Monocarboxylate Transporter 1 Mediates DL-2-Hydroxy-(4-Methylthio)Butanoic Acid Transport across the Apical Membrane of Caco-2 Cell Monolayers[1]

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Abstract

The methionine hydroxy analogue DL-2-hydroxy-(4-methylthio)butanoic acid (DL-HMB) is a supplementary source of methionine commonly added to commercial animal diets to satisfy the total sulfur amino acid requirement. In this study, we characterized DL-HMB transport across the apical membrane of Caco-2 cells to identify the transport mechanism involved in the intestinal absorption of this methionine source. DL-HMB transport induced a significant decrease in intracellular pH (pH_i) and was inhibited in the presence of the protonophore carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone. Moreover, both Na^+ removal and 5-(*N*-ethyl-*N*-isopropyl)amiloride, an inhibitor of apical Na^+/H^+ exchanger (NHE3), significantly reduced substrate uptake and pH_i recovery, suggesting cooperation between H^+-dependent DL-HMB transport and NHE3 activity. *cis*-Inhibition experiments with L-Ala, β-Ala, D-Pro, betaine, or glycyl-sarcosine excluded the participation of systems proton amino acid transporter 1 and peptide transporter 1. In contrast, α-cyano-4-hydroxycinnamate, phloretin, L-lactate, β-hydroxybutyrate, butyrate, and pyruvate, inhibitors and substrates of monocarboxylate transporter 1 (MCT1), significantly reduced DL-HMB uptake. Dixon plot analysis of L-lactate transport in the presence of DL-HMB revealed a competitive interaction (inhibition constant, 17.5 ± 0.11 mmol/L), confirming the participation of system MCT1. The kinetics of DL-HMB uptake was described by a model involving passive diffusion and a single low-affinity, high-capacity transport mechanism (K_D, 1.9 nL/μg protein; K_m, 13.1 ± 0.04 mmol/L; and V_{max}, 43.6 ± 0.14 pmol/μg protein) compatible with MCT1 kinetic characteristics. In conclusion, the methionine hydroxy analogue is transported in Caco-2 cell apical membrane by a transport mechanism with functional characteristics similar to those of MCT1.

Introduction

The methionine hydroxy analogue, DL-2-hydroxy-(4-methyl-thio)butanoic acid (DL-HMB),[4] is a synthetic source of methi-onine that is commonly added to commercial animal diets to ensure that the nutritional requirement for this essential amino acid is satisfied. The biological utilization of DL-HMB relies on its conversion to L-Met, a process that already starts in the intestine upon absorption (1,2). The bioefficacy of DL-HMB has been compared with DL-Met in several studies, but differences between them are still subject of debate. Taking into account the utilization of this analogue in animal production, it is important to establish the role of intestinal absorption in DL-HMB bioefficacy. Commercial DL-HMB contains a large proportion of nonmonomeric forms (3,4), which have a lower biopotency than the monomeric form (5). However, we have recently shown that the presence of oligomers in DL-HMB is not the limiting factor in its absorption, because the hydrolytic capacity of the chicken small intestine is high (6).

The characteristics of DL-HMB transport in the small intestine are quite different from those reported for L- and D-Met (7–13). In the chicken, DL-HMB is transported across the apical membrane of enterocytes by a nonstereospecific, Na^+-independent (9) and H^+-dependent carrier-mediated mechanism (6,11,14), in addition to passive diffusion. Here we attempted to identify the transport mechanism involved in DL-HMB uptake across Caco-2 cell apical membrane. The results may contribute to the design of new dietary strategies to improve animal growth and performance. Cultures of differentiated Caco-2 cells form a highly polarized epithelium with many of the properties of intestinal villous absorptive cells (15). This in vitro experimental model is currently used for the characterization of intestinal transport mechanisms and their regulation by dietary compo-nents (16). Given the H1 dependence of DL-HMB transport, we considered systems proton amino acid transporter 1 (PAT1), pep-tide transporter 1 (PepT1), and monocarboxylate transporter 1 (MCT1) the best candidates to mediate the uptake of the Met analogue. These transport mechanisms are expressed in the api-cal membrane of Caco-2 cells, as in the intestine (17–19). They are low-affinity, high-capacity transport mechanisms (20–22) like DL-HMB transport (9,11), and they show structural sub-strate requirements that a priori are compatible with DL-HMB recognition (22–24). The PAT family has 4 members (P_iAT1–PAT4), of which only PAT1 is expressed in the apical membrane of the small intestine (19). This transport system, member of the solute carrier SLC36 family (21), recognizes short-chain a-amino acids, b-amino acids, and g-amino acids (19,25). It also transports osmolytes such as betaine, sarcosine, and taurine, in addition to some D-amino acids (D-Ala, D-Pro, and D-Ser) (19) and the SCFA acetate, propionate, and butyrate, thus indicating that a free amino group is not essential for binding (24).

Abbreviations used: BCECF, 2#,7#-bis(2-carboxyethyl)-5(6-carboxyfluorescein); CHC, α-cyano-4-hydroxycinnamate; ChoCl, choline chloride; DL-HMB, DL-2-hydroxy-(4-methylthio)butanoic acid; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; FCCP, carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone; Gly-Sar, glycyl-sarcosine; K_i, inhibition constant; MCT1, monocarboxylate transporter 1; NHE3, apical Na^+/H^+ exchanger; PAT1, proton amino acid transporter 1; PepT1, peptide transporter 1; pH_i, intracellular pH; SLC, solute carrier; TER, transepithelial electrical resistance.

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PepT1, member of the SLC15 family (26), mediates intestinal absorption of di- and tri-peptides and several pharmacologically active substrates (27–29). The minimal structural substrate re-quirement for binding is the presence of 2 ionized groups sep-arated by at least 4 methylene groups; 5-amino pentanoic acid is thus the smallest substrate that can be recognized (23). The re-moval of the peptide bond is tolerated without a significant loss of substrate affinity (23).

The MCT family, member of the SLC16 family (22), includes a large number of isoforms. In the intestine, MCT1 expression is mainly confined to the apical membrane (18), whereas MCT3, 4, and 5 are basolaterally localized (30). MCT1 activity is essen-tial for the transport of many monocarboxylates and pharma-cologically active compounds. The substrates recognized are, in general, weak monovalent organic acids with the carboxyl group attached to a relatively short side chain that is either hydrophobic or hydrophilic (31). Substitutions on C2 and C3, such as in pyruvate, L-lactate, acetoacetate, and β-hydroxybutyrate, are tolerated or even preferred (22).

Materials and Methods

Materials. DMEM, trypsin, penicillin, and streptomycin were supplied by GIBCO. Nonessential amino acids, fetal bovine serum, bovine serum albumin, sterile PBS, D-glucose, HEPES, 2-(*N*-morpholino)ethan sulfonic acid, phloretin, α-cyano-4-hydroxycinnamate (CHC), 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP), 2′,7′-bis(2-carboxyethyl)-5(6-carboxyfluorescein) (BCECF), and choline chloride (ChoCl) were supplied by Sigma. Tissue culture sup-plies, including Transwells and clusters, were obtained from Costar. Unlabeled DL-HMB was kindly provided by Adisseo France SAS. DL-[1-^{14}C]-2-hydroxy-4-methylthio-butanoic acid (specific activity 55 mCi/mmol) and L-[U-^{14}C]-lactic acid (specific activity 165 mCi/mmol) were purchased from ARC and PerkinElmer, respectively. Filtron-X was supplied by National Diagnostics.

Cell culture. Caco-2 cells were kindly provided by Dr. David Thwaites at the School of Cell and Molecular Biosciences, University of Newcastle upon Tyne, and cultured following Thwaites et al. (17). The cells (passages 112–121) were routinely grown in plastic flasks at a density of 5 104 cells/cm2 and cultured in DMEM containing 4.5 g/L D-glucose and 2 mmol/L L-Gln and supplemented with 1% (v:v) nonessential amino acids, 10% (v:v) heat-inactivated fetal bovine serum, 100 kU/L penicillin, and 100 mg/L streptomycin at 37C in a modified atmosphere of 5% CO2 in air. For transport experiments, cells were cultured at a density of 4 105 cells/cm2 onto polycarbonate filters with a pore size of 0.4 mm (Transwells, 12 mm diameter). Uptake was measured in cultures 19–21 d after seeding. Cell differentiation was assessed from trans-epithelial electrical resistance (TER) and sucrase activity (32).

Uptake experiments. Transport experiments were performed follow-ing Thwaites et al. (17) as previously described (33). After TER deter-mination, monolayers grown on filters were incubated at 37°C for 5 min, unless otherwise stated, in modified Krebs buffer (composition in mmol/L: NaCl, 137; KCl, 5.4; $CaCl_2$, 2.8; $MgSO_4$, 1.0; NaH_2PO_4, 0.3; KH_2PO_4, 0.3; D-glucose, 10; and HEPES/Tris, 10; pH 7.4) containing 0.2 mCi/L DL-[1-^{14}C]-2-hydroxy-4-methylthiobutanoic acid or L-[U-^{14}C]-lactic acid and the appropriate concentration of unlabeled substrate in the apical compartment. For the experiments performed at pH 5.5, HEPES was replaced by 2-(*N*-morpholino)ethan sulfonic acid. In some experiments, apical and basolateral NaCl was replaced by an equimolar concentration of ChoCl and NaH_2PO_4 was omitted. Monolayers incubated in the absence of Na^+ were washed in modified Krebs buffer with ChoCl instead of NaCl and NaH_2PO_4. The filters were removed from the insert and dissolved in 4 mL scintillation cocktail (Filtron-X) to be counted in a Packard 1500 Tri-Carb counter.

Protein assay. Monolayers were washed in PBS and incubated overnight with 750 μL 0.5 mol/L NaOH. The protein concentration was determined in the homogenate by the Bradford method using the Bio-Rad protein assay kit with bovine serum albumin as standard.

Intracellular pH measurement. Intracellular pH (pH_i) was measured in Caco-2 cells grown on filters using the pH-sensitive fluorescent dye BCECF, as previously described (34). After TER determination, cell monolayers were preloaded from both apical and basolateral sides with 7.5 μmol/L BCECF (modified Krebs buffer, pH 7.4) for 40 min at 37°C. The preloaded monolayers were then washed in modified Krebs buffer (pH 7.4), placed in clusters, and incubated at apical pH of 5.5 or 7.4 in the absence or presence of 20 mmol/L unlabeled DL-HMB. The ratio of fluorescence of the intracellular accumulated BCECF was determined with excitation at wavelengths of 440/485 nm and emission at 535 nm using a luminescence spectrometer (Wallac 1420 Victor^3, Perkin-Elmer). To measure pH_i, the BCECF excitation fluorescence ratios were calibrated using the K^+/nigericin method, as previously described (35). The calibration curve demonstrated that the fluorescence ratios were a linear function of pH_i from 6.0 to 8.0 ($R^2 = 0.947$).

Kinetic and statistical analysis. To estimate the kinetic parameters of apical DL-HMB uptake, the rates of mediated transport were analyzed following the strategy described in Soriano-García et al. (12), assuming either a 1-system or a 2-system model by nonlinear regression from plots generated by the Enzfitter statistical package (Biosoft). The best fit was assigned to the fit with the lowest significantly different residual sums of squares ($P < 0.05$), following the criteria established by Motulsky and Ransnas (36). Apparent inhibition constant (K_i) value was determined from Dixon plot by linear regression analysis.

Results are given as means ± SEM. Data were analyzed by 1-way ANOVA followed by Dunnett's post hoc test (**Table 1**) or Student's *t* test using SPSS software. Differences of $P < 0.05$ were considered significant.

Results

DL-HMB influx (100 μmol/L) was determined in the presence or absence of an imposed H^+-gradient (apical pH, 5.5 or 7.4; basolateral pH, 7.4) and no significant differences were detected after 5-min incubation, either in the presence or in the absence of Na^+ (Table 1). However, incubation with the protonophore

FCCP (50 μmol/L) significantly reduced substrate uptake, suggesting that DL-HMB transport is associated with the H^+-gradient. Na^+ removal and EIPA (1 mmol/L), a specific inhib-itor of apical Na^+/H^+ exchanger (NHE3) (37), significantly reduced DL-HMB transport. Moreover, incubation of cells with 20 mmol/L DL-HMB significantly reduced pH_i (**Fig. 1**) and after substrate removal, pH_i recovery was significantly inhibited in the presence of EIPA, thus confirming NHE3 involvement. To investigate only the imposed H^+ gradient and to minimize the capacity of NHE3 to generate the H^+ gradient, as suggested by Anderson et al. (38), we studied DL-HMB transport during a short incubation period (1 min). Transport was greater at an apical pH of 5.5 (128.5 $\pm$ 7.3) than at pH 7.4 (95.3 $\pm$ 3.2 fmol/μg protein, $n = 3$, $P < 0.05$). Moreover, during this short incubation period, EIPA did not have an effect (pH 5.5 + EIPA: 120.6 6 10.7 fmol/mg protein, n ¼ 3), confirming the reduced NHE3 contribution after 1-min incubation.

To characterize carrier substrate specificity, *cis*-inhibition experiments were performed. First, we examined the contribu-tion of PAT1 and PepT1. The presence of 20 mmol/L D-Pro, L-Ala, β-Ala or betaine, specific substrates of system PAT1 (17,19,25), and glycyl-sarcosine (Gly-Sar), a specific substrate of PepT1 (39), did not affect DL-HMB transport (Table 1).

The potential role of MCT1 in DL-HMB transport was assayed with specific inhibitors of this transport system: phloretin (0.2 mmol/L) and CHC (1 mmol/L) (22). Both inhib-itors significantly reduced DL-HMB uptake (Table 1). In ad-dition, *cis*-inhibition experiments showed a significant decrease in DL-HMB transport in the presence of L-lactate, pyruvate, β-hydroxybutyrate, and butyrate, MCT1 substrates, but not with D-lactate (Table 1). Nevertheless, because pH did not affect DL-HMB uptake after 5-min incubation, substrate transport was further characterized in the absence of the imposed H^+ gradient. Both CHC and L-lactate induced a significant reduc-tion in DL-HMB uptake (Table 1), similar to that detected at pH 5.5.

The kinetic parameters of apical DL-HMB uptake were calculated from initial DL-HMB influx rates at a range of sub-strate concentrations of 0–40 mmol/L. The nonmediated com-ponent was measured in the presence of CHC (K_D, 1.9 nL/μg protein) and subtracted from total DL-HMB uptake. The best fit for the mediated component (**Fig. 2**) was obtained by consid-ering the participation of a single transport system with a K_m of 13.1 $\pm$ 0.04 mmol/L and a V_{max} of 43.6 $\pm$ 0.14 pmol/μg protein. The interaction between DL-HMB and L-lactate was also kinetically characterized. In these experiments, the uptake of L-lactate (1, 2, and 3 mmol/L) was determined in the presence of increasing unlabeled DL-HMB concentrations (0, 5, 10, and 15 mmol/L). The Dixon plot analysis (**Fig. 3**) supported a competitive inhibition with a K_i of 17.5 $\pm$ 0.11 mmol/L.

Discussion

Met is a limiting essential amino acid in commercial animal diets. To supply this nutritional requirement, dietary supple-mentation with synthetic sources of Met, such as DL-Met or DL-HMB, is common practice in animal feed production. Because of the economic implications, many studies have been performed to evaluate the relative biological efficacy of DL-HMB compared with Met; however, there is still some debate. Because intestinal absorption is the first step in Met analogue utilization, the identification of the transport mechanisms involved in DL-HMB uptake is worthwhile to study and may allow the design of new dietary strategies to better valorise the hydroxy-analog of Met.

DL-HMB was tested as substrate of PAT1, because the structural requirements for this transport mechanism establish that a free amino group is not essential for binding (24). How-ever, the cis-inhibition experiments showed no interaction be-tween PAT1 substrates and the hydroxy analog. This was as expected, because replacement of the amino group in L-Ala, which is substrate of PAT1, by a hydroxyl group, giving lactate, completely reduces its binding capacity (40). The same would be expected to occur with DL-HMB, which has a hydroxyl group in the place of the amino group but in addition, the corresponding amino acid, L-Met, is not recognized by PAT1 (41). cis-Inhibition experiments with Gly-Sar, a specific substrate of PepT1, indi-cated no effect on DL-HMB transport. Although the smallest substrate to be transported by this mechanism is 5-amino pentanoic acid (23), the presence of either the methylthio group at C4 or the hydroxyl group at C2 might prevent substrate recognition.

In contrast, the results obtained with specific MCT1 inhib-itors and substrates suggest the interaction of DL-HMB with this transport mechanism. Brachet and Puigserver (9) reported the inhibition of DL-HMB transport with a-cyanocinnamate, pro-pionate, L-lactate, and pyruvate in chicken intestine. In addition, Friedrich et al. (42) described the mutual inhibition of L-lactate and L-Leu hydroxy analog transport in rabbit intestine. More-over, both groups described the uptake of both hydroxy analogs as not stereoselective. MCT1 shows stereoselectivity for 2-hydroxy-substituted monocarboxylates such as lactic acid and mandelic acid (43) but not for b-hydroxybutyrate (44). In this sense, the results presented here, indicating no effect of D-lactate on DL-HMB uptake, confirm MCT1 substrate spec-ificity but give no additional information from the carrier stereo-selectivity for the hydroxy analog.The kinetic analysis of DL-HMB transport in Caco-2 cells indicates the participation of a single low-affinity, high-capacity transport system. Brachet and Puigserver (9) and Maenz and Engele-Schaan (11) calculated lower Km values (1.7 and 1.13 mmol/L, respectively) for L-HMB transport in chicken brush border membrane vesicles. In contrast, Friedrich et al., (42) found a Km of 15.4 mmol/L for L-Leu hydroxy analog trans-port in brush border membrane vesicles from rabbit intestine. Nevertheless, although revealing differences, all these values are in the range of the affinity constants reported for MCT1 activity (1–16 mmol/L) (45). Moreover, the Vmax calculated for DL-HMB uptake is also similar to the data reported in Caco-2 cells for MCT1 substrates such as lactate and butyrate (43, 46,47). The kinetic parameters calculated here for DL-HMB transport are thus compatible with the participation of system MCT1. The nonmediated DL-HMB component is similar to that reported for L-Met in Caco-2 cells (48), although Maenz and Engele-Schaan (11) found higher KD values for L-HMB than for L-Met, whereas Brachet and Puigserver (9) reported the opposite.

Dixon plot analysis shows that DL-HMB inhibition of L-lactate transport is competitive, supporting the view that both substrates share the same transport mechanism. Brachet and Puigserver (9) also described the inhibition of L-HMB transport by L-lactate as a competitive interaction with an inhibition constant (0.55 mmol/L) lower than the Km that they found for L-HMB transport. Because substrates acting as competitive inhibitors have Ki values that closely match their Km values (45), the inhibition constant obtained in Caco-2 cells for DL-HMB (17.5 6 0.11 mmol/L) provides further evidence of the carrier affinity for this substrate. This is consistent with previous data reporting Km values for L-lactate transport (31) lower than that reported here for DL-HMB.

All these results provide evidence of MCT1 participation inintestinal DL-HMB absorption. However, the imposed H^+ gradient did not stimulate DL-HMB transport, despite the fact that FCCP significantly reduced substrate transport and pH_i was lowered by DL-HMB uptake. The driving force for H^+-dependent transport systems is the acidic microclimate adjacent to the brush border membrane, maintained by NHE3, which exchanges intracellular H^+ and extracellular Na^+. The inward H^+ movement associated with substrate uptake activates apical NHE3 activity, which maintains pH_i and the H^+ transmem-brane electrochemical gradient (49). The lack of differences in DL-HMB uptake between pH conditions might thus be explained in terms of the capacity of NHE3 to generate and maintain the H^+ electrochemical gradient. Accordingly, NHE3 inactivation (EIPA or Na^+ removal) and H^+-gradient dissipa-tion (FCCP) significantly reduced hydroxy analog transport at a pH of 7.4. Moreover, in the absence of an imposed H^+ gradient, CHC and L-lactate also inhibited DL-HMB transport. In fact, the experiments of Brachet and Puigserver (8,9), who described a shared transport system with L-lactate, were performed at pH 7.4, although these authors already suggested the possible con-tribution of the H^+ gradient on DL-HMB transport. To further investigate the apparent capacity of MCT1 to operate at a pH of 7.4, the CHC effect was tested after 5-min incubation in the absence of NHE3 activity. There was no effect (EIPA, 161.6 ± 2.4; EIPA + CHC, 170.8 ± 16.5 fmol/μg protein, $n = 3$), confirming that the capacity of MCT1 to transport DL-HMB in the absence of an imposed H^+ gradient is due to the high contribution of NHE3. Anderson et al. (38) observed that PAT1 activity in Caco-2 cells showed partial Na^+ dependence, which increased with incubation time. At short incubation periods, the imposed H^+-gradient maintained substrate transport, but dur-ing long incubation periods, the activity of NHE3 was needed to maintain the gradient. This hypothesis was checked for DL-HMB transport; if 5-min incubation was sufficient for NHE3 to generate an H^+ gradient and to reduce differences between pH conditions, DL-HMB transport at a short incubation period would only reflect the effect of the imposed H^+ gradient. The fact that after 1 min incubation DL-HMB uptake was higher in the presence of an H^+ gradient confirms both the pH depen-dence of DL-HMB transport across the apical membrane and the high contribution of NHE3 in this process.

In **conclusion**, the data reported here on DL-HMB transport across the apical membrane of Caco-2 cells suggest the partic-ipation of a transport mechanism with many of the properties of system MCT1. Moreover, the results are consistent with the coop-erative functional relation described between H1-dependent transport systems and apical NHE3 (38,50). Taking into account the importance of this analog in animal production, knowledge of the transport mechanism involved would open further studies concerning the regulation of this mechanism by dietary contents.

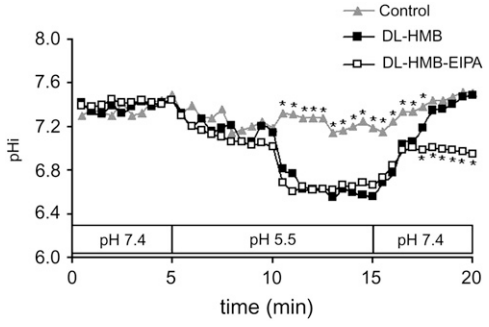

Figure 1 pH_i in Caco-2 cells loaded with the pH-sensitive fluorochrome BCECF. The preloaded monolayers were incubated at the indicated apical pH. At min 10, DL-HMB (20 mmol/L) was added to the apical compartment (□,■) and after 5-min incubation, the substrate was removed and the cells were then incubated in the absence (▲,■) or presence (□) of 1 mmol/L EIPA in the apical compartment. Values are means, $n = 3$; SEM values were less than 2% of the mean. *Different from the other conditions at that time, $P < 0.05$ (Student's t test).

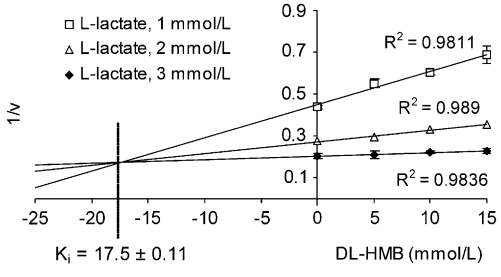

Figure 3 Dixon plot of DL-HMB inhibition of L-lactate transport across Caco-2 cell apical membrane. Transport of 1, 2, and 3 mmol/L L-[^{14}C]-lactic acid (0.2 mCi/L) was measured for 5 min in the presence of 0, 5, 10, and 15 mmol/L unlabeled DL-HMB (apical pH, 5.5; basolateral pH, 7.4). Values are means ± SEM, $n = 3$ monolayers. The apparent K_i determined by linear regression analysis from the Dixon plot was 17.5 ± 0.11 mmol/L.

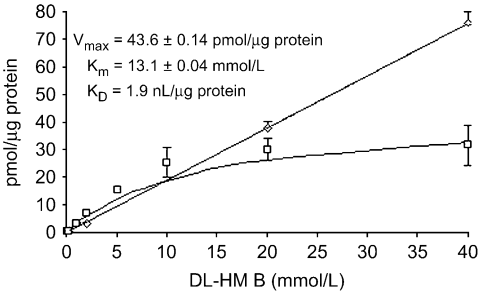

Figure 2 Substrate concentration dependence of DL-HMB transport across Caco-2 cell apical membrane. Transport of DL-[1-^{14}C]-2-hydroxy-4-methylthio-butanoic acid (0.2 mCi/L) was measured for 5 min in the presence of an H^+ gradient and increasing unlabeled DL-HMB concentrations (0–40 mmol/L). The nonmediated component (◇) was estimated ($R^2 = 0.999$) in the presence of CHC (1 mmol/L) and subtracted from total DL-HMB uptake. The best fit for the mediated component (□) was obtained by considering a model of a single transport system. Values are means ± SEM, $n = 3$–4 monolayers.

TABLE 1 DL-HMB transport across Caco-2 cell apical membrane at pH 5.5 and 7.4[1]

	Apical pH 5.5	Apical pH 7.4
	fmol/µg protein	
Control	431.1 ± 9.9	429.9 ± 12.2
ChoCl	354.1 ± 19.4*	354.8 ± 30.8*
FCCP, *50 µmol/L*	134.6 ± 11.1*	114.2 ± 4.6*
EIPA, *1 mmol/L*	185.5 ± 19.2*	163.4 ± 24.4*
Control	425.9 ± 9.9	—
D-Proline, *20 mmol/L*	421.8 ± 35.9	—
L-Alanine, *20 mmol/L*	432.4 ± 30.1	—
β-Alanine, *20 mmol/L*	414.8 ± 20.4	—
Betaine, *20 mmol/L*	406.8 ± 54.0	—
Gly-Sar, *20 mmol/L*	434.1 ± 55.8	—
Control	424.8 ± 8.5	416.5 ± 12.5
Phloretin, *0.2 mmol/L*	181.2 ± 10.9*	—
CHC, *1 mmol/L*	139.4 ± 17.2*	156.4 ± 6.6*
L-Lactate, *20 mmol/L*	240.9 ± 15.4*	322.1 ± 35.5*
D-Lactate, *20 mmol/L*	416.0 ± 7.9	—
Pyruvate, *20 mmol/L*	346.1 ± 23.5*	—
β-OHbut[2], *20 mmol/L*	377.8 ± 4.2*	—
Butyrate, *20 mmol/L*	276.7 ± 5.7*	—

[1] Values are means ± SEM, $n = 3$ monolayers except for control and ChoCl, $n = 4$. *Different from the corresponding control, $P < 0.05$ (Dunnett's test).
[2] β-OHbut, β-hydroxybutyrate.

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