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Functional Activity of a Monocarboxylate Transporter, MCT1, in the Human Retinal Pigmented Epithelium Cell Line, ARPE-19

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Abstract: The purpose of this study was to identify and characterize the functional activity of monocarboxylic acid transporter 1 (MCT1) on the human retinal pigmented epithelium (RPE) cell line, ARPE-19, and to evaluate whether the cell line can function as an in vitro screening tool for intravitreally administered drugs/prodrugs targeted to the MCT1 expressed in RPE. Uptake studies were carried out at 37 °C, for 30 s, with ARPE-19 cells. [^{14}C]L-Lactic acid was selected as a substrate for this transporter. Uptake of [^{14}C]L-lactic acid by ARPE-19 cells was found to exhibit saturable kinetics ($K_m = 3.1 \pm 0.6$ mM and $V_{max} = 63.1 \pm 4.1$ pmol/min/mg of protein). Monocarboxylic acids, such as benzoic acid, salicylic acid, and pyruvic acid, inhibited the uptake of [^{14}C]L-lactic acid whereas di- and tricarboxylic acids, such as phthalic, succinic, and citric acids, did not demonstrate any inhibitory effect. Uptake was stereospecific where D-lactic acid was less effective in inhibiting [^{14}C]L-lactic acid uptake than unlabeled L-lactic acid. ELISA indicated the expression of only MCT1, MCT4, and MCT8 isoforms by ARPE-19 cells. Increase in [^{14}C]L-lactic acid uptake was observed as the uptake medium pH was lowered from 7.4 to 5.0. Moreover, inhibition of [^{14}C]L-lactic acid uptake was observed in the presence of the protonophore 2,4-dinitrophenol. Uptake was significantly decreased in the presence of sodium azide, ouabain, *p*-chloromercuribenzoic acid (pCMBA), *N*-ethylmaleamide, dithiothreitol, and *p*-chloromercuribenzene sulfonate (pCMBS). However, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and L-thyroxine did not inhibit [^{14}C]L-lactic acid. RT-PCR studies and sequence analysis of the PCR product confirmed the expression of MCT1 by ARPE-19 cells. Our results indicate that MCT1 is functionally active and is the only MCT isoform involved in the apical uptake of monocarboxylates by ARPE-19 cells. This cell line may thus be used as an effective screening tool for intravitreally administered drugs/prodrugs targeted toward MCT1 expressed on the RPE.

Keywords: Lactic acid; monocarboxylic acid transporters; retina; ARPE-19; RPE

Introduction

A number of studies have reported the polarized expression of monocarboxylate transporters (MCTs) on the mouse and rat retinal pigmented epithelium (RPE).[1,2] These studies indicate that MCT1 and MCT3 are expressed on the apical and basolateral membranes of RPE, respectively. MCTs play

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an important physiological role in the RPE. Muller cells use glucose for ATP production and in the process generate considerable amounts of lactic acid. This lactic acid is pumped out of the muller cells and is used by the photoreceptor cells for energy generation. The remaining quantity of lactic acid enters into the subretinal space. Unless the excess lactic acid is quickly and efficiently cleared from the subretinal space, a drop in pH would occur, affecting the functioning of the ocular tissues. Through the polarized expression, coupled with simultaneous activity of MCTs, the RPE pumps out the lactic acid from the subretinal space and maintains the retinal environment. This physiological role of the MCTs may be utilized to target drugs/prodrugs to the intracellular compartment of the RPE, following intravitreal administration. MCT1 targeted drugs/prodrugs could be quickly and efficiently picked up by the transporter and translocated into the intracellular compartment, where prodrugs will be cleaved by cytoplasmic enzymes generating the parent drug, and lead to higher intracellular drug concentrations.

Selection of a lead candidate from a number of promising intravitreally administered MCT1 targeted compounds requires an effective in vitro screening tool. A number of cell lines have been investigated with respect to the expression of monocarboxylate transporters. However, none of these are of retinal origin. Concentrations of enzymes, transporters, and ion channels differ from one tissue to another. Thus, in order to effectively screen for prodrugs targeted toward the RPE it is essential that the cell line selected originates from native RPE.

ARPE-19 cells, a cell line of human RPE origin, have been extensively employed as an in vitro model of human RPE. Numerous studies have shown that this cell line displays characteristic epithelial morphology and mimics the native RPE tissue with respect to polarization and expression of various ion channels and transporters.[3−8] Although Philp et al. presented biochemical evidence for the expression of MCT1 on the apical membrane of ARPE-19 cells,[9] studies investigating functional activity and delineating transport kinetics of MCTs in the ARPE-19 cell line have not yet been reported. For transporter targeted drug screening studies it is imperative that the transporter be functionally active in the in vitro model selected. A recent study conducted by Ocheltree et al. reports that whereas biochemical studies indicated the expression of peptide transporters in ARPE-19 cells, functional studies demonstrated that peptide transporters were not active in the cell line.[10] This report underscores the importance of establishing both functional activity and biochemical expression of a transporter in a cell culture model prior to its use in drug screening studies. The primary objective of this study was to evaluate whether ARPE-19 can be used as an in vitro model for screening intravitreally administered drugs/prodrugs targeted toward the MCT1 system expressed in the RPE.

Experimental Section

Materials. ARPE-19 cells were purchased from American Type Culture Collection (Manassas, VA) at passage number 21. [^{14}C]L-Lactic Acid (131 mCi/mmol) was procured from Perkin-Elmer Inc. (Boston, MA). D-MEM/F-12 and fetal bovine serum (FBS) were purchased from Gibco-BRL-Invitrogen (Grand Island, NY). Affinity-purified rabbit anti-human MCT1, MCT3, MCT4, MCT5, MCT6, MCT7, and MCT8 primary antibodies were purchased from Chemicon International, Inc. (Temecula, CA). Affinity-purified rabbit anti-human MCT2 antibody was procured from Alpha Diagnostic International, Inc. (San Antonio, TX). *p*-Chloromercuribenzene sulfonate (pCMBS) was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Culture plates were obtained from Costar (Corning, NY). Anti-rabbit IgG peroxidase conjugate secondary antibody and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification.

Cell Culture. ARPE-19 cells were utilized between passages 22 and 30. Culture media consisted of D-MEM/F-12 supplemented with 10% fetal bovine serum, 15 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 29 mM sodium bicarbonate, penicillin (100 μg/mL), and streptomycin (100 μg/mL). Medium was replaced every other day. Cells were maintained at 37 °C, in a humidified atmosphere of 5% CO_2 and 90% relative humidity. For uptake studies, cells were plated at a density of 500000 cells/well on 12-well culture plates and incubated at 37 °C.

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Enzyme Linked Immunosorbent Assay (ELISA). ELISA was performed on ARPE-19 cells grown in 96-well tissue culture plates 28−30 days postseeding. At the end of the culture period medium was aspirated and the cells were washed twice with cold phosphate buffered saline (PBS). The cells were then fixed with 4% paraformaldehyde in PBS at 4 °C for 20 min. Following fixation, cells were subjected to graded treatment with ethanol (50%, 75%, 95%, 75%, and 50% ethanol, respectively). At the end of the ethanol treatment, cells were washed in PBS for 5 min and treated with 5% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20 (PBST) for 1 h at 37 °C to reduce nonspecific binding. BSA solution was aspirated at the end of 1 h, and the cells were washed with PBST. Human MCT specific primary antibodies (1:1000 dilution in PBST) were then added and incubated for 2 h at 37 °C (only PBST was added to control wells). At the end of the 2 h incubation period, the primary antibody solutions were removed and the cells washed thrice with PBST. Following this, a solution of secondary antibodies (anti-rabbit IgG peroxidase conjugate, 1:30000 dilution) was added and allowed to incubate for 2 h at 37 °C. Subsequently the cells were washed in PBST three times and then incubated with the peroxidase substrate tetramethylbenzidine (1-Step Ultra TMB−ELISA (Pierce, Rockford, IL)) for 15 min. The reaction was stopped with the addition of 2 M sulfuric acid, and the solution color was measured using SpectraFluor Plus (Tecan NC) at 450 nm.

Concentration Dependence of L-Lactic Acid Uptake. Uptake studies were conducted based on our earlier published methods[11] with slight modifications. Briefly, 28−30 days postseeding medium was removed and cells were washed twice with Dulbecco's phosphate buffered saline (DPBS), pH 7.4, containing 130 mM NaCl, 2.5 mM KCl, 7.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$ and 5 mM glucose. L-Lactic acid solutions were prepared at different concentrations (0.01−15 mM) in DPBS, pH 6.0 (pH adjusted with 2-(*N*-morpholino)ethanesulfonic acid (MES)). The solutions were spiked with 0.5 μCi/mL of [^{14}C]-L-lactic acid. Uptake was initiated by incubating the cells with two milliliters of each solution for a predetermined time at 37 °C. Initial experiments revealed that L-lactic acid uptake was linear for at least 60 s. Longer uptake times resulted in intracellular acidification and nonlinear kinetic profiles. Moreover, for studying uptake of compounds that are metabolized by intracellular enzymes, uptake time must be kept as short as possible, ideally less than 1 min, to reduce metabolism driven uptake. All subsequent experiments were therefore carried out with a 30 s incubation period.

At the end of the incubation period, solution was aspirated off and cells were washed twice with 2 mL of ice-cold stop solution (0.52 g/L HEPES and 15.64 g/L KCl), to arrest cellular uptake. One milliliter of 0.3 N NaOH containing 0.1% Triton-X solution was then added to each well and left overnight to solubilize the cells. Five hundred microliters of that solution was then transferred to scintillation vials containing 5 mL of scintillation cocktail. Cellular radioactivity was quantified using a scintillation counter (model LS-6500, Beckman Coulter (Fullerton, CA)). Twenty microliters of the solution from each well was then taken to measure the protein content in each sample using the method of Bradford[12] with bovine serum albumin as the standard (Bio-Rad protein estimation kit, Hercules, CA). Nonspecific binding was corrected by carrying out the uptake at 4 °C.

pH Dependence Studies. Solution pH was varied from 5.0 to 7.4 for pH dependence studies (pH adjusted with MES). In all cases, uptake of [^{14}C]L-lactic acid (0.5 μCi/mL) was conducted according to the procedure described earlier.

Competitive Inhibition Studies. To delineate the structural requirements for interaction with the carrier system, uptake experiments were carried out using various carboxyl group containing compounds (mono-, di-, and tricarboxylic acids). Cells were incubated simultaneously with [^{14}C]L-lactic acid (0.5 μCi/mL) and 10 mM unlabeled carboxylic acid solutions (DPBS, pH 6.0), and uptake experiments were conducted as described earlier. Stereoselectivity of the carrier system was also determined by adding both L- and D-lactic acid (unlabeled) as inhibitors.

Studies investigating effect of metabolic inhibitors, protonophores, anion transporter inhibitors, and sulfhydryl-group modifying agents were carried out by preincubating the cells for 10 min with buffer alone (control) or in the presence of various inhibitors. Uptake was then examined with [^{14}C]L-lactic acid (0.5 μCi/mL) in pH 6.0 buffer, as described earlier.

RT-PCR. Total RNA from ARPE-19 was extracted by the acid guanidium thiocyanate−phenol−chloroform extraction method.[13] Random hexamers (Promega Corporation, Madison, WI) were used to reverse transcribe 5 μg of total RNA at 42 °C for 60 min using SuperScript II RT (Invitrogen Corporation). The reaction was terminated by heating at 70 °C for 10 min, followed by the addition of RNase H inhibitor (Promega Corporation, Madison, WI). Two microliters of RT reaction solution were used to amplify MCT1 using PCR. The reaction was carried out using primers designed from the published human MCT cDNA sequences.[14] The amplification conditions were one cycle at 94 °C for 2 min, followed by 35 cycles at 94 °C for 45 s, annealing at 68 °C for 1 min, and extension at 72 °C for 2 min for MCT1 cDNA.[14] Final extension was carried out

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at 72 °C for 10 min. The primers used for amplification were 5′-CCA CAT GCC CAG TAT GTG TAT TTG-3′ (reverse primer) and 5′-CTT GCT ACT AAA CTG CTG TGT TGC-3′ (forward primer). Caco-2 cells, a human colon carcinoma cell line which is known to express MCT1, was selected as the positive control.[14] The resultant product was sequenced from both directions by SeqWright using an automated Perkin-Elmer Applied Biosystems 3730xl PrismTM DNA sequencer to establish its molecular identity.

Computer Analysis. Nucleotide sequence homology matching was performed using basic local alignment tool (BLAST) via on-line connection to the National Center of Biotechnology Information (NCBI). Multiple nucleotide sequence comparisons were done using CLUSTAL W (1.81) multiple sequence alignment tool from Swiss-Prot.

Data Analysis. Uptake data was fitted to the classical Michaelis−Menten equation denoted by eq 1. Lineweaver−Burke and Eadie−Hofstee plots were constructed using eq 2 and eq 3, respectively. V is the total rate of uptake,

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

$$\frac{1}{V} = \frac{K_m}{V_{max}C} + \frac{1}{V_{max}} \quad (2)$$

$$V = V_{max} - K_m\frac{V}{C} \quad (3)$$

V_{max} is the maximum uptake rate for the carrier-mediated process, K_m (Michaelis−Menten constant) is the concentration at half the maximum velocity, and C is the substrate concentration.

Data were fitted to eq 1 with the aid of a nonlinear least-squares regression analysis program (Kaleida Graph V3.09, Synergy Software).

Statistical Analysis. All experiments were conducted at least in quadruplicate, and results are expressed as mean ± standard deviation. Michaelis−Menten parameters K_m and V_{max}, are expressed as mean ± SE. Statistical analysis between two groups was carried out with Student's t test. A difference between mean values was considered statistically significant if the p value was ≤ 0.05.

Results

Concentration-Dependent Uptake of L-Lactic Acid. To determine concentration dependency, uptake studies were conducted by spiking 0.5 μCi/mL of [^{14}C]L-lactic acid to various concentrations of unlabeled L-lactic acid (0.1−15 mM). Uptake of L-lactic acid was found to display saturable kinetics (Figure 1A). Data were fitted to eq 1 and values for the kinetic constants. K_m and V_{max} for L-lactic acid uptake were determined to be 3.1 ± 0.6 mM and 63.1 ± 4.1 pmol/min/mg of protein, respectively. Similar values for the kinetic parameters were obtained using a Lineweaver−Burke plot (K_m and V_{max} were 3.5 mM and 61.7 pmol/min/mg of protein, respectively) (eq 2, Figure 1B) and an Eadie−Hofstee plot

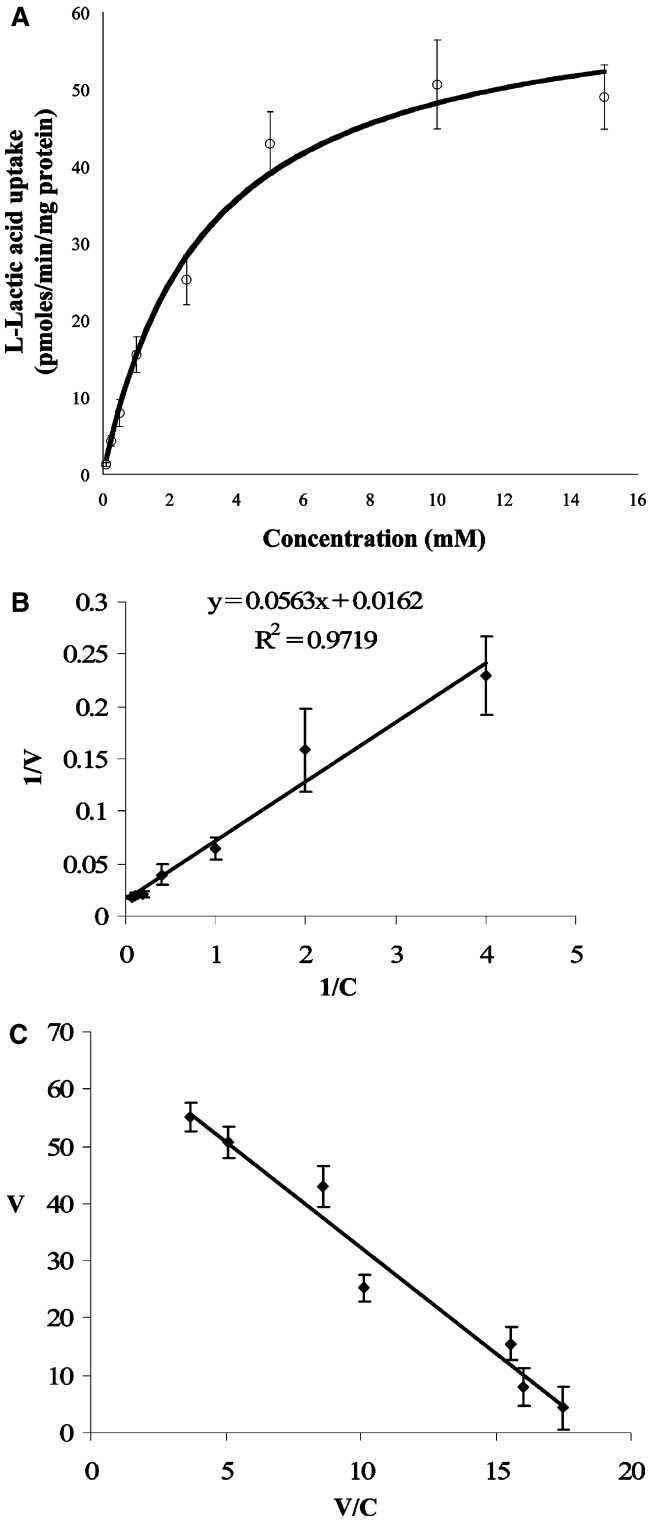

Figure 1. Concentration dependent uptake of L-lactic acid. Cells were incubated in the presence of various concentrations of L-lactic acid. Uptake solutions were spiked with 0.5 μCi/mL of [^{14}C]L-lactic acid, and total uptake was calculated. V represents the velocity of uptake (pmol/min/mg of protein) and C the substrate concentration (mM). A: Michaelis−Menten plot. B: Lineweaver−Burke plot. C: Eadie−Hofstee plot. Results are expressed as mean ± standard deviation (n = 4−6).

Table 1. Uptake of [^{14}C]L-Lactic Acid in the Presence of Mono-, Di-, and Tricarboxylic Acids and L-Thyroxine[a]

inhibitors	uptake of [^{14}C]L-lactic acid as a % of control	SD	
monocarboxylic acids			
L-lactic acid (10 mM)	5.69	2.16	*
D-lactic acid (10 mM)	34.05	3.14	*
benzoic acid (10 mM)	19.10	2.86	*
salicylic acid (10 mM)	8.45	1.13	*
pyruvic acid (10 mM)	19.77	2.98	*
dicarboxylic acids			
glutaric acid (10 mM)	110.12	8.11	
fumaric acid (10 mM)	89.62	6.50	
phthalic acid (10 mM)	92.14	5.80	
succinic acid (10 mM)	86.00	6.80	
tricarboxylic acid			
citric acid (10 mM)	94.20	5.80	
L-thyroxine	102.58	3.70	

[a] Results are expressed as mean ± standard deviation ($n = 4$). Asterisks (*) represent significant difference from control ($p \leq 0.05$).

(K_m and V_{max} were 3.7 mM and 69.2 pmol/min/mg of protein, respectively) (eq 3, Figure 1C).

Eadie−Hofstee transformation of the data (eq 3) pointed to the involvement of a single carrier protein ($r^2 > 0.96$) in the uptake process (Figure 1C).

Effect of Mono-, Di-, and Tricarboxylic Acids and L-Thyroxine on [^{14}C]L-Lactic Acid Uptake. Various mono-, di-, and tricarboxylic acids were evaluated for their inhibitory effect on [^{14}C]L-lactic acid uptake by ARPE-19 cells. Studies were conducted by spiking 0.5 μCi/mL of [^{14}C]L-lactic acid to the donor solutions in the presence or absence of an inhibitor. Solution pH was adjusted to 6.0 following preparation of the inhibitor solution. Table 1 depicts the effect of mono-, di-, and tricarboxylic acids (10 mM) on [^{14}C]L-lactic acid (0.5 μCi/mL) uptake. Monocarboxylic acids produced significant inhibition of [^{14}C]L-lactic uptake by ARPE-19 cells. L-Lactic acid, benzoic acid, salicylic acid, and pyruvic acid decreased lactic acid uptake by approximately 94%, 80%, 90%, and 80%, respectively. On the other hand, di- and tricarboxylic acids did not demonstrate any measurable inhibition of L-lactic acid uptake.

Additionally, inhibition by unlabeled L-lactic (94% inhibition) was significantly higher compared to D-lactic acid (65% inhibition), indicating that the transporter is stereoselective (Table 1).

Uptake of L-lactic acid was not affected in the presence of 100 μM L-thyroxine (Table 1).

Effect of pH and Metabolic Inhibitors on [^{14}C]L-Lactic Acid Uptake. Uptake of [^{14}C]L-lactic acid was investigated as a function of the medium pH. Rate of L-lactic acid uptake was observed to be maximum (22.72 pmol/30 s/mg) when the pH was 5.0. Uptake rate at pH 5.0 was 7-fold higher than that at pH 7.4 and 1.4 times higher than that at pH 6.0 (Figure 2).

Metabolic inhibitors ouabain (a Na^+/K^+ ATPase inhibitor) and sodium azide (an uncoupler of oxidative phosphorylation) both inhibited lactic acid uptake by ARPE-19 cells. These studies were carried out by preincubating the cells with the inhibitor solutions (prepared in DPBS pH 7.4) for

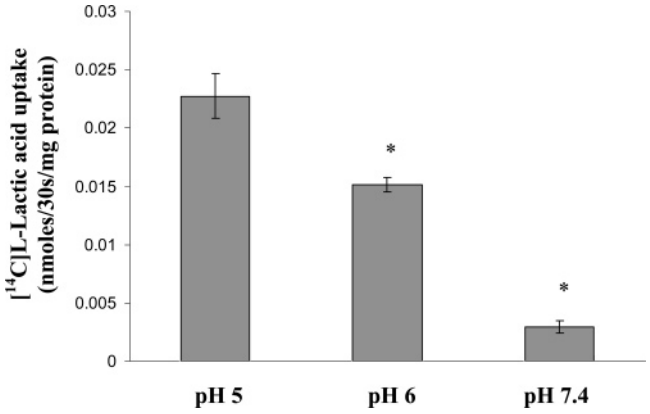

Figure 2. [^{14}C]L-Lactic acid uptake by ARPE-19 as a function of pH. Results are expressed as mean ± standard deviation ($n = 4-6$). Asterisks (*) represent significant difference from rate of uptake at pH 5.0 ($p \leq 0.05$).

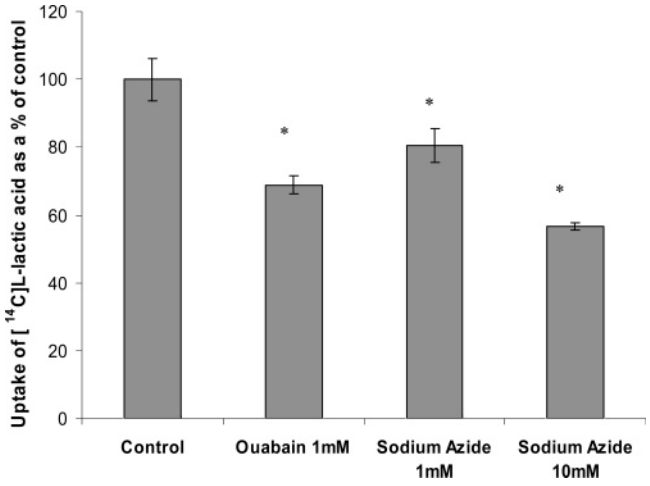

Figure 3. Uptake of [^{14}C]L-lactic acid by ARPE-19 cells in the presence or absence (control) of metabolic inhibitors. Results are expressed as mean ± standard deviation ($n = 4-6$). Asterisks (*) represent significant difference from control ($p \leq 0.05$).

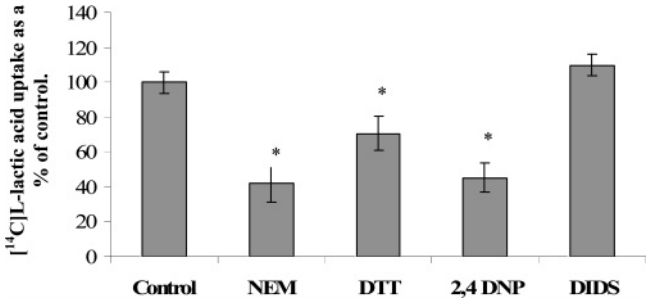

Figure 4. Effect of protonophores, sulfhydryl-group modifying agents, and stilbene derivatives on [^{14}C]L-lactic acid uptake by ARPE-19 cells. Results are expressed as mean ± standard deviation ($n = 5-6$). Asterisks (*) represent significant difference from control ($p \leq 0.05$).

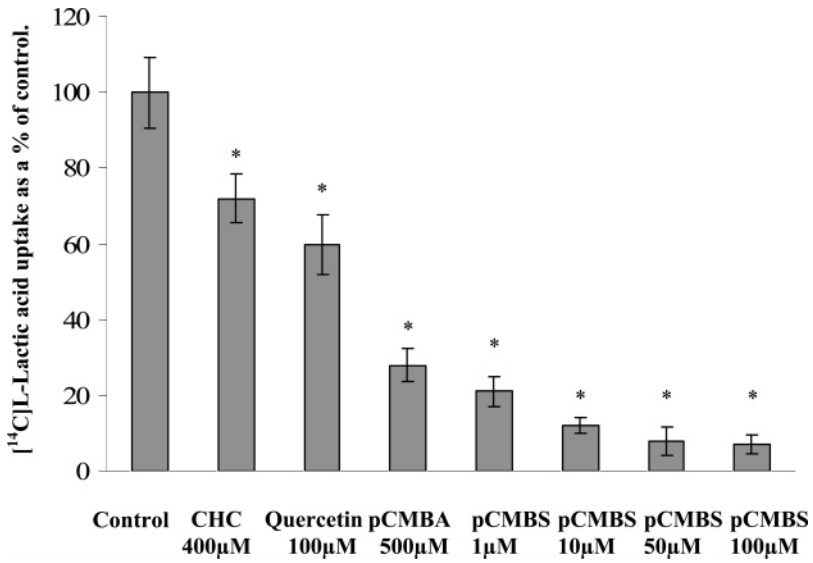

Figure 5. Effect of specific MCT inhibitors on [^{14}C]L-lactic acid uptake by ARPE-19 cells. ARPE-19 cells were preincubated for 10 min in the presence of the inhibitors before initiation of uptake. Results are expressed as mean ± standard deviation ($n = 4-5$). Asterisks (*) represent significant difference from control ($p \leq 0.05$).

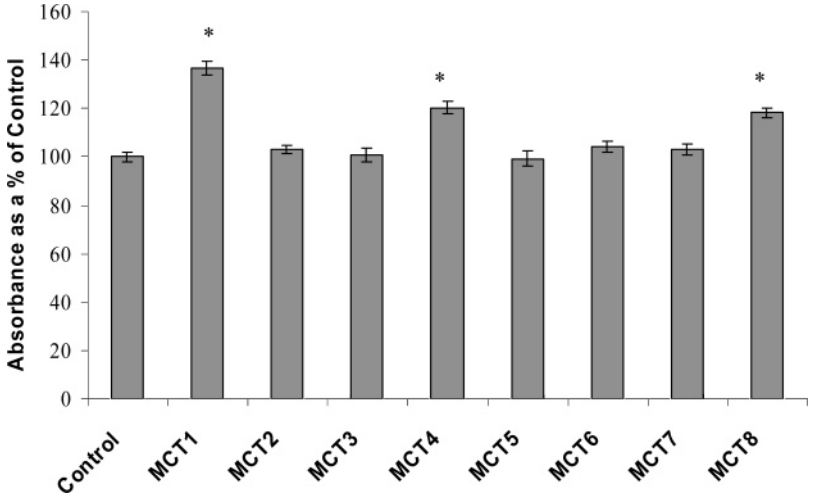

Figure 6. Response of ARPE-19 cells to ELISA with rabbit anti-human MCT1 through MCT8 antibodies. Control cells did not receive any antibody. Results are expressed as mean ± standard deviation ($n = 12$). Asterisks (*) represent significant difference from control ($p \leq 0.05$).

10 min prior to conducting the uptake studies. [^{14}C]L-Lactic acid (0.5 μCi/mL in DPBS pH 6.0) uptake was inhibited to the extent of 20%, 43%, and 31% in the presence of 1 mM sodium azide, 10 mM sodium azide, and 1 mM ouabain, respectively (Figure 3).

Effect of Protonophores and Organic Anion Transporter Inhibitors on [^{14}C]L-Lactic Acid Uptake. Further studies were carried out to investigate the effect of protonophores on the uptake of L-lactic acid. ARPE-19 cells were preincubated with 500 μM 2,4-dinitrophenol (DNP). DNP produced 55% inhibition of L-lactic acid uptake (Figure 4). The organic anion transporter inhibitor DIDS did not produce any significant effect on L-lactic acid uptake (Figure 4).

Effect of Amino Acid Modifying Agents on L-Lactic Acid Uptake. To study the effect of amino acid modifying agents on the uptake of L-lactic acid, cells were preincubated with the inhibitors for 10 min prior to an uptake experiment. *N*-Ethylmaleimide (NEM, 500 μM) and dithiothreitol (DTT, 500 μM) reduced uptake of L-lactic acid by 58% and 30%, respectively (Figure 4). These results indicate the involvement of carrier-protein sulfhydryl groups in the translocation process.

Effect of Specific Monocarboxylic Acid Transporter Inhibitors. To delineate whether [^{14}C]L-lactic acid uptake involves a monocarboxylic acid transporter, the effect of specific monocarboxylate transporter inhibitors quercetin (100 μM), α-cyano-4-hydroxycinnamate (CHC, 400 μM), pCMBA (500 μM), and pCMBS on uptake was studied. Prior to an experiment being conducted, cells were incubated for 10 min with the inhibitors. All the inhibitors produced significant decrease in the uptake of L-lactic acid by ARPE-19 cells. Quercetin decreased uptake by 40% whereas

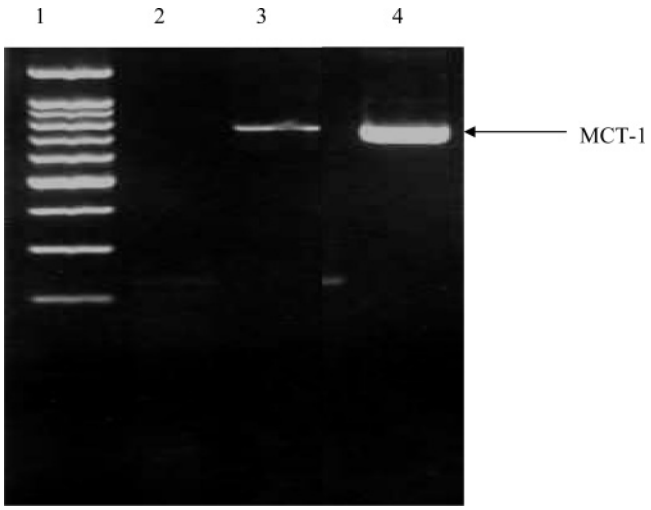

Figure 7. MCT1 cDNA was generated by reverse transcription-PCR amplification of total RNA from ARPE-19 cells (lane 3) and Caco-2 (lane 4). Caco-2 cells were used as a positive control. Aliquots of PCR products were analyzed by gel electrophoresis on 3.0% agarose ($n = 4$). A major ~707-nt band corresponding to the amplified human MCT1 cDNA (lanes 3, 4) is observed. Negative control (lane 2), without any RNA, did not show any background interaction. A 1 kbp DNA ladder is shown on the left (lane 1).

pCMBA and CHC decreased uptake by 71% and 28%, respectively (Figure 5).

pCMBS, an inhibitor of MCT1 but not of MCT2, exhibited concentration dependent inhibition of L-lactic acid uptake by ARPE-19 cells. Uptake was reduced by 79% and 93% in the presence of 1 and 100 μM pCMBS, respectively (Figure 5).

Stock solutions of quercetin and pCMBS were prepared in dimethyl sulfoxide (DMSO). Final concentration of DMSO in the uptake medium was less than 5%. Control studies containing 5% DMSO did not show any effect of DMSO on L-lactic acid uptake (data not presented).

ELISA. ARPE-19 cells responded positively to ELISA with affinity-purified rabbit anti-human MCT1, MCT4, and MCT8 antibodies. Figure 6 compares the absorbance obtained with the various MCT specific antibodies.

RT-PCR. PCR products were analyzed by gel electrophoresis on 3.0% agarose gel. A major band, corresponding to the amplified human MCT1 cDNA, was observed in both ARPE-19 and Caco-2 cells (Figure 7). The PCR product was sequenced in both directions. The sequence obtained showed maximum homology to hMCT1 using the BLAST search program (NCBI).

Discussion

The objective of this study was to determine whether the ARPE-19 cell line functionally mimics the native human RPE with respect to expression of MCTs, and whether it can serve as an in vitro screening tool for prodrugs targeted toward this transporter.

L-Lactic acid uptake by ARPE-19 cells is saturable and involves a single transporter as indicated by the Eadie−Hofstee Plot. Uptake appears to be proton-coupled as decreasing the pH of the uptake medium resulted in higher uptake of L-lactic acid. MCT1−MCT4 require binding of a proton to the transporter initially followed by a lactate anion. The proton and lactate are then translocated across the membrane followed by their release on the other side.[15] In the presence of a protonophore, the transporter is not activated by proton binding and hence transcellular flux of lactic acid is decreased. When DNP, a protonophore, was added in the extracellular medium, uptake of L-lactic acid by ARPE-19 cells diminished. These results confirm that lactic acid transport across the apical membrane of ARPE-19 cells is proton-coupled. Our observations are consistent with previous reports demonstrating proton-coupled MCT-mediated translocation of monocarboxylates.[14,15]

Sodium azide (uncoupler of oxidative phosphorylation) and ouabain (Na^+/K^+ ATPase inhibitors) significantly inhibited uptake of L-lactic acid. Inhibition of lactate transport by ouabain is consistent with the uniqueness of the Na^+/K^+ ATPase polarization on the apical membrane of ARPE-19 cells.[16,17] Normally Na^+/K^+ ATPase pump is expressed on the basolateral surface of epithelial cells. Na^+/K^+ ATPase pump acts in conjunction with the Na^+/H^+ exchanger and maintains the intracellular pH through influx of Na^+ and efflux of H^+ when a proton gradient exists. Na^+/K^+ ATPase is involved in sodium homeostasis and generation of the inwardly directed Na^+ gradient required to drive the Na^+/H^+ exchange process.[18] Thus, inhibition of Na^+/K^+ ATPase pump interferes with proton efflux from the intracellular compartment, and consequently the pH gradient between the intracellular and extracellular compartments is disrupted and uptake of L-lactic acid decreases.

L-Lactic acid uptake was significantly inhibited by all the monocarboxylic acids tested. However, the di- and tricarboxylic acids did not produce any significant inhibition confirming that the transporter involved in the uptake of L-lactic acid by ARPE-19 cells is a monocarboxylate transporter.[15,19−24] Quercetin and CHC, classical inhibitors of MCTs,[14,15,21] decreased uptake of L-lactic acid by

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ARPE-19 cells, further confirming the involvement of MCTs in the translocation of lactate across the ARPE-19 apical cell membrane.

ELISA studies using affinity-purified anti-MCT1 through MCT8 specific antibodies indicated the expression of MCT1, MCT4, and MCT8 proteins in the ARPE-19 cells. In an earlier study, Philp et al.[9] investigated the expression of MCT isoforms 1, 3, and 4 in ARPE-19 cells and reported the expression of MCT1 on the apical and MCT4 on the basolateral membrane under culture conditions identical to those employed by us in this study. MCT3 protein was not expressed in ARPE-19 cells.[9] Our results are thus consistent with the results of Philp et al. However, in this study we find that MCT8 is also expressed in ARPE-19 cells, and this is the first time that the possible presence of this MCT isoform has been reported in retinal cells. Thyroid hormones play a critical part in the differentiation and development of retinal cells,[25] and MCT8, a specific thyroid hormone transporter,[26] may play a significant role in maintaining thyroid hormone homeostasis in the retina. Further studies are warranted to confirm and characterize the expression of MCT8 in ARPE-19 cells and native RPE.

To confirm that MCT2 is not involved in the translocation of L-lactic acid across the apical membrane of the ARPE-19 cells, pCMBS was used as an inhibitor. pCMBS is a potent inhibitor of MCT1 but does not produce any inhibition in MCT2-mediated lactic acid uptake.[15,27] L-Lactic acid uptake by ARPE-19 cells was drastically inhibited in the presence of pCMBS (93% inhibition in the presence of 100 μM pCMBS), indicating that the MCT2 isoform is not involved in the uptake process.

Taken together, the above results suggest that only MCT isoforms 1, 4, and/or 8 can be involved in the uptake of L-lactic acid by ARPE-19 cells. Transport kinetics of L- and D-lactic acid is reported to be similar for the MCT1 and MCT4 isoforms, the only known difference being that the affinity of MCT4 for L-lactate is much lower than that for MCT1. The apparent K_m for L-lactic acid ranges between 10 and 22 mM for MCT4. The K_m associated with L-lactic acid uptake by ARPE-19 cells was determined to be 3.1 mM. This value is in the range associated with MCT1 (3.5−5 mM).[15] Moreover, Eadie−Hofstee transformation of the data indicates the involvement of a single transporter in the uptake process ($r^2 > 0.96$). Considering the K_m value of the uptake process, involvement of MCT4 can be ruled out. This conclusion is consistent with earlier reports demonstrating the localization of MCT4 on the basolateral membrane of ARPE-19 cells under identical culture conditions.[9]

MCT8 is reported to be a thyroid hormone transporter, and it has been suggested that MCT8 is not involved in the translocation of monocarboxylic acids.[26] To delineate the role of MCT8 in the uptake of L-lactic acid by ARPE-19 cells, L-thyroxine, a high-affinity substrate of MCT8,[26] was used in conjunction with L-lactic acid. L-Thyroxine did not exhibit any inhibitory effect on the uptake of L-lactic acid, indicating that MCT8 is not involved in the uptake of L-lactic acid by ARPE-19 cells. It is thus evident that MCT1 is the only transporter involved in the translocation of L-lactic acid across the apical membrane of ARPE-19 cells.

MCT1 is known to be steroselective,[15] with L-lactic acid displaying higher affinity for MCT1 compared to D-lactic acid. In this study, inhibition of [^{14}C]L-lactic acid uptake was significantly greater with the L-isomer compared to the D-isomer of unlabeled lactic acid. Moreover, when sulfhydryl group modifying agents, NEM and DTT, were added to the uptake medium, significant decrease in lactic acid uptake was observed, suggesting the involvement of sulfhydryl groups in the translocation of lactic acid and the involvement of MCT1 in the apical uptake process.[28] These results support our earlier observation that MCT1 is involved in the apical transport of L-lactic acid in ARPE-19 cells.

RT-PCR studies were carried out to confirm the expression of MCT1 in ARPE-19 cells. A major band corresponding to the amplified human MCT1 cDNA, in ARPE-19 cells, as well as in the positive control (Caco-2), was observed. Sequencing of the PCR product and subsequent BLAST search (NCBI) revealed that the PCR product demonstrated maximum homology to hMCT1, confirming the expression of the MCT1 isoform in ARPE-19 cells.

MCT1-mediated transport of L-lactic acid across the apical membrane of ARPE-19 cells is consistent with earlier reports on polarized expression of MCTs in mouse and rat RPE. ARPE-19 can thus serve as a valuable tool for screening intravitreally administered prodrugs and drugs targeted toward MCT1 expressed in the RPE.

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Expression of MCT1 in endothelial cells of the retinal blood vessels was earlier demonstrated by Hosoya et al.[29] Drug design and/or prodrug derivatization, for intravitreal administration, targeting the MCTs may provide a viable strategy to enhance drug concentrations both in the RPE and in retinal endothelial cells, and may lead to higher therapeutic efficacy in cytomegalovirus (CMV) retinitis (both RPE and retinal endothelial cells are active sites of CMV replication) and other retinal diseases. Considering the bidirectional nature of MCT-mediated transport, it may also be possible to deliver therapeutic drug concentration into the RPE or retinal endothelial cells following systemic administration. However, when the prodrug is systemically administered, MCT3 (MCT isoform expressed on the basolateral membrane of native RPE) will assume greater prominence. ARPE-19 cells will not be a good model for such studies as the basolateral MCT expressed by this cell line is MCT4 and not MCT3.

In conclusion, this study demonstrates, for the first time, that MCT1 expressed on the apical membrane of ARPE-19 cells is functionally active and is the only MCT isoform involved in the apical uptake of monocarboxylates by ARPE-19 cells. MCT1 polarization and functional activity in the ARPE-19 cell line parallel those on native RPE. This cell line may thus be used as an effective screening tool for intravitreally administered drugs/prodrugs targeted toward MCT1 expressed on the RPE.

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