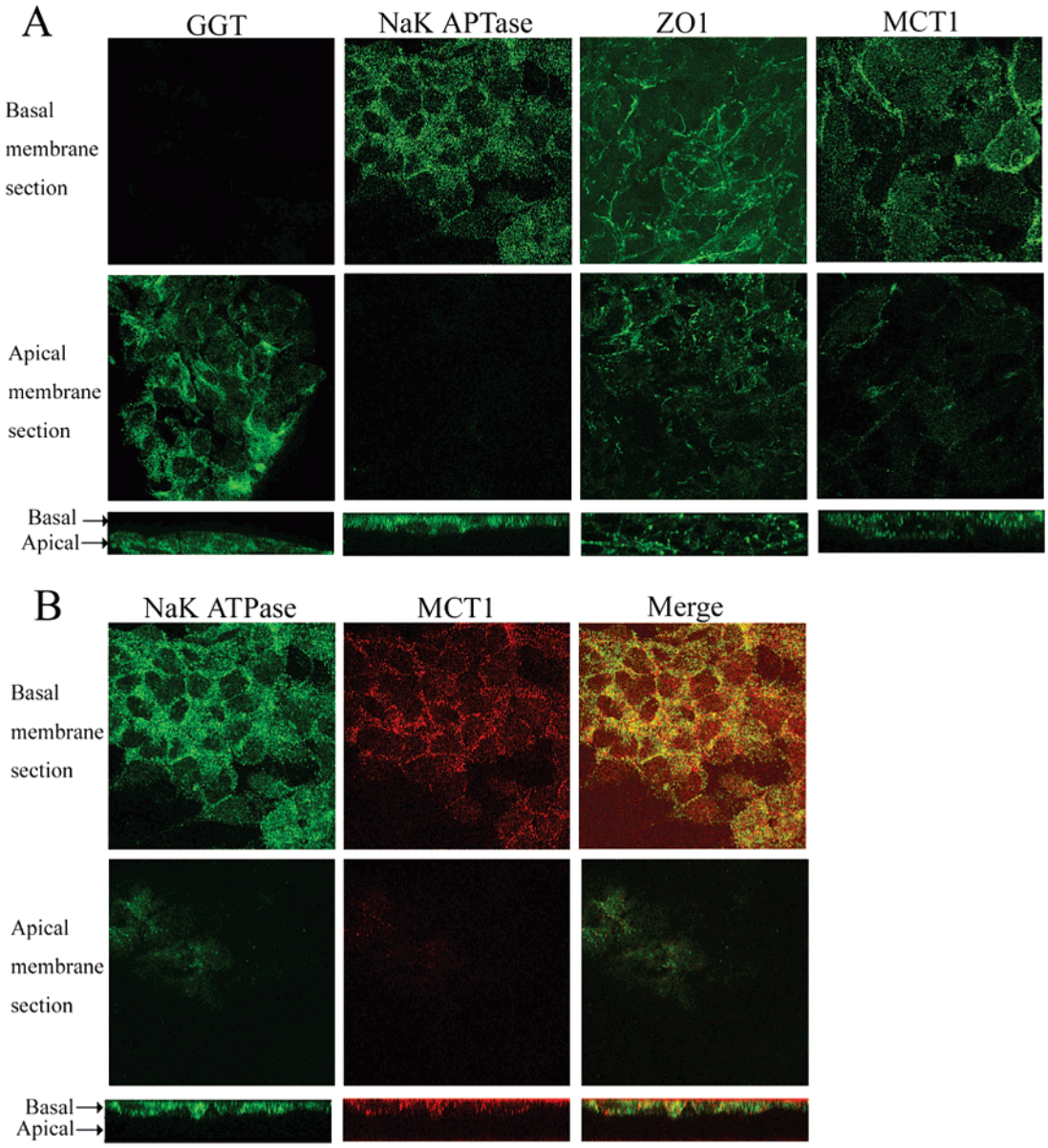

Figure 3. Protein distribution of Na^+/K^+-ATPase, GGT, ZO1, and MCT1 in HK-2 cells. The top two panels are representative *XY* sections of confocal microscopy; the bottom panels are representative *XZ* sections. (A) Na^+/K^+-ATPase is predominantly localized on the basal membrane of HK-2 cells; GGT is predominantly localized on the apical membrane of HK-2 cell; ZO-1 protein, a marker for tight junctions, is mainly localized between cell junctures; MCT1 (FITC-conjugated antibody) is distributed predominantly on the basal membrane of HK-2 cells. (B) The overlay of MCT1 (red, stained with rhodamine-conjugated antibody) and Na^+/K^+-ATPase (green). The two proteins are distributed on the same side of the cell membrane, and they are partially colocalized.

pH on D-lactate or butyrate uptake by HK-2 cells was examined by incubating cells with buffers of different pH values containing [^{3}H]-D-lactate (25 nM) or [^{14}C]-butyrate (0.1 mM) for 10 min or 5 min at room temperature, respectively. The uptake rates of D-lactate or butyrate at pH 5.5, 6.0, 6.5, and 7.0 were all statistically significantly higher than that at pH 7.5 (Figure 5). The maximal uptake rates were observed at pH 5.5. Uptake of D-lactate or butyrate was slightly decreased in the absence of sodium ions, at either pH 7.5 or pH 6.0, but the differences were not statistically significant (data not shown).

Concentration Dependence of D-Lactate and Butyrate Uptake. The concentration-dependent uptake of D-lactate or butyrate by HK-2 cells was determined at an extracellular pH of 6.0 over a concentration range of 0.1−50 mM (Figure 4C,D). The Eadie−Hofstee plot for D-lactate suggested a single transporter-mediated process (Figure 4E); however, the Eadie−Hofstee plot for butyrate suggested two transporters or one transporter plus a non-transporter-mediated process (Figure 4F). These conclusions were supported by nonlinear regression analysis of the data. D-Lactate uptake data was best fitted to a single Michaelis−Menten equation (eq 1) with fitted K_m and V_{max} values of 26.5 ± 2.2 mM and 72.0 ± 14.5 nmol mg^{-1} min^{-1}, respectively. Butyrate uptake was

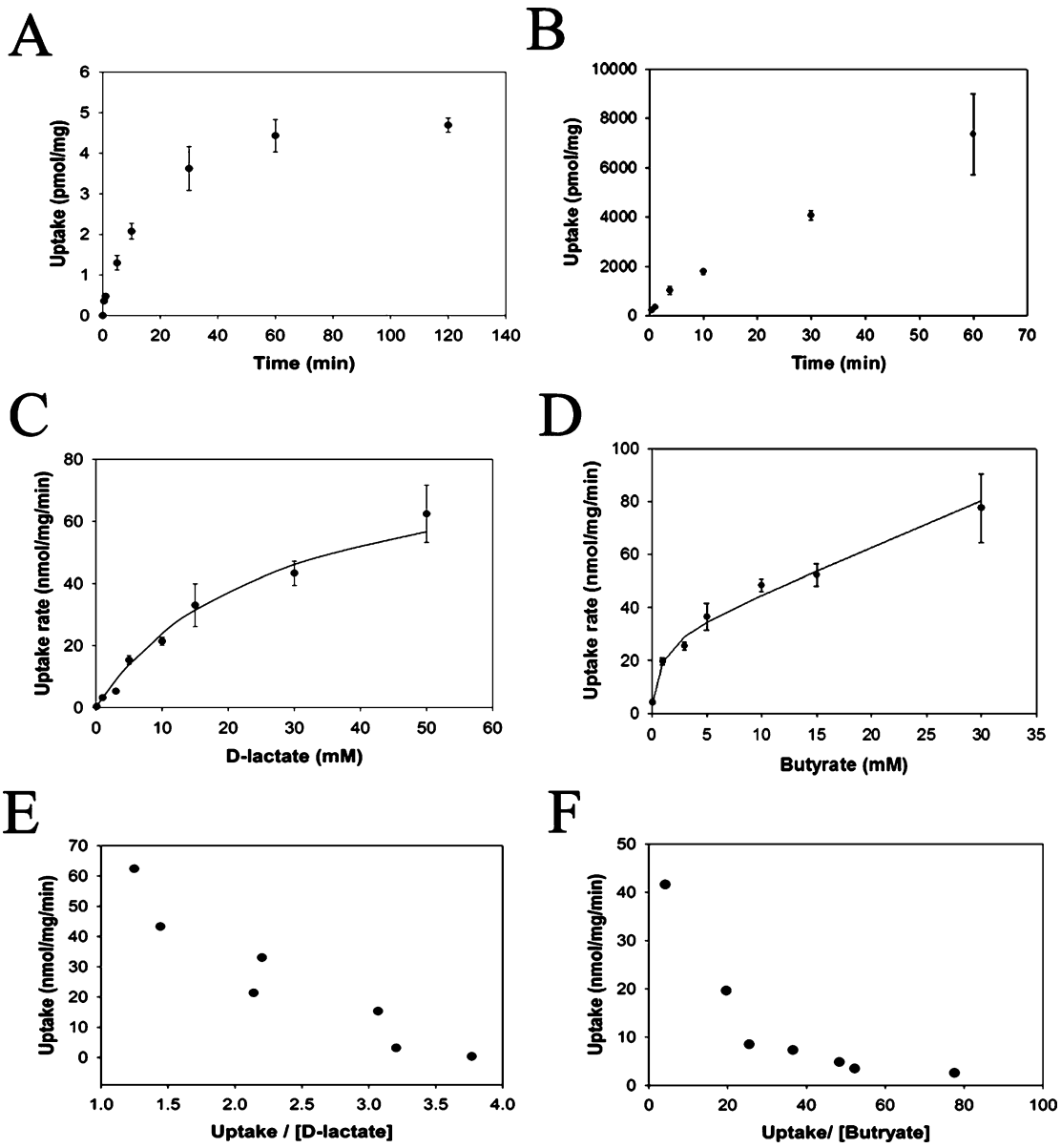

Figure 4. D-Lactate and butyrate uptake in HK-2 cells. (A) Time course of D-lactate uptake by HK-2 cells. (B) Time course of butyrate uptake by HK-2 cells. (C) Concentration-dependent uptake of D-lactate by HK-2 cells. (D) Concentration-dependent uptake of butyrate by HK-2 cells. Data were presented as mean ± SD; —, fitted line; $N = 9-12$. (E) Eadie−Hofstee plot for D-lactate uptake in HK-2 cells (representative plot of triplicate studies). (F) Eadie−Hofstee plot for butyrate uptake in HK-2 cells (representative plot of triplicate studies).

best described by a Michaelis−Menten equation plus a nonsaturable clearance (eq 2) with K_m of 0.8 ± 0.3 mM, V_{max} of 29.3 ± 3.5 nmol mg^{-1} min^{-1}, and a nonsaturable clearance P of 2.1 ± 0.4 μL mg^{-1} min^{-1}.

Effects of Inhibitors on D-Lactate and Butyrate Uptake. The effects of various inhibitors on [^{3}H]-D-lactate (0.1 mM) or [^{14}C] butyrate (0.1 mM) uptake by HK-2 cells were examined at an extracellular pH of 6.0 at room temperature. Cells were incubated with 0.1 mM [^{3}H]-D-lactate or 0.1 mM [^{14}C] butyrate in the presence or absence of CHC (2 mM), DIDS (2 mM), phloretin (0.5 mM), sodium butyrate (5 mM), TEA (5 mM), rifampicin (0.5 mM), probenecid (0.5 mM), and L-lactate (5 mM). The uptake of D-lactate by HK-2 cells was significantly inhibited by CHC, phloretin, butyrate, probenecid, rifampicin, DIDS, and L-lactate, but not by TEA (Figure 6A). The uptake of butyrate by HK-2 cells was significantly inhibited by CHC, phloretin, probenecid, D-lactate, and L-lactate, but not by rifampicin, DIDS, and TEA (Figure 6B). The MCT inhibitor pCMB could inhibit the uptake of D-lactate (Figure 6C) or butyrate (Figure 6D) to a similar degree as phloretin and CHC.

The Results of Directional Flux Study with D-Lactate. The apparent permeability of D-lactate was measured as follows: $P_{app,BA}$ (apparent permeability of basal to apical) was 0.96 ± 0.13 × 10^{-5} cm/s; and $P_{app,AB}$ (apparent permeability of apical to basal) was 0.38 ± 0.29 × 10^{-5} cm/s. The ratio $P_{app,BA}/P_{app,AB}$ for D-lactate was 3.7 ± 2.3; however, that for mannitol was 1.0 ± 0.2.

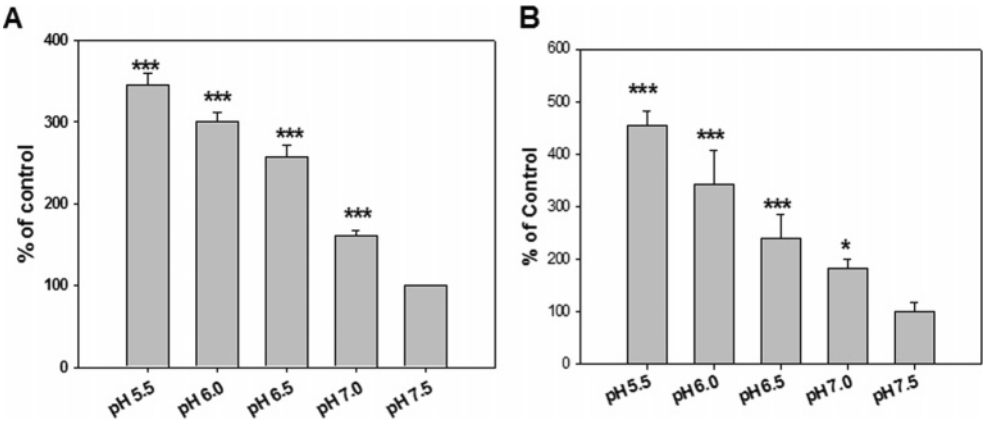

Figure 5. pH-dependence of butyrate (2.5 nM) (A) and D-lactate (0.1 mM) (B) uptake by HK-2 cells. All data were normalized to control (pH 7.5) and plotted as mean ± SD. (***) $p < 0.001$, (*) $p < 0.1$, one-way ANOVA followed by Dunnett's test ($N = 3-6$).

The Results of MCT1 siRNA Treatment on HK-2 Cells. The two siRNA used in the study decreased the protein expression of MCT1 in HK-2 cells (Figure 7A), with the MCT1 protein almost completely absent following #1 siRNA treatment. Using D-lactate as the substrate, the uptake was significantly suppressed in two siRNA treatment groups (Figure 7B). Similar results were observed when butyrate was used as the substrate (Figure 7C).

Discussion

The HK-2 cell line was established from normal human kidney cells immortalized by the human papilloma virus E6/E7 genes.[21] These cells retain a phenotype representative of well-differentiated proximal tubule cells, including epidermal growth factor dependent growth, presence of proximal tubule cell enzymes (alkaline phosphatase, γ-glutamyl transpeptidase, leucine aminopeptidase, and acid phosphatase), as well as sodium-dependent/phlorizin-sensitive glucose transport.[21] We have also studied these enzymes and the sodium-dependent/phlorizin-sensitive glucose transport in our laboratory and confirmed the results from the literature (Y.L. and M.E.M., unpublished data). The kidney proximal tubule cell enzymes, GGT and Na^+/K^+-ATPase, were also identified in HK-2 cells in this study. We observed that GGT was expressed predominantly on the apical membrane of HK-2 cells, while Na^+/K^+-ATPase was expressed on the basal membrane of HK-2 cells. GGT has been reported to be localized to the apical membrane of normal kidney proximal tubule cells, with little or no expression in the distal tubule, while Na^+/K^+-ATPase is distributed on the basolateral membrane of normal kidney proximal tubule cells.[31,32] Thus, our data demonstrates that HK-2 cells form cell monolayers with polarized protein distribution on cell membranes, similar to that found in kidney proximal tubule cells.

This investigation represents the first report examining the expression and function of MCTs in HK-2 cells and comparing MCT expression in HK-2 cells and human kidney cortex. We found that mRNA and protein expression of MCT1 and MCT2 are present in both human kidney cortex and HK-2 cells. MCT1 and MCT2 has been detected in mammalian kidney using indirect immunofluorescence;[4,33] recent studies also demonstrated the protein expression of MCT1 and MCT2 in human kidney.[29] Using confocal imaging, we observed that MCT1 was colocalized with Na^+/K^+-ATPase on the basal membrane of HK-2 cells. This finding is supported by the results of studies examining the directional flux of D-lactate across HK-2 cells monolayer. We observed that basal-to-apical permeability was greater than apical-to-basal permeability with a permeability ratio of 3.7, suggesting facilitated uptake at the basal membrane. This observation is similar to a previous study, where MCT1 has been shown, using immunohistochemical methods, to be on the basal membrane of rat kidney cortex tissues.[33] In that study, the authors also identified that MCT2 was distributed mainly on the basal membrane of distal tubule cells, not proximal tubule cells;[33] however, MCT2 protein was also detected on the basal membrane fraction of kidney cortex tissues in that study. In our investigation, we detected MCT2 protein expression in HK-2 cells. We have detected the mRNA of MCT3 and MCT4 in both human kidney cortex and HK-2 cells. It was surprising to detect mRNA of MCT3 in kidney cortex tissues and HK-2 cells, since its tissue distribution has been reported to be restricted to the basal membrane of retinal pigment epithelium and choroid plexus;[13] however, the protein expression of MCT3 was not examined in this study. MCT4 has been reported to be present mainly in white skeletal muscles mediating the efflux of lactate,[14] but has not been detected in human kidney by Northern blot.[20] The discrepancy between this study and the previous one may be due to the sensitivity of the two methods

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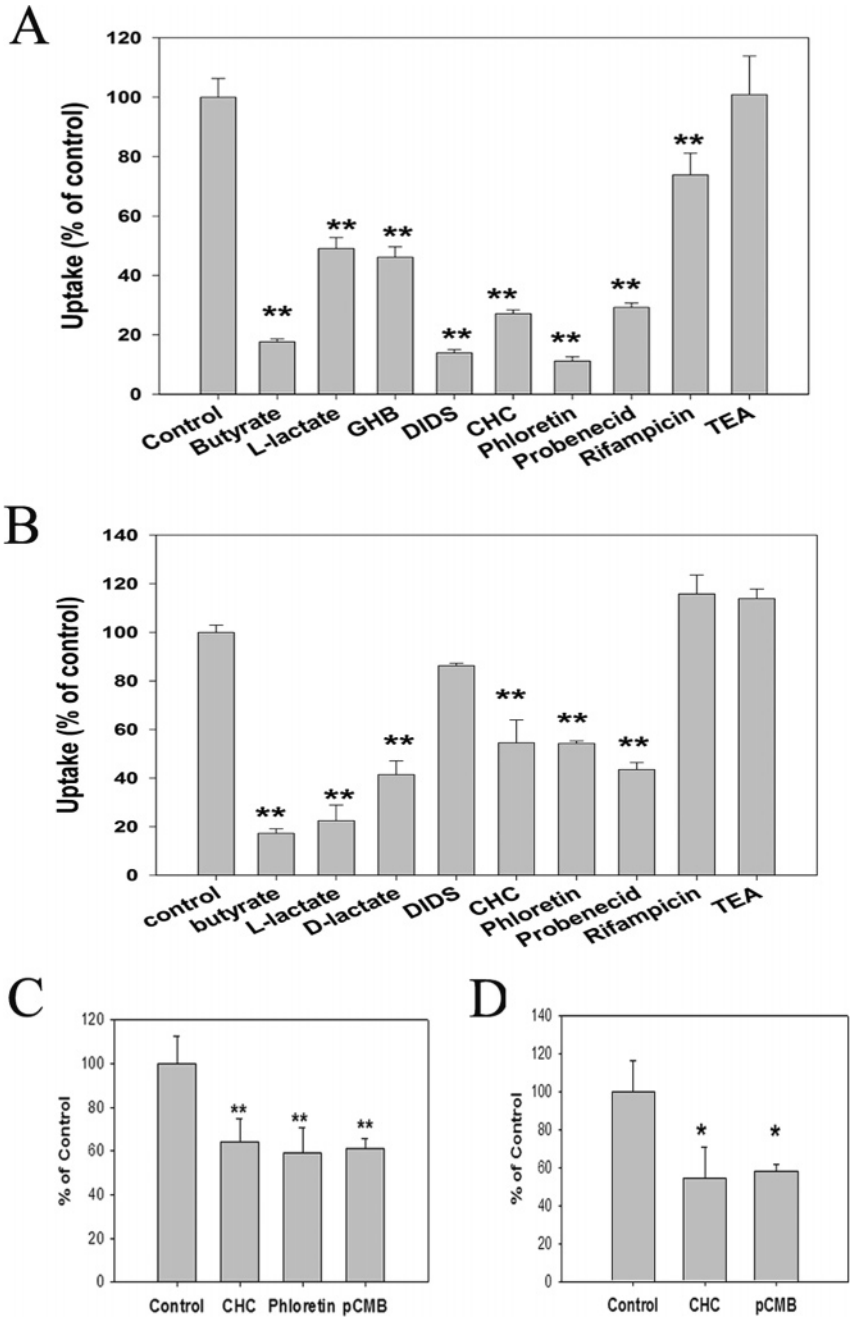

Figure 6. Inhibitors of D-lactate and butyrate uptake. (A) [^{3}H]-D-Lactate (0.1 mM) uptake in the presence or absence of inhibitors; the uptake was performed at pH 6.0 for 10 min, at room temperature. (B) [^{14}C]-Butyrate (0.1 mM) uptake in the presence or absence of inhibitors; the uptake was performed at pH 6.0 for 5 min, at room temperature. (C) Effects of pCMB, CHC, and phloretin on the uptake of [^{3}H]-D-lactate (0.1 mM); the uptake time was 1 min. (D) Effects of pCMB and CHC on the uptake of [^{14}C]-butyrate (0.1 mM); the uptake time was 5 min. Each bar represents the mean ± SD, $N = 3-6$ (representative plot of duplicate studies); one-way ANOVA followed by Dunnett's test, (***) $p < 0.001$.

used, namely, RT-PCR and Northern blot, respectively. Additionally, recent studies have detected MCT4 protein in human kidney tissues,[29,30] and our Western blot results in HK-2 cells agreed with these findings.

The transport of D-lactate has been shown to be both pH- and sodium-dependent in the literature.[34] A sodium-dependent transport of L-lactate and D-lactate has been found also in the rabbit kidney, and has recently been confirmed to be mediated by solute carrier gene 5A8 (SLC5A8).[35] In our studies, only pH-dependent transport was observed, with little or no sodium-dependent transport based on the uptake results. However, we could not rule out the contribution of a sodium-

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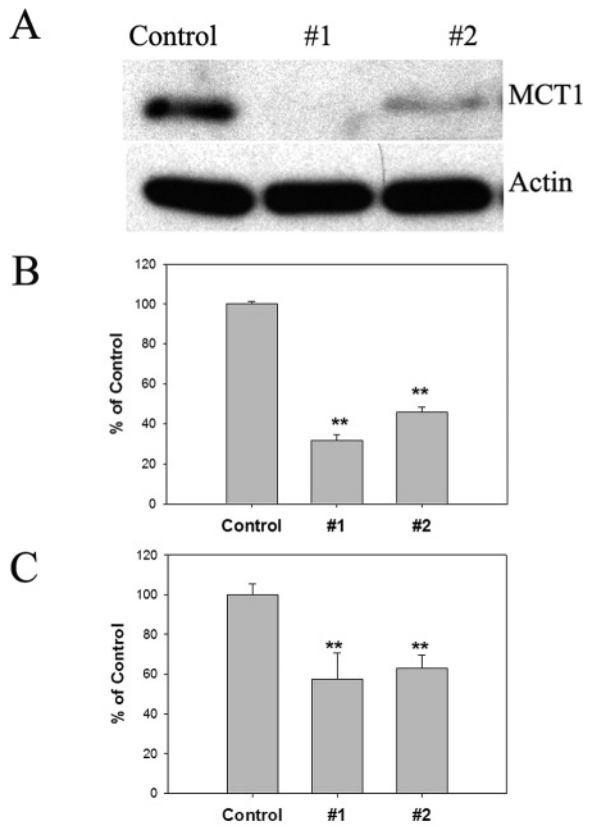

Figure 7. RNA interference of MCT1: (A) protein expression of MCT1 after RNA interference with β-actin used as loading control; (B) [^{3}H]-D-lactate (0.1 mM) uptake after siRNA treatment; (C) [^{14}C]-butyrate (0.1 mM) uptake after siRNA treatment. #1 and #2 represent two transfection experiments using two different siRNA for MCT1, respectively. Each bar represents the mean ± SD, $N = 3-6$, and the graph shown here is the result of one representative experiment out of at least three independent transfection experiments with similar results; one-way ANOVA followed by Dunnett's test, (**) $p < 0.01$.

dependent transporter to the total uptake, due to the lack of specific sodium-dependent transport inhibitors used in this study. We characterized the transport kinetics of D-lactate in HK-2 cells in this investigation, and its transport was well-described by an equation incorporating a single Michaelis−Menten equation. This interpretation was also supported by the Eadie−Hofstee plot of D-lactate uptake. D-Lactate, unlike its L-isomer, is not directly metabolized by human kidney cells,[36] so the kinetic profile represents transport and is not complicated by cellular metabolism. Butyrate was also studied as a MCT1 substrate;[5,37] the characterization of butyrate kinetics in this study required the use of a nonsaturable clearance term along with the Michaelis−Menten equation, indicating more passive permeability than observed with D-lactate, and this interpretation was supported by Eadie−Hofstee plot of butyrate uptake. We have found that the K_m of D-lactate in HK-2 cells was very similar to that previously reported for its MCT1-mediated transport.[2] We also evaluated the kinetics of L-lactate using a short uptake time (1 min) in order to minimize the contribution of cellular metabolism; the K_m for L-lactate uptake was 6.5 mM (unpublished data in this laboratory), which was significantly smaller than that of D-lactate (26 mM). This stereoselectivity for lactate transport has been reported previously in the literature for MCT1.[2] The K_m for L-lactate is similar to that reported in the literature for its MCT1-mediated transport, but not MCT2-mediated transport.[38] The affinities of MCT1 for L-lactate and pyruvate are several times less than that of MCT2 for these substrates,[2,3] while MCT4 has a much lower affinity for monocarboxylates than MCT1.[14,39] Although we could not demonstrate MCT2 activity from the kinetic profile, it was possible that MCT2 plays a role in monocarboxylate transport in HK-2 cells. We have tried to evaluate the role of MCT2 and MCT4 in the transport of D-lactate and L-lactate in HK-2 cells using RNA interference assays, and the results suggested that MCT2 and MCT4 play minor roles in the transport of these two substrates in HK-2 cells, which could not be detected and separated using kinetic assays (unpublished data in our laboratory). The K_m value of butyrate determined here was similar to that obtained in Caco-2 cells, where MCT1 appears to be the major transporter for butyrate.[5] However, it is likely that significant amounts of butyrate diffuse into cells, as suggested by the need to incorporate a diffusional clearance in fitting the uptake data of butyrate, which contrasts with that observed with L-lactate and D-lactate; this observation agrees with published results in the literature.[40]

The relatively specific MCT inhibitors, CHC and phloretin, inhibited the uptake of D-lactate and butyrate. This fact further supported our hypothesis that MCTs were involved in the uptake of D-lactate and butyrate. It has been reported that the transport of L-lactate and D-lactate by MCT2 was not sensitive to pCMB.[38] The inhibition of D-lactate and butyrate uptake by pCMB was similar to the inhibition by CHC and phloretin, which suggested that MCT2 might not be a major transporter for both substrates. Additionally, MCT3 may not be important for the uptake of D-lactate and butyrate, since this transporter has been reported to be insensitive to CHC and phloretin.[12] Probenecid, an inhibitor for OAT, OATPs, and also an inhibitor for MCT1 and MCT2,[2] also inhibited the uptake of both D-lactate and butyrate. However, there were differences in the inhibitory effects of DIDS and rifampicin on the two substrates: both DIDS and rifampicin inhibited the uptake of D-lactate significantly, but they did not inhibit the uptake of butyrate.

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DIDS has been previously reported in the literature not to be an inhibitor for butyrate uptake in Caco-2 cells.[5] The differing effects observed may be due to the greater binding affinity of butyrate compared with D-lactate, the possibility of more than one binding site on MCT1, the involvement of different MCT isoforms in the transport of these substrates, or the possibility of transport by additional organic anion transporters. It must be pointed out that a low concentration of DIDS (0.1 mM) has no effect on D-lactate uptake (data not shown), which suggested little contribution from an anion exchanger, since low concentrations of DIDS could inhibit the anion-exchanger-mediated transport of lactate.[41] The uptake of both D-lactate and butyrate by HK-2 cells was significantly decreased in cells transfected with siRNA for MCT1. The uptake of two substrates with #1 siRNA treatment was inhibited to a similar level as that of chemical inhibitors CHC and phloretin. This suggested that MCT1 represents a major transporter of D-lactate and butyrate uptake in HK-2 cells.

In summary, RT-PCR and Western blot results demonstrated the presence of members of the MCT family in both HK-2 cells and human kidney cortex. Characterization of the transport kinetics, driving force, and inhibition of the transport of D-lactate and butyrate in HK-2 cells suggested that MCTs play an important role in the kidney transport of these substrates. The RNA interference study indicated that MCT1 may be responsible, at least in part, for the uptake of D-lactate and butyrate by HK-2 cells. Our results also support the usefulness of HK-2 cells as an in vitro model for the study of monocarboxylate transport in renal proximal tubule cells.

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