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ORIGINAL ARTICLE

Glutamate metabolism in a human intestinal epithelial cell layer model

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Abstract

Plasma glutamate concentrations are constant despite dynamic changes in diets. Most likely, virtually all the dietary glutamate is metabolized in the gut. The present study investigated permeability and metabolism of dietary glutamate in a Caco-2 intestinal epithelial cell layer model by tracing the fate of [U-^{13}C] or [^{15}N]glutamate added to the apical medium. For comparison, several other labelled essential and non-essential amino acids were tested as well. Almost all the labelled glutamate in the apical medium (98% and 96% at 24 h of the culture, respectively) was incorporated in the cell layer, while it barely appeared at the basolateral side, indicating an almost complete utilization of glutamate. Indeed, the ^{13}C was incorporated into alanine, proline, ornithine, and glutamine, and the ^{15}N was incorporated into alanine, glutamine, ornithine, proline, branched chain amino acids and also found as ammonia indicative of oxidation. In contrast, substantial apical-to-basolateral transport of amino acids (8–85% of uptake) other than glutamate and aspartate was evident in studies using amino acid tracers labelled with ^{13}C, ^{15}N or D. These results suggest that the intestinal epithelial cell monolayer utilizes dietary glutamate which adds to maintaining glutamate homeostasis in the body.

Keywords Metabolic barrier · Intestinal mucosa · Stable isotope · Amino acid · Glutamic acid

Introduction

Glutamate, a nonessential amino acid, not only is a building block of proteins but has also a variety of other roles in the body. Since its amino moiety is readily exchanged with other amino acids by the corresponding aminotransferase, glutamate metabolism is linked to both the catabolism and anabolism of other amino acids (Sakai et al. 2004). Since deamination of glutamate generates α-ketoglutarate, a TCA cycle metabolite, this process is an important anaplerotic pathway. In addition, exchange of the amino moieties of glutamate and aspartate is an essential component of the malate-aspartate shuttle, which transfers NADH across mitochondrial membranes. Extracellular glutamate also has unique functions. Recently, it was suggested that glutamate is a signalling molecule also in nonneuronal tissues, since glutamate receptors are expressed in various types of cells (Du et al. 2016). To fulfil the function of glutamate as a signalling molecule, extracellular concentrations of glutamate would be maintained at low levels. Indeed, concentration deference of intracellular glutamate in intestinal mucosa from that of plasma are reportedly more than 100 (Adibi and Mercer 1973).

The postprandial concentration of circulating glutamate is also maintained at a low level despite of the dynamic changes in dietary glutamate input. Glutamate is one of the most abundant amino acids in alimentary proteins (Hou et al. 2019; Li et al. 2011); thus, glutamate intake is more than 15 g/person on average, accounting for 20% of the total amino acid intake, according to a national survey in the US (NHANES III) (Food and Nutrition Board 2005). This is not the case for other amino acids, such as branched chain amino acids (BCAAs) and alanine, since their concentrations in the

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blood are elevated after a meal despite their lower dietary intake than glutamate (Sakai et al. 2004). Dietary glutamate is metabolized during the first pass through the gut, which is why the circulating glutamate level is not elevated after the ingestion of glutamate (Reeds et al. 1996; Riedijk et al. 2007; Nakamura et al. 2013; Hou and Wu 2018). Dietary glutamate carbon (C) is utilized for the synthesis of alanine or lactate or catalysed to CO_2 for energy production in the gut, and only a minor proportion of dietary glutamate is taken up by the blood (Reeds et al. 2000). Our study using ^{15}N-labelled glutamate also showed that the majority of dietary glutamate is utilized in the gut as a nitrogen (N) source to synthesize alanine, aspartate, proline, and ornithine (Nakamura et al. 2017).

The gut consists of a variety of organized tissue types, such as smooth muscle, nervous system tissue, and the epithelial mucosa, and cells, such as secretory cells, immune cells, and interstitial cells. Although previous studies suggest that the intestine plays a role in maintaining glutamate homeostasis in the circulation, which intestinal cell types limit direct entry of dietary glutamate into the portal vein has not been elucidated. There are numbers of studies indicating glutamate uptake by primary culture of enterocytes and intestinal epithelial cell lines (Mordrelle et al. 2000; Broer and Fairweather 2018). Intestinal metabolic capacity for glutamate is presumably high, since glutamine is known to be one of major energy source for these cells through its catabolism to glutamate (Fleming et al. 1991; Wu et al. 1995). Thus, it is believed that intestinal epithelial cell layer metabolizes dietary glutamate and limits its direct entry into the circulation (Blachier et al. 2009; Wu 1998). However, direct evidences are limited. Although Oba et al. have shown that enterocytes metabolized glutamate to CO_2 and lactate, their study did not quantify either entire fate of glutamate taken up by the cells or its permeability across these cells (Oba et al. 2004). Nicklin et al. have evaluated glutamate transport across intestinal cell layer in in vitro model system using radiolabelled glutamate by measuring radioactivity (Nicklin et al. 1995). However, they evaluated neither glutamate metabolism in the epithelial cells nor its transport itself. Therefore, the present study aimed to quantify glutamate transport across intestinal monolayer epithelial cells in addition to its metabolism during the first pass.

Caco-2 cells, a human intestinal cell line derived from colon cancer, have been shown to differentiate on the membrane filter in transwell culture into cells that exhibit both morphological and biochemical features characteristic of intestinal epithelial cells. The cells develop mucosal villi on the apical membrane and intercellular gap junctions (Hidalgo et al. 1989). An apparent polarity in transport velocity has also been shown in this model (Hidalgo and Borchardt 1990a, b). Thus, Caco-2 cells cultured in transwells are often used to assess intestinal transport and the permeability of the intestine to amino acids (Hidalgo and Borchardt 1990a; Nicklin et al. 1995; Thwaites et al. 1996, 1994; Satsu et al. 2009) as well as to food components (Steensma et al. 2004; Yasuda et al. 2015) and drugs (Artursson and Karlsson 1991). In the present study, we investigated glutamate transport and fates of glutamate-C and N in this intestinal epithelial cell layer model using [U-^{13}C] and [^{15}N] glutamate. Furthermore, we compared glutamate transport across the epithelial cell layer with those of other amino acids. This study demonstrates that monolayer intestinal epithelial cells metabolized almost all the glutamate taken up from the apical medium and limited its entry into the basolateral side.

Materials and methods

Cell culture

Caco-2 cells were purchased from the European Collection of Authenticated Cell Culture (ECACC; Salisbury, UK). The cells were seeded on polycarbonate cell culture inserts (pore size, 0.4 µm; diameter, 24 mm) in a 12-well transwell plate (Corning; NY, USA). The cells were grown and differentiated as described previously (Yasuda et al. 2015) in E-MEM (FUJIFILM Wako, Osaka, Japan) supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 ng/mL), and Gibco™ MEM nonessential amino acid solution (final concentrations of glycine, L-alanine, L-asparagine, L-aspartate, L-glutamate, L-proline and L-serine: 100 µM each) at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 for 21 days. Apical and basolateral media (0.5 and 1.5 mL, respectively) were exchanged twice a week. The transepithelial electrical resistance (TEER) value of the monolayers was monitored during culture using a Millicell-ERS instrument (Millipore Corp., Bedford, MA, USA). After confirming epithelial cell differentiation by the TEER value (> 400 Ω cm^2), the epithelial layer model was used for experiments.

Experimental procedures

Basolateral and apical media were refreshed, and apical media were supplemented with 300 µM [U-^{13}C] or [^{15}N] glutamate in experiments 1 and 2, respectively. The cells were cultured for 1, 3, 6, 10, and 24 h, and apical and basolateral media were collected. In experiment 3, apical media were supplemented with 1) 400 µM [1-^{13}C]leucine, 400 µM [1-^{13}C]isoleucine, 400 µM [1-^{13}C]valine, 400 µM [1-^{13}C]lysine hydrochloride, 200 µM [1^{-13}C]phenylalanine, 50 µM [1-^{13}C]tryptophan and 2000 µM [U-^{13}C] glutamine; 2) 200 µM [ring-2-^{13}C]histidine hydrochloride monohydrate, 200 µM [1-^{13}C]serine, 200 µM [1-^{13}C]

proline, 400 μM [U-^{13}C]threonine, 100 μM [1-^{13}C]methionine and 600 μM [guanido-$^{13}C_2$]arginine hydrochloride; 3) 200 μM [^{15}N]aspartate; 4) 200 μM [$^{15}N_2$]asparagine monohydrate; 5) 200 μM [1-^{13}C]glycine, 200 μM [ring-3,5-D_2] tyrosine and 400 μM [U-^{13}C]cysteine; or 6) 200 μM [2,3-$^{13}C_2$]alanine and 200 μM [1-^{13}C]glutamate at the time of medium change. The culture media were harvested after 24 h of culture and stored at –80 °C before analysis. All the amino acids labelled with stable isotopes were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA).

Amino acid analysis

The concentrations of amino acids in the media were determined using an amino acid analyser (L-8000, Hitachi, Tokyo, Japan) after deproteinization with 5% trichloroacetic acid. Briefly, amino acids separated by cation-exchange chromatography were detected spectrophotometrically after post-column reaction with ninhydrin reagent.

The isotopic enrichment of amino acids and ammonia in the media was measured by liquid chromatography–tandem mass spectrometry (LC–MS/MS; Agilent 1200 Series LC System and a 6400 Series Triple Quad LC/MS; Agilent Technologies, Santa Clara, CA, USA) after derivatization as reported previously (Nakamura et al. 2015). Briefly, samples of media were deproteinized with methanol, derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC; Waters Corporation, MA, USA), and then injected into a high-performance LC (HPLC) column (L-column ODS; Chemicals Evaluation and Research Institute, Japan). Positive ions generated by electrospray ionization (ESI) were analysed in multiple reaction monitoring (MRM) mode. The following precursor ions (m/z) were used to detect individual amino acids and ammonia: glutamate: 318, glutamine: 317, aspartate: 304, asparagine: 303, alanine: 260, proline: 286, serine: 276, glycine: 246, arginine: 173, ornithine: 473, leucine and isoleucine: 302, valine: 288, phenylalanine: 336, tyrosine: 352, tryptophan: 375, methionine: 320, cystine: 291, threonine: 290, lysine: 487, histidine: 326 and ammonia: 188. To detect their isotopomers, the m/z values were increased by the corresponding numbers. The collision energy was set to 30 eV for ornithine, 10 eV for arginine and 25 eV for other amino acids, and a common ion (m/z 171) generated from AQC derivatives was detected as a product ion. Regarding [U-^{13}C]cysteine, M_3 and M_6 isotopomers of cystine were measured, since isotope enrichments in cysteine and cystine are considered to be equal due to their rapid exchange in physiological solutions through thiol-disulfide reactions (Lash and Jones 1985).

Calculation

The isotopic enrichment (*IE*: molecular percent excess) of each amino acid was calculated from the tracer/tracee ratio (*TTR*) as follows:

$$\mathrm{IE(mol\%)} = \mathrm{TTR}(1 + \mathrm{TTR})$$

When multiple ^{13}C-labels appeared, the tracer/tracee ratio and the isotopic enrichment of the isotopomer with an additional molecular weight of *i* (*TTRi* and *IEi*, respectively) were calculated as follows:

$$\mathrm{TTR}_i(\mathrm{mol\%}) = \left(M_i/M_0\right)_{\mathrm{sample}} - \left(M_i/M_0\right)_{\mathrm{back}} - \left(\mathrm{TTR}_{i-1} \times \left(M_1/M_0\right)_{\mathrm{back}} - \mathrm{TTR}_{i-2} \times \left(M_2/M_0\right)_{\mathrm{back}} - \cdots\right)$$

$$\mathrm{IE}_i(\mathrm{mol\%}) = \mathrm{TTR}_i/(1 + \sum_{(x=0)}^{n} \mathrm{TTR}_x).$$

Here, M_i is the abundance of the isotopomer with an additional molecular weight of *i*. Thus, $(M_i/M_0)_{\mathrm{sample}}$ and $(M_i/M_0)_{\mathrm{back}}$ are the relative abundance of the amino acid in the sample and background, respectively. *n* is the number of carbons in the amino acid. TTR_x is the tracer/tracee ratio of the isotopomer with an additional molecular weight of *x*. The average ^{13}C enrichment of all the carbons in the amino acids (*AIE*) was calculated as reported (Nakamura et al. 2013) using the following equation:

$$\mathrm{AIE(mol\%)} = \sum_{i=1}^{n}(\mathrm{MPE}_i \times i)/n.$$

Then, the total ^{13}C content (C_{13C}) of the amino acid in each medium was calculated as follows:

$$C_{13C}(\mathrm{nmol}) = \mathrm{AIE} \times C \times V.$$

Here, *C* is the amino acid concentration (μM) in the medium, and *V* is the volume of the medium (mL). The ^{15}N content of each amino acid and ammonia was calculated in a similar manner with the following equation:

$$C_{15N}(\mathrm{nmol}) = \mathrm{IE} \times C \times V.$$

Here, *IE* is the ^{15}N-enrichment (mol%) of each amino acid.

Uptake (nmol) of each labelled amino acid by the cells from the apical medium was estimated as the difference in the labelled amino acid content in the apical medium before and after culture. The transport ($T_{A\text{-}B}$, *% of Up*) of the labelled amino acid was calculated as follows:

$$T_{A-B}(\%) = \mathrm{IE} \times C \times V/Up.$$

In this case, *IE* and *C* are isotopic enrichment (mol%) and concentration (μM) of a target amino acid in the basolateral medium. *V* is volume (mL) of basolateral medium and *Up* is uptake of the target amino acid from the apical medium (nmol). Fractional distribution (*% of Up*) of each label (i.e., ^{13}C or ^{15}N) into both apical and basolateral amino acids are calculated similarly.

Statistics

Differences in concentration before and after culture and on the apical and basolateral sides were analysed by Sidak's multiple comparisons test after 1-way analysis of Variance (ANOVA). Changes from baseline were analysed by Dunnett's multiple comparisons test. Differences between apical and basolateral were analysed by paired student's *t* test. Amino acid uptake from the apical medium and transport from apical to basolateral compartment were analysed after logarithmic transformation, since there were more than 100 folds variations among the groups. Then statistical differences from glutamate were analysed by Dunnett's test after one-way ANOVA. A probability less than 0.05 indicated statistical significance. All the data are expressed as the means ± standard deviations (SDs).

Results

Amino acid concentrations in the apical and basolateral media (Exp. 1)

Concentrations of individual amino acids in the apical and basolateral media changed to different extents over 24 h of culture (Fig. 1a, b; Table 1). In the apical medium, the concentrations of glutamate, aspartate, lysine and arginine decreased significantly, while the alanine and leucine concentrations increased during culture. In addition, changes in the basolateral medium were different from those in the apical medium. For example, there was no decrease in the glutamate, aspartate, lysine or arginine concentrations in the basolateral medium. This caused the apical/basolateral concentration ratios for glutamate, lysine and arginine, leucine and alanine to be significantly different from 1.0 (Fig. 1c), which indicates the polarity of Caco-2 cells in transwell culture in the metabolism of these amino acids.

Fate of glutamate-C (Exp. 1)

The glutamate concentration on the apical side decreased in a time-dependent manner (Fig. 2a; Table 1), which was accompanied by a time-dependent decrease in ^{13}C content of apical glutamate (Fig. 2b). At the end of the culture period, ^{13}C-glutamate in the apical medium was almost depleted

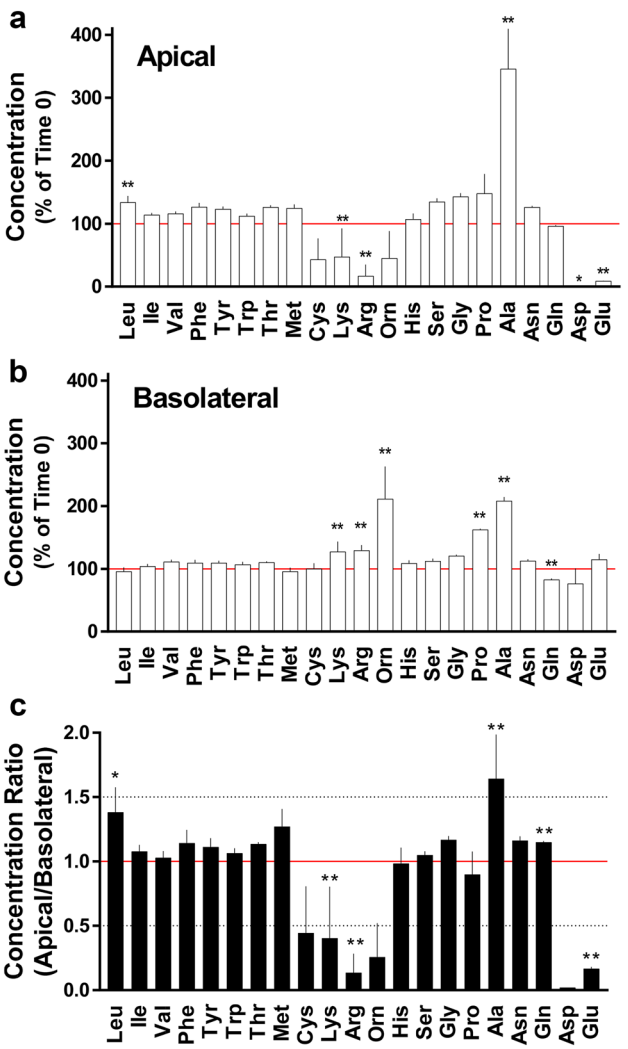

Fig. 1 Amino acid concentrations in the apical and basolateral media (Exp. 1). **a** and **b**: Amino acid concentrations in the apical and basolateral media after 24 h of culture. Amino acid concentrations are expressed as percentages of those before culture. **c** Ratios of amino acid concentrations in the apical and basolateral media after 24 h of culture. All data are expressed as the means ± SDs ($N=4$). *: $p<0.01$, **: $p<0.001$ before vs. after culture in **a** and **b** and apical vs. basolateral media in **c** (Sidak's test)

(Fig. 2b), indicating that almost all the [U-^{13}C]glutamate added to the apical medium had been taken up by the cells. However, the basolateral concentration of glutamate was maintained during culture (Fig. 2a; Table 1), and the [U-^{13}C] glutamate in the basolateral medium after 24 h of culture was only 1.1 ± 0.7% of the amount added.

To trace the fate of ^{13}C originating from [U-^{13}C]glutamate, the ^{13}C contents in other amino acids in the media were measured. Significant ^{13}C increases over the natural abundances were detected in proline, alanine and ornithine, and the contents elevated steadily during culture (Fig. 3a; Table 2). After 24 h of culture, the recovered ^{13}C contents

Table 1 Amino acid concentrations before and during the culture in Exp. 1

	Apical amino acid (μM)						Basolateral amino acid (μM)					
Time (h)	0	1	3	6	10	24	0	1	3	6	10	24
Leu	466	512	566#	613#	635#	622#	472	491	467*	466*	449*	453*
	(11)	(30)	(54)	(75)	(39)	(48)	(7)	(11)	(6)	(18)	(18)	(30)
Ile	393	418	436#	437#	438#	448#	399	422#	408	423#	415	416
	(8)	(24)	(18)	(12)	(15)	(12)	(7)	(7)	(4)	(10)	(11)	(15)
Val	422	454	480#	481#	479#	489#	428	459#	450#	469#	464#	476#
	(11)	(25)	(19)	(10)	(18)	(15)	(6)	(9)	(5)	(11)	(14)	(15)
Phe	215	233	250#	262#	266#	272#	219	234#	227*	234#	230#,*	239#
	(5)	(12)	(14)	(17)	(4)	(14)	(4)	(3)	(2)	(6)	(5)	(10)
Tyr	204	217	230#	238#	242#	251#	207	221#	214	223#	218#,*	226#,*
	(5)	(12)	(12)	(12)	(3)	(8)	(3)	(4)	(1)	(5)	(5)	(8)
Trp	58	60	63#	64#	62	65#	57	61#	59*	60	59	61*
	(1)	(4)	(2)	(3)	(2)	(2)	(2)	(1)	(2)	(2)	(2)	(3)
Thr	394	419	444#	455#	464#	498#	398	423#	414*	432#	426#,*	438#,*
	(7)	(22)	(19)	(13)	(10)	(12)	(6)	(10)	(2)	(9)	(10)	(7)
Met	105	114	122#	128#	131#	131#	108	113	108	109	105*	104*
	(3)	(7)	(9)	(11)	(4)	(7)	(2)	(2)	(1)	(3)	(3)	(7)
Cys	80	79	76	67	57	34#	82	86	82	86*	84	82
	(3)	(5)	(2)	(9)	(19)	(27)	(1)	(1)	(2)	(3)	(4)	(7)
Lys	445	462	457	399	328	211#	452	500	492	530	537#	575#
	(7)	(28)	(18)	(74)	(154)	(200)	(13)	(11)	(21)	(35)	(52)	(73)
Arg	567	543	479	325#	224#	95#	573	628*	631	700#,*	709#,*	741#,*
	(13)	(35)	(75)	(167)	(197)	(103)	(7)	(12)	(30)	(53)	(67)	(49)
Orn	65	67	65	56	45	29#	66	81*	83*	100#,*	109#	139#,*
	(2)	(3)	(4)	(14)	(26)	(28)	(1)	(5)	(7)	(12)	(19)	(34)
His	189	197	207	207	204	203	190	202	197	206	200	207
	(7)	(12)	(9)	(5)	(11)	(17)	(2)	(4)	(2)	(5)	(5)	(9)
Ser	159	194#	210#	217#	214#	214#	181	193#	190*	197#,*	196#,*	204#,*
	(24)	(9)	(10)	(6)	(3)	(9)	(2)	(4)	(2)	(4)	(6)	(7)
Gly	207	229#	253#	268#	277#	296#	210	227#	224#	237#,*	237#,*	254#,*
	(4)	(13)	(13)	(9)	(7)	(11)	(3)	(2)	(2)	(5)	(6)	(4)
Pro	163	179	189	197	211#	241#	165	186#	186#	201#	211#	268#
	(7)	(9)	(5)	(12)	(26)	(50)	(7)	(4)	(2)	(8)	(5)	(3)
Ala	275	340	401	512#	628#	951#	279	332	339	380#,*	406#,*	581#,*
	(7)	(22)	(45)	(75)	(93)	(175)	(4)	(2)	(7)	(14)	(12)	(18)
Asn	116	118	126#	133#	138#	146#	111	117	116	119#,*	120#,*	125#,*
	(7)	(4)	(7)	(1)	(4)	(3)	(4)	(3)	(1)	(3)	(3)	(3)
Gln	1765	1808	1860	1860	1854	1697	1779	1819	1740	1754	1660#	1476#,*
	(81)	(114)	(105)	(84)	(42)	(32)	(47)	(35)	(15)	(29)	(56)	(27)
Asp	139	122#	84#	26#	3#	0#	141	146*	136*	147*	135*	108#,*
	(4)	(10)	(4)	(3)	(1)	(0)	(2)	(6)	(8)	(7)	(14)	(34)
Glu	503	468#	463#	334#	131#	45#	237*	246*	241*	260	270#,*	273#,*
	(8)	(11)	(17)	(22)	(14)	(1)	(19)	(12)	(2)	(13)	(12)	(22)
NH3	307	333	412#	488#	581#	881#	302	332	352*	414#,*	491#,*	670#,*
	(70)	(17)	(2)	(7)	(11)	(87)	(76)	(21)	(16)	(24)	(2)	(14)

Amino acid concentrations in apical and basolateral media before and after the culture in Exp. 1. Initial glutamate concentration in the apical medium was higher than in basolateral medium, since [U-^{13}C]glutamate was added to the apical medium. Data were expressed as Means and SDs (in the parentheses) of 4 individual transwell cultures at each time point. #: $p < 0.05$ v.s. time 0 h (Dunnett's test), *: $p < 0.05$ v.s. apical (paired *t*-test)

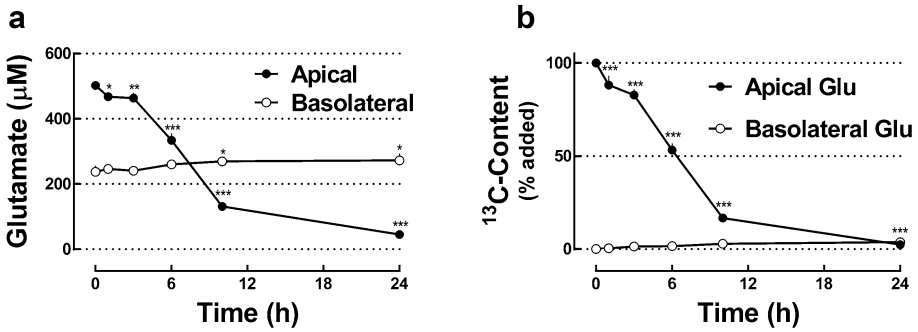

Fig. 2 Time-dependent changes in the concentrations, U-^{13}C enrichment and ^{13}C content of glutamate in the apical and basolateral media (Exp. 1). Concentrations (**a**) and total ^{13}C content (**b**) of glutamate in the apical (closed circle) and basolateral (open circle) media during the 24 h of culture were plotted. The ^{13}C content is expressed as a percentage of the amount added (means ± SDs (N = 4)). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ vs. before culture (Dunnett's test)

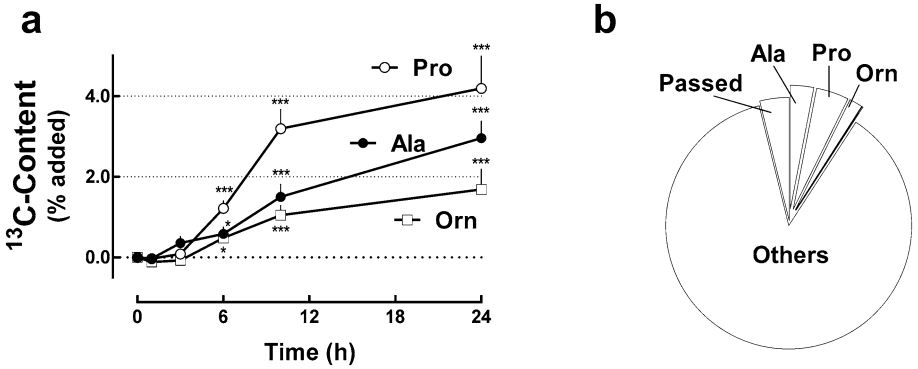

Fig. 3 Fate of glutamate-C (Exp. 1). **a** The combined total ^{13}C content in proline (open circle), alanine (closed circle) and ornithine (open square) in the apical and basolateral media during the 24 h of culture. The values are expressed as a percentage of the amount added (means ± SDs (N = 4)). *: $p < 0.05$, ***: $p < 0.001$ vs. before culture (Dunnett's test). **b** Fractional distribution of glutamate-C (% of uptake of glutamate-C by the cells from the apical medium) after 24 h of culture. "Others" indicates the unidentified fraction and "Passed" indicates ^{13}C released into the basolateral medium in the form of glutamate. Glutamine was not analysed in Exp. 1

in alanine, proline, ornithine and aspartate were 3.1%, 4.3%, 1.7% and 0.1%, respectively, of the ^{13}C taken up (Fig. 3b). Although 3.9% of the ^{13}C was released into the basolateral medium in the form of glutamate, the fraction released directly (i.e., ^{13}C released in the form of [U-^{13}C]glutamate) was less than 30% of the total ^{13}C in the basolateral glutamate, since other isotopomers such as M_1, M_2, M_3 were also released into the basolateral medium (Table 2). The fate of the majority of the ^{13}C (87%) was not identified, presumably due to its metabolism to CO_2 and other metabolites, such as glutamine and nucleic acids.

Fate of glutamate-N (Exp. 2)

Glutamate concentrations on the apical side decreased in a time-dependent manner, as was observed in Exp. 1 (Fig. 4a; Table 3), which was accompanied by a time-dependent decrease in ^{15}N-glutamate content (Fig. 4b). After 10 h of culture, the [^{15}N]glutamate in the apical medium was almost depleted (Fig. 4b). However, the basolateral concentration of glutamate did not change during the culture period (Fig. 4a and Table 3), and only a minor proportion of the [^{15}N]glutamate (2.3% of the amount added) was found in the basolateral medium after 24 h of culture.

To trace the fate of glutamate-^{15}N added to the apical medium, ^{15}N-labelled amino acids in the medium were analysed (Table 4). The ^{15}N content in alanine, glutamine, ornithine, proline, BCAAs and ammonia increased in a time-dependent manner until 10 h after [^{15}N] glutamate had been added to the apical medium (Fig. 5a). Then, the ^{15}N content in these amino acids remained almost constant until the end (24 h) of the culture period. After 24 h of culture, the recovered ^{15}N contents in alanine, glutamine, ornithine, proline,

Table 2 Isotopic enrichments of apical and basolateral amino acids before and during the culture in Exp. 1

	Apical amino acid						Basolateral amino acid					
Time (h)	0	1	3	6	10	24	0	1	3	6	10	24
	Isotopic enrichment (mol%)						Isotopic enrichment (mol%)					
Glu M_1	−0.04	−0.03	−0.04	0.09	0.33#	0.31#	0.00	0.01	0.01	0.06	0.17*	0.18
	(0.01)	(0.01)	(0.02)	(0.06)	(0.05)	(0.19)	(0.14)	(0.13)	(0.13)	(0.12)	(0.01)	(0.08)
Glu M_2	− 0.01	0.00	− 0.01	0.09#	0.22#	0.07#	0.00	− 0.02	0.02	0.06	0.07#,*	0.12#
	(0.01)	(0.02)	(0.02)	(0.02)	(0.04)	(0.02)	(0.02)	(0.01)	(0.01)	(0.03)	(0.04)	(0.04)
Glu M_3	0.11	0.11	0.14	0.27#	0.57#	0.18#	0.00*	− 0.01*	0.01*	0.06#,*	0.14#,*	0.20#
	(0.01)	(0.01)	(0.01)	(0.03)	(0.05)	(0.03)	(0.00)	(0.01)	(0.01)	(0.02)	(0.04)	(0.04)
Glu M_4	2.66	2.50#	2.40#	2.12#	1.73#	0.55#	0.00*	0.01*	0.03*	0.03*	0.05#,*	0.06#,*
	(0.03)	(0.09)	(0.03)	(0.07)	(0.04)	(0.03)	(0.00)	(0.00)	(0.02)	(0.01)	(0.01)	(0.04)
Glu M_5	54.45	51.67	49.07#	43.79#	34.74#	14.37#	0.00*	0.17*	0.53*	0.52*	0.87#,*	1.12#,*
	(0.10)	(2.45)	(0.39)	(0.69)	(0.57)	(5.06)	(0.01)	(0.05)	(0.33)	(0.17)	(0.14)	(0.66)
Ala M_3	0.00	0.00	0.02	0.12#	0. 24#	0.20#	0.00	0.00	0.03	0.04#,*	0.10#,*	0.15#,*
	(0.00)	(0.00)	(0.01)	(0.03)	(0.06)	(0.02)	(0.00)	(0.00)	(0.04)	(0.00)	(0.01)	(0.02)
Pro M_5	0.00	0.01	0.20	0.90#	2.08#	1. 99#	0.00	0.00	0.05*	0.27#,*	0.66#,*	1.02#,*
	(0.00)	(0.01)	(0.02)	(0.04)	(0.25)	(0.09)	(0.00)	(0.00)	(0.01)	(0.08)	(0.20)	(0.19)
Orn M_5	−0.01	0.00	−0.05	0.12	0.39#	0.47#	0.00	−0.05	0.01	0.39#	0.82#	1.00#
	(0.03)	(0.03)	(0.02)	(0.13)	(0.34)	(0.19)	(0.01)	(0.02)	(0.02)	(0.07)	(0.13)	(0.13)

Initial isotopic enrichment of M_3, M_4 and M_5 isotopmers of glutamate in the apical medium were higher than natural abundance, since [U-^{13}C] glutamate was added to the apical medium. Data were expressed as Means and SDs (in the parentheses) of 4 individual transwell cultures at each time point. Data at time 0 h are derived from 4 analyses of one sample both in apical and basolateral media. #: $p<0.05$ v.s. time 0 h (Dunnett's test), *: $p<0.05$ v.s. apical (paired *t*-test)

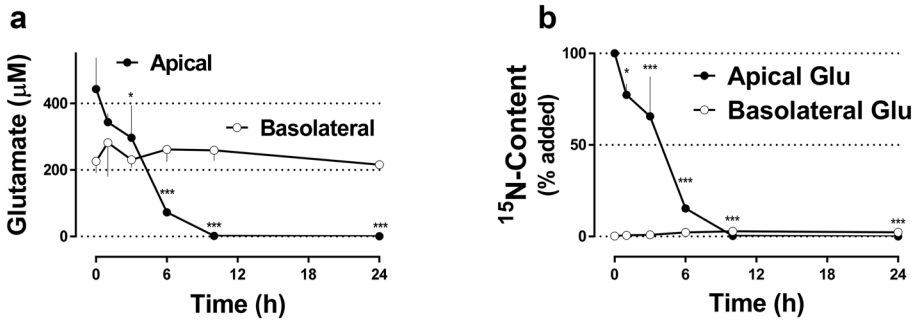

Fig. 4 Time-dependent changes in the concentrations, ^{15}N enrichment and ^{15}N content of glutamate in the apical and basolateral media (Exp. 2). Glutamate concentrations (**a**) and ^{15}N content (**b**) in glutamate in the apical (closed circle) and basolateral (open circle) media during the 24 h of culture. The ^{15}N content is expressed as a percentage of the amount added (means ± SDs ($N=4$)). *: $p<0.05$, ***: $p<0.001$ vs. before culture (Dunnett's test)

BCAAs, asparagine, serine and ammonia were 14.4%, 7.7%, 7.0%, 4.0%, 5.6%, 0.3%, 0.5% and 18.1%, respectively, of the glutamate ^{15}N taken up by the cells, and only a minor part (2.3%) of the ^{15}N was released into the basolateral medium in the form of glutamate (Fig. 5b). The fate of approximately 40% of the ^{15}N was not identified, presumably due to the volatilization of ^{15}N-ammonia and utilization of the ^{15}N for the synthesis of other metabolites.

Permeability of the cell layer to other amino acids and their metabolism (Exp. 3)

Twenty amino acids labelled with stable isotopes (^{13}C, ^{15}N or D) were added to apical media, and their metabolism was compared with that of glutamate. Amino acid concentrations and enrichments of each tracers supplemented are shown in Tables 5 and 6. The uptake of each amino acid by the

Table 3 Amino acid concentrations before and during the culture in Exp. 2

	Apical amino acid (μM)						Basolateral amino acid (μM)					
Time (h)	0	1	3	6	10	24	0	1	3	6	10	24
Leu	388	369	394	410	419	428	375	370	376*	365*	355*	327*
		(34)	(5)	(5)	(7)	(40)		(4)	(8)	(5)	(4)	(15)
Ile	372	348	363	362	348	322	360	361	370	367*	363*	349
		(31)	(5)	(6)	(6)	(30)		(3)	(8)	(4)	(3)	(16)
Val	391	368	386	390	378	355	380	382	391	389	387	379
		(34)	(5)	(5)	(7)	(34)		(3)	(8)	(6)	(2)	(17)
Phe	188	177	186	190	192	192	179	180	186	183	183*	177
		(15)	(4)	(5)	(4)	(18)		(1)	(4)	(3)	(2)	(8)
Tyr	193	182	191	192	191	191	183	184	191	188	189	183
		(15)	(4)	(5)	(4)	(18)		(2)	(4)	(2)	(3)	(9)
Trp	41	40	42	45	41	46	40	42	42	42	42	41
		(4)	(1)	(7)	(1)	(8)		(0)	(1)	(1)	(1)	(3)
Thr	381	356	373	383	379	373	364	367	375	374*	372	366
		(34)	(6)	(4)	(7)	(36)		(4)	(8)	(5)	(2)	(17)
Met	89	87	91	94	96	99	86	87	88*	86*	86*	80*
		(6)	(2)	(2)	(2)	(9)		(1)	(2)	(1)	(1)	(4)
Cys	87	80	82	79	72	56	85	84	86*	85*	85*	81*
		(6)	(1)	(1)	(1)	(5)		(1)	(2)	(1)	(1)	(4)
Lys	359	337	350	344	317	261	347	354	359	360*	365*	379*
		(31)	(5)	(4)	(4)	(27)		(3)	(8)	(8)	(5)	(19)
Arg	527	468	467	423	328	162	508	522	540*	551*	565*	595*
		(45)	(9)	(9)	(4)	(19)		(4)	(11)	(7)	(5)	(25)
Orn	36	37	41	45	45	42	36	51*	62*	79*	95*	138*
		(4)	(1)	(1)	(1)	(4)		(1)	(2)	(2)	(2)	(7)
His	165	160	167	167	161	157	163	167	166	165	164	166
		(14)	(3)	(3)	(2)	(17)		(5)	(6)	(6)	(5)	(15)
Ser	131	122	127	127	122	114	126	127	129	126	127*	127*
		(12)	(2)	(1)	(2)	(11)		(1)	(3)	(2)	(0)	(6)
Gly	172	163	169	169	168	161	167	167	171	170	169*	164
		(15)	(2)	(2)	(4)	(15)		(2)	(4)	(3)	(1)	(8)
Pro	128	124	132	116	120	156	130	132	151	148	157*	193*
		(12)	(4)	(19)	(3)	(24)		(3)	(16)	(4)	(5)	(26)
Ala	204	219	256	310	400	574	198	219	238*	259*	297*	404*
		(22)	(5)	(1)	(12)	(63)		(1)	(5)	(4)	(4)	(20)
Asn	92	87	93	97	98	95	90	89	91	90*	90*	86*
		(8)	(2)	(1)	(1)	(9)		(1)	(2)	(2)	(0)	(4)
Gln	1386	1264	1317	1350	1295	1150	1258	1191	1206*	1168*	1090*	892*
		(122)	(23)	(21)	(5)	(110)		(32)	(23)	(31)	(9)	(49)
Asp	127	78	29	4	2	2	117	120*	123*	122*	124*	122*
		(8)	(2)	(1)	(0)	(1)		(1)	(2)	(3)	(1)	(6)
Glu	585	491	379	134	28	41	252	260*	274*	279*	291*	288*
		(48)	(11)	(8)	(10)	(8)		(5)	(8)	(4)	(6)	(16)
NH3	602	590	631	632	690	735	651	755*	775*	780*	869*	938*
		(60)	(11)	(13)	(11)	(72)		(12)	(41)	(24)	(40)	(51)

Amino acid concentrations in apical and basolateral media before and after the culture in Exp. 2. Initial glutamate concentration in the apical medium was higher than in basolateral medium, since [^{15}N]glutamate was added to the apical medium. Data were expressed as Means and SDs (in the parentheses) of 4 (at 1, 3, 6, 10 h) or 8 (at 24 h) individual transwell cultures. Data at 0 h are derived from a single analysis. *: $p < 0.05$ v.s. apical (paired t -test)

Table 4 ^{15}N-enrichments of apical and basolateral amino acids before and during the culture in Exp. 2

	Apical amino acid						Basolateral amino acid					
Time (h)	0	1	3	6	10	24	0	1	3	6	10	24
	Isotopic enrichment (mol%)						Isotopic enrichment (mol%)					
Leu + Ile	−0.01	0.22	0.61$^{\#}$	1.50$^{\#}$	2.13$^{\#}$	1.77$^{\#}$	0.06	0.11	0.31$^{\#,*}$	0.43$^{\#,*}$	0.67$^{\#,*}$	0.83$^{\#,*}$
	(0.10)	(0.12)	(0.07)	(0.16)	(0.27)	(0.16)	(0.05)	(0.11)	(0.09)	(0.04)	(0.05)	(0.09)
Val	−0.06	0.05	0.03	0.27$^{\#}$	0.37$^{\#}$	0.67$^{\#}$	0.08	0.00	− 0.09	0.05*	0.06*	0.14*
	(0.12)	(0.06)	(0.06)	(0.08)	(0.19)	(0.20)	(0.10)	(0.13)	(0.18)	(0.01)	(0.04)	(0.09)
Orn	−0.54	−0.18	1.19	1.21	5.00$^{\#}$	1.66	0.51	2.11*	4.62$^{\#}$	8.51$^{\#,*}$	8.78$^{\#}$	6.51$^{\#,*}$
	(1.37)	(1.30)	(3.65)	(2.35)	(3.86)	(2.95)	(1.29)	(1.23)	(0.58)	(1.36)	(0.58)	(1.04)
Ser	0.05	0.12	0.08	0.21	0.26$^{\#}$	0.20	0.11	0.11	0.16	0.27$^{\#}$	0.38$^{\#}$	0.28$^{\#}$
	(0.07)	(0.12)	(0.10)	(0.10)	(0.10)	(0.11)	(0.15)	(0.06)	(0.05)	(0.09)	(0.03)	(0.07)
Pro	0.23	0.18	0.63$^{\#}$	1.26$^{\#}$	2.27$^{\#}$	1.96$^{\#}$	0.02	0.01	0.30$^{\#}$	0.83$^{\#,*}$	1.22$^{\#,*}$	1.44$^{\#,*}$
	(0.11)	(0.14)	(0.12)	(0.13)	(0.12)	(0.11)	(0.18)	(0.05)	(0.19)	(0.05)	(0.22)	(0.09)
Ala	0.04	0.43$^{\#}$	1.54$^{\#}$	3.95$^{\#}$	4.69$^{\#}$	3.29$^{\#}$	-0.04	0.16*	0.67$^{\#,*}$	1.94$^{\#,*}$	2.86$^{\#,*}$	2.48$^{\#,*}$
	(0.11)	(0.11)	(0.10)	(0.15)	(0.43)	(0.11)	(0.09)	(0.16)	(0.11)	(0.24)	(0.09)	(0.14)
Asn	0.16	0.14	0.16	0.38	0.50	0.44	0.12	0.01	0.24	0.18	0.21	0.26*
	(0.22)	(0.15)	(0.11)	(0.20)	(0.26)	(0.18)	(0.09)	(0.24)	(0.10)	(0.03)	(0.02)	(0.15)
Gln	−0.05	0.00	0.13	0.52$^{\#}$	0.86$^{\#}$	0.88$^{\#}$	−0.04	−0.08	0.06	0.32$^{\#,*}$	0.52$^{\#,*}$	0.58$^{\#,*}$
	(0.08)	(0.06)	(0.07)	(0.06)	(0.14)	(0.13)	(0.07)	(0.14)	(0.07)	(0.07)	(0.06)	(0.08)
Asp	0.15	0.17	1.03$^{\#}$	–	–	–	−0.02	0.09	−0.16*	0.13	0.01	0.19
	(0.06)	(0.30)	(0.50)				(0.11)	(0.18)	(0.28)	(0.13)	(0.08)	(0.16)
Glu	59.1	59.0	57.9	55.3$^{\#}$	36.0$^{\#}$	3.8$^{\#}$	0.09*	0.21*	0.34*	0.72$^{\#,*}$	0.95$^{\#,*}$	0.90$^{\#,*}$
	(0.2)	(0.2)	(0.2)	(0.3)	(2.3)	(2.0)	(0.22)	(0.11)	(0.07)	(0.13)	(0.16)	(0.12)
NH3	0.01	0.48$^{\#}$	2.30$^{\#}$	4.30$^{\#}$	2.89$^{\#}$	1.72$^{\#}$	−0.02	0.07*	0.69$^{\#,*}$	1.94$^{\#,*}$	2.45$^{\#,*}$	1.95$^{\#,*}$
	(0.03)	(0.03)	(0.09)	(0.20)	(0.12)	(0.09)	(0.02)	(0.04)	(0.06)	(0.18)	(0.07)	(0.04)

Data were expressed as Means and SDs (in the parentheses) of 4 (at 1, 3, 6, 10 h) or 8 (at 24 h) individual transwell cultures. Data at 0 h are derived from 4 analyses of one sample both in apical and basolateral media. -: not determined because the signals were too small. $^{\#}$: $p < 0.05$ v.s. time 0 h (Dunnett's test), *: $p < 0.05$ v.s. apical (paired *t*-test)

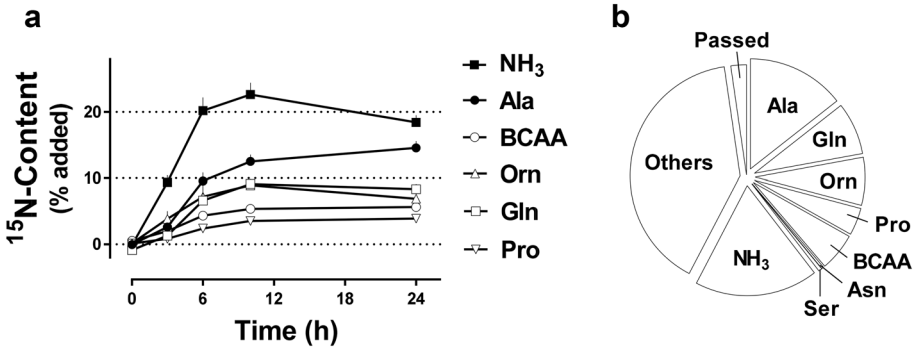

Fig. 5 Fate of glutamate-N (Exp. 2). **a** The combined total ^{15}N content in proline (open circle), alanine (closed circle) and ornithine (open square) in the apical and basolateral media during the 24 h of culture. The values are expressed as a percentage of the amount added (means ± SDs ($N = 4$)). The increase in the content of each ^{15}N-amino acid after culture was significant ($p < 0.0001$) (Dunnett's test). **b**: Fractional distribution of glutamate-N (% of uptake of glutamate-N by the cells from the apical medium) after 24 h of culture. "Others" indicates the unidentified fraction and "Passed" indicates ^{15}N released into the basolateral medium in the form of glutamate

Table 5 Amino acid concentrations before and after the culture in Exp. 3

	Apical amino acid (μM)		Basolateral amino acid (μM)	
Time (h)	0	24	0	24
Leu	728	806 ± 10	369	327 ± 18
Ile	697	672 ± 7	354	352 ± 19
Val	742	730 ± 7	375	380 ± 20
Phe	338	36 ± 31	172	175 ± 9
Tyr	323	333 ± 3	176	181 ± 1
Trp	73	78 ± 10	38	42 ± 2
Thr	727	763 ± 7	366	344 ± 24
Met	165	191 ± 3	81	70 ± 5
Cys	163	115 ± 0	80	81 ± 1
Lys	727	575 ± 8	348	406 ± 20
Arg	1064	479 ± 9	502	611 ± 46
His	332	350 ± 10	161	151 ± 11
Ser	314	288 ± 7	126	119 ± 9
Gly	352	335 ± 2	166	167 ± 1
Pro	295	315 ± 6	129	161 ± 16
Ala	429	870 ± 14	196	394 ± 3
Asn	269	266 ± 4	86	87 ± 2
Gln	2776	2862 ± 45	1103	866 ± 50
Asp	277	0 ± 0	124	121 ± 17
Glu	429	0 ± 0	243	244 ± 3

Six kinds of culture media supplemented with either 1) 400 μM [1-^{13}C]leucine, 400 μM [1-^{13}C]isoleucine, 400 μM [1-^{13}C]valine, 400 μM [1-^{13}C]lysine hydrochloride, 200 μM [1^{-13}C]phenylalanine, 50 μM [1-^{13}C]tryptophan and 2000 μM [U-^{13}C]glutamine, 2) 200 μM [ring-2-^{13}C]histidine hydrochloride monohydrate, 200 μM [1-^{13}C] serine, 200 μM [1-^{13}C]proline, 400 μM [U-^{13}C]threonine, 100 μM [1-^{13}C]methionine and 600 μM [guanido-$^{13}C_2$]arginine hydrochloride; 3) 200 μM [^{15}N]aspartate; 4) 200 μM [$^{15}N_2$]asparagine monohydrate; 5) 200 μM [1-^{13}C]glycine, 200 μM [ring-3,5-D_2]tyrosine and 400 μM [U-^{13}C]cysteine; or 6) 200 μM [2,3-$^{13}C_2$]alanine and 200 μM [1-^{13}C] glutamate were prepared as described in Materials and Methods. Each culture medium supplemented with mixture of amino acid tracer was added to inner chamber (apical compartment) of 4 transwells and basolateral medium was refreshed with basal medium containing no tracer amino acids. Amino acid concentrations after supplementation of each amino acid was shown as an initial value in apical medium. Initial concentrations in basolateral medium are those in the basal medium and common to all the groups. After 24 h culture, concentrations in apical and basolateral media were measured to see the change after supplementation of each amino acid. Data were expressed as Means and SDs (in the parentheses) of 4 individual transwell cultures. Data at time 0 h are derived from a single analysis

Table 6 Isotopic enrichments of the tracer amino acid before and after the culture in Exp. 3

	Apical amino acid		Basolateral amino acid	
Time (h)	0	24	0	24
Leu (M1)	49.2 ± 0.1	30.7 ± 0.4*	0.00 ± 0.06	6.77 ± 0.08*
Ile (M_1)	49.9 ± 0.0	46.4 ± 0.5*	0.00 ± 0.07	0.96 ± 0.12*
Val (M_1)	49.3 ± 0.1	46.7 ± 0.4*	0.00 ± 0.07	0.70 ± 0.04*
Phe (M_1)	49.0 ± 0.1	37.6 ± 0.4*	0.00 ± 0.12	4.57 ± 0.16*
Tyr (M_2)	43.8 ± 0.1	36.3 ± 0.3*	0.00 ± 0.03	2.77 ± 0.07*
Trp (M_1)	47.6 ± 0.1	41.7 ± 0.3*	0.00 ± 0.35	2.23 ± 0.43*
Thr (M_4)	49.1 ± 0.1	42.9 ± 0.3*	0.00 ± 0.00	2.37 ± 0.09*
Met (M_1)	50.9 ± 0.2	35.9 ± 0.2*	0.00 ± 0.16	4.70 ± 0.29*
Cys (M_3)$^{\#}$	52.2 ± 0.1	48.8 ± 0.2*	0.00 ± 0.02	5.33 ± 0.10*
Lys (M_1)	52.4 ± 0.3	49.3 ± 0.2*	0.00 ± 0.23	5.66 ± 0.48*
Arg (M_2)	44.9 ± 0.1	41.3 ± 0.3*	0.00 ± 0.03	12.45 ± 0.26*
His (M_1)	53.2 ± 0.7	49.8 ± 0.5*	0.00 ± 0.46	1.09 ± 0.45*
Ser (M_1)	60.4 ± 0.1	47.9 ± 0.9*	0.00 ± 0.07	2.24 ± 0.19*
Gly (M_1)	52.8 ± 0.1	44.8 ± 0.4*	0.00 ± 0.04	0.55 ± 0.07*
Pro (M_1)	59.7 ± 0.1	41.9 ± 1.1*	0.00 ± 0.07	5.45 ± 0.16*
Ala (M_2)	54.6 ± 0.2	13.7 ± 0.4*	0.00 ± 0.02	1.68 ± 0.06*
Asn (M_2)	67.5 ± 0.2	61.9 ± 0.3*	-0.06 ± 0.17	2.00 ± 0.44*
Gln (M_5)	60.1 ± 0.1	54.3 ± 0.4*	0.00 ± 0.00	1.94 ± 0.08*
Asp (M_1)	59.0 ± 0.1	–	0.00 ± 0.06	0.26 ± 0.14*
Glu (M_1)	49.3 ± 0.4	–	0.00 ± 0.17	0.71 ± 0.17*

Six kinds of culture media supplemented with either 1) 400 μM [1-^{13}C]leucine, 400 μM [1-^{13}C]isoleucine, 400 μM [1-^{13}C]valine, 400 μM [1-^{13}C]lysine hydrochloride, 200 μM [1^{-13}C]phenylalanine, 50 μM [1-^{13}C]tryptophan and 2000 μM [U-^{13}C]glutamine, 2) 200 μM [ring-2-^{13}C]histidine hydrochloride monohydrate, 200 μM [1-^{13}C] serine, 200 μM [1-^{13}C]proline, 400 μM [U-^{13}C]threonine, 100 μM [1-^{13}C]methionine and 600 μM [guanido-$^{13}C_2$]arginine hydrochloride; 3) 200 μM [^{15}N]aspartate; 4) 200 μM [$^{15}N_2$]asparagine monohydrate; 5) 200 μM [1-^{13}C]glycine, 200 μM [ring-3,5-D_2]tyrosine and 400 μM [U-^{13}C]cysteine; or 6) 200 μM [2,3-$^{13}C_2$]alanine and 200 μM [1-^{13}C] glutamate were prepared as described in Materials and Methods. Each culture medium supplemented with mixture of amino acid tracer was added to inner chamber (apical compartment) of 4 transwells and basolateral medium was refreshed with basal medium containing no tracer amino acids. Isotopic enrichments of each tracer amino acid in apical media at time 0 h and 24 h were measured. The enrichments in the corresponding basolateral media were also measured. Their initial enrichments of basolateral amino acids were zero since no tracer amino acids were supplemented to the medium. Data were expressed as Means and SDs (in the parentheses) of 4 individual transwell cultures. Data at time 0 h are derived from 4 analyses of one sample both in apical and basolateral media -: not determined because the signals were too small

Caco-2 cells from the apical medium was estimated from the decrease in the labelled amino acid content in the apical medium. While almost 100% of the added glutamate and aspartate was taken up by the cells, significantly less of the other amino acids was taken up (Fig. 6a). However, virtually no glutamate or aspartate crossed the Caco-2 cell layer into the basolateral medium, while other amino acids taken up by the cells were transferred substantially across the basolateral membrane into the medium (Fig. 6b).

The fates of glutamate-C ([1-^{13}C]glutamate), aspartate-N ([^{15}N]aspartic acid), asparagine-N ([$^{15}N_2$]asparagine), glutamine-C ([U-^{13}C]glutamine), alanine-C ([2,3-$^{13}C_2$]alanine), proline-C ([1-^{13}C]proline), serine-C ([1-^{13}C]serine) and glycine-C ([1-^{13}C]glycine) added to the apical medium

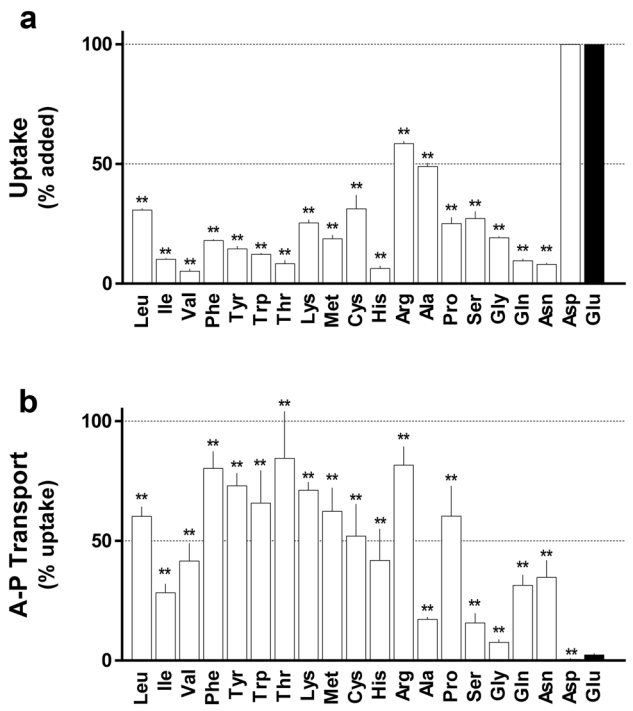

Fig. 6 Comparison of uptake and transport of 20 kinds of amino acids (Exp. 3). **a** Uptake of individual amino acids labelled with stable isotopes (^{13}C, ^{15}N or D) from the apical medium was calculated as the difference between the amount added and the content remaining in the apical medium after 24 h of culture and is expressed as a percent of the amount added. **b** Apical-to-basolateral transport (A-P transport) of each amino acid from apical to basolateral compartment across the Caco-2 cell layer was estimated from the content of each labelled amino acid in the basolateral medium after 24 h of culture and is expressed as the percent of the uptake (means ± SDs ($N=4$)). **: $p<0.001$ vs. glutamate (Dunnett's test)

were estimated. The distributions of ^{13}C and ^{15}N of these amino acids differed (Table 7). Although glutamate-C and glutamine-C were distributed to glutamine, ornithine and proline, the transport of glutamine-C from apical to basolateral compartment was much higher than that of glutamate-C (Fig. 7a and b). Both aspartate-N and asparagine-N were distributed to alanine, glutamine, ornithine, BCAAs and ammonia (Fig. 7c and d). However, apical-to-basolateral transport of asparagine-N was much higher than that of aspartate-N. Alanine-C was distributed to glutamine, ornithine and proline, while proline-C was distributed to ornithine (Fig. 7e and f). Serine-C was detected in glycine, while glycine-C was detected in serine (Fig. 7g and h).

Discussion

We studied the metabolism of glutamate in transwell cultures of Caco-2 cells, a model for an intestinal epithelial cell layer, and revealed its barrier function against apical-to-basolateral entry of glutamate. Although almost all glutamate added to the apical medium disappeared and had been incorporated into the epithelial cell layer, it barely appeared in the basolateral medium, independent of kinds of tracers used. The results indicate that virtually all the glutamate taken up from the apical medium was metabolized in the cells. Indeed, labelled C and N atoms, derived from glutamate were found in other amino acids in the medium. Glutamate-C was shown to be incorporated into alanine, glutamine, proline, ornithine and aspartate, while glutamate-N was incorporated into alanine, glutamine, proline, ornithine, BCAAs and ammonia. In vivo studies using [U-^{13}C] and [^{15}N]glutamate have also shown that virtually all dietary glutamate is metabolized in the gut to alanine, proline, ornithine, and ammonia by measuring the portal-arterial balance of ^{13}C and ^{15}N (Reeds et al. 1996, 2000; van der Schoor et al. 2001; Nakamura et al. 2013). However, these in vivo studies did not identify the intestinal cell type that contributes to the metabolism of dietary glutamate, although various cell types exist in the gut. Since the present study investigated the metabolism of glutamate only in epithelial cells, the results strongly suggest the contribution of the epithelial cell monolayer to this unique intestinal metabolism of dietary glutamate.

Although apical and/or dietary glutamate-C and N were metabolized to similar amino acids and metabolites both in epithelial cells in vitro and in the gut in vivo (Reeds et al. 2000; Nakamura et al. 2013), their quantitative distributions differed. In vivo studies indicated that approximately half of dietary glutamate-C was metabolized to CO_2, while the present in vitro study did not investigate its metabolism to CO_2, since the cells were cultured in a CO_2 incubator. However, the following results provide appropriate evidence that substantial glutamate-C was also metabolized to CO_2 in the present in vitro study. First, the utilization of glutamate-N for the synthesis of ammonia, alanine, and BCAAs indicates the generation of α-ketoglutarate, a TCA cycle metabolite, suggesting the utilization of glutamate-C to generate CO_2 via TCA cycle metabolism. Indeed, multiple ^{13}C-labels of glutamate were generated from [U-^{13}C]glutamate (Table 2), indicating the metabolism of glutamate-C via the TCA cycle. Second, the utilization of glutamate-C for the synthesis of alanine and aspartate also indicates the metabolism of glutamate-derived α-ketoglutarate to oxaloacetic acid via the TCA cycle. Glutamate-N metabolism also differed quantitatively in these studies: a large proportion of glutamate-N was metabolized to ammonia in this in vitro study, while it was utilized mainly for the production of alanine in vivo (Nakamura et al. 2013). This difference might be due to the features of the Caco-2 cells used in the present study, since the cells were originally derived from the colon cancer. The expression of ornithine transcarbamylase, an enzyme that uses ammonia via carbamoyl phosphate for the synthesis of citrulline, was reported to be lower in the colon than

Table 7 Distribution of Glu-C, Gln-C, Asp-N, Asn-N, Ala-C, Pro-C, Ser-C and Gly-C into apical and basolateral amino acids in Exp. 3

	Tracer added to the apical media															
	[^{13}C]Glu		[$^{13}C_5$]Gln		[^{15}N]Asp		[$^{15}N_2$]Asn		[$^{13}C_2$]Ala		[^{13}C]Pro		[^{13}C]Ser		[^{13}C]Gly	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
	Isotopic enrichment (mol%)															
Glu M_1						**0.44** (0.09)		**0.03** (0.04)				**0.07** (0.05)				
Glu M_2										**0.0** (0.03)						
Glu M_5				**0.24** (0.03)												
Gln M_1	**0.86** (0.05)	**0.32*** (0.09)			**0.65** (0.08)	**0.27*** (0.03)	**0.05** (0.01)	**0.06** (0.02)			**0.02** (0.04)	**0.08** (0.07)				
Gln M_2									**0.12** (0.01)	**0.01*** (0.02)						
Asp M_1								**0.04** (0.15)								
Asn M_1					**0.34** (0.15)	**0.01** (0.19)										
Ala M_1					**2.22** (0.03)	**1.60*** (0.03)	**0.22** (0.07)	**0.07*** (0.04)								
Ala M_3			**0.25** (0.01)	**0.1*** (0.01)												
Pro M_1	**2.19** (0.04)	**1.08*** (0.11)			**1.21** (0.07)	**0.65*** (0.08)	**0.05** (0.06)	**0.11*** (0.11)								
Pro M_2									**0.29** (0.03)	**0.07*** (0.02)						
Pro M_5			**4.78** (0.48)	**0.8*** (0.03)												
Orn M_1	**0.86** (1.74)	**2.97** (0.52)			**1.64** (1.72)	**3.34** (0.32)	**0.49** (1.04)	**0.89** (0.39)			**1.20** (0.33)	**1.33** (1.10)				
Orn M_2									**0.34** (0.32)	**0.22** (0.30)						
Orn M_5			**2.07** (0.42)	**1.0*** (0.21)												
Ser M_1					**0.17** (0.06)	**0.19** (0.11)	**0.02** (0.05)	**0.05** (0.06)							**5.46** (0.41)	**1.57*** (0.08)
Gly M_1					**0.08** (0.03)	**0.02** (0.08)	**0.02** (0.03)	**0.01** (0.05)					**4.09** (0.19)	**0.19*** (0.01)		
Leu M_1					**1.03** (0.10)	**0.37*** (0.04)	**0.12** (0.04)	**0.08** (0.01)								
Ile M_1					**1.07** (0.13)	**0.44*** (0.04)	**0.09** (0.06)	**0.04** (0.10)								
Val M_1					**0.42** (0.07)	**0.10*** (0.03)	**0.04** (0.08)	**0.02** (0.03)								
NH_3 M_1					**1.13** (0.03)	**1.16** (0.05)	**0.25** (0.04)	**0.20** (0.02)								

Isotopic enrichments of indicated isotopomers of apical (**a**) and basolateral (**b**) amino acids were analyzed to evaluate distribution of Glu-C, Gln-C, Asp-N, Asn-N, Ala-C, Pro-C, Ser-C and Gly-C in Exp. 3. Major fate of each labeled atom was determined theoretically from the metabolic map to quantify its distribution to other amino acids and ammonia. Data were expressed as Means and SDs (in the parentheses) of 4 individual transwell cultures. *: $p<0.05$ v.s. apical (paired *t*-test)

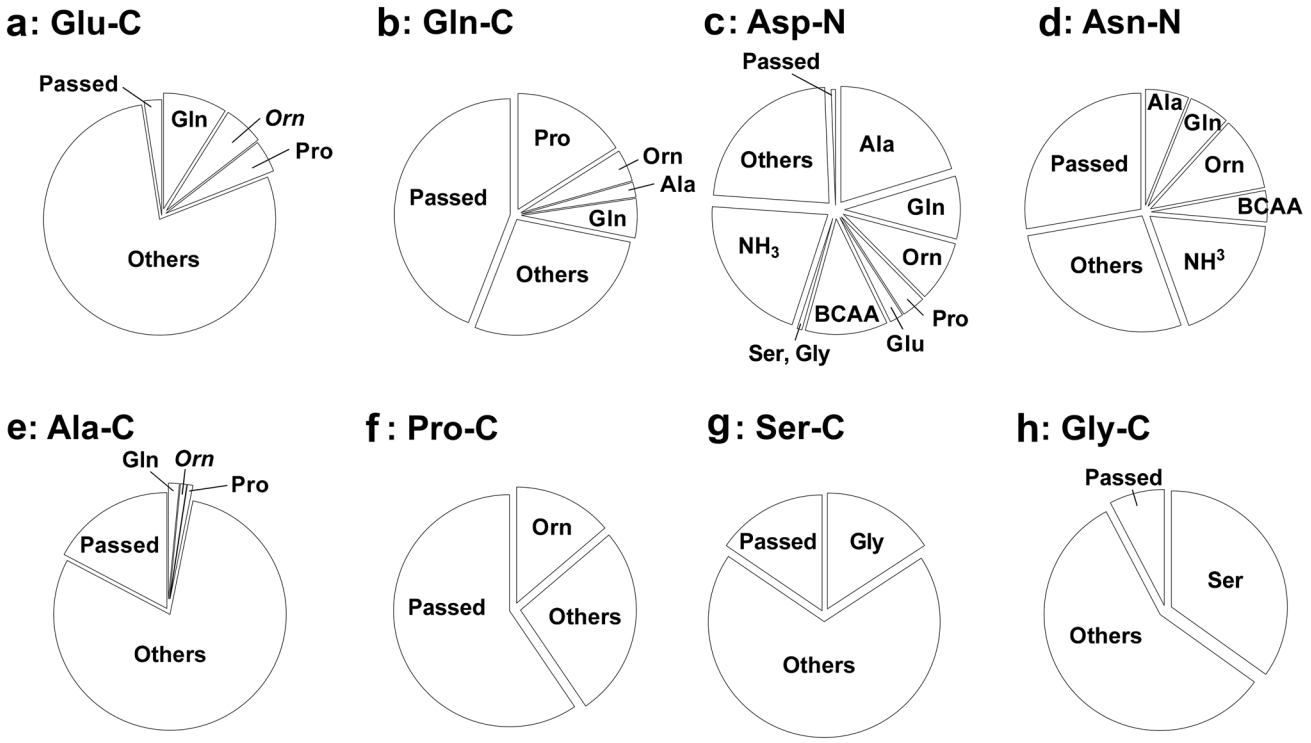

Fig. 7 Fates of other amino acids-C and N (Exp. 3). Fractional distributions (% of uptake from the apical media) of glutamate-C (**a**), glutamine-C (**b**), aspartate-N (**c**), asparagine-N (**d**), alanine-C (**e**), proline-C (**f**), serine-C (**g**) and glycine-C (**h**) after 24 h of culture were estimated from concentrations and isotopic enrichments of amino acids in apical and basolateral media in Exp. 3 as described in the Materials and Methods section. "Others" indicates unidentified fractions, and "Passed" indicates fractions released into the basolateral medium in the form added

in the small intestine (Hamano et al. 1988), which might have increased ammonia production in the present study. In addition, the expression of alanine aminotransferase, which produces alanine from glutamate and pyruvic acid, is also lower in the colon than in the small intestine (Yang et al. 2009), which might have decreased alanine production in the present in vitro study. This difference might also be due to the secondary metabolism of glutamate-C and N after their metabolism in the epithelial cell layer. Although the fates of dietary and/or apical glutamate-C and N were quantitatively different between in vitro and in vivo studies, the qualitative similarity in their fates suggests the substantial impact of intestinal epithelial cells on the metabolism of dietary glutamate.

Glutamate metabolism in the intestinal epithelial cell layer model is unique compared to other amino acids. The cell layer passed hardly any glutamate to the basolateral side, as mentioned above. However, the cell layer transported significant amounts of other amino acids to the basolateral side, except in the case of aspartate, although the uptake of the other amino acids by the layer was less than that of glutamate. Similar differences in the transport of amino acids were found in the gut in vivo. Postprandial arterial-portal differences in amino acids in piglets and rats indicated that the net uptake of glutamate by the portal vein was much less than that of other amino acids (Reeds et al. 1996; Nakamura et al. 2017). Although the net uptake of glutamine was negative in these studies, a study using both [2-^{15}N] and [5-^{15}N]glutamine indicated the entry of significant dietary glutamine into the circulation (Nakamura et al. 2013). Thus, the present results indicate the impact of intestinal epithelial cells on the metabolism of dietary amino acids and the regulation of amino acid entry in the circulation. Indeed, the fates of the apical amino acids-C and N were similar to those in vivo. For example, aspartate-N was distributed in alanine, glutamate, glutamine, ornithine, and ammonia in both studies. Bidirectional metabolism between serine and glycine was also evident in both studies.

In all the experiment in the present study, enrichment of labelled amino acid in apical media were decreased during the culture (Tables 2, 4, 6). The decreases of the enrichments indicate isotope dilution due to entry of non-labelled amino acids from the cells into the apical media. Thus, present study suggests that bidirectional transports of amino acids take place in this intestinal epithelial cell layer model. Detailed analysis of bidirectional transport in various experimental setting, for example, at different amino acid concentrations, might provide important information to

understand functions and cooperation of multiple transporters expressed in intestinal epithelial cells (Broer and Fairweather 2018). Although transwell culture of Caco-2 cells are widely used as an intestinal epithelial cell layer model because of its similarities in morphology, functional characteristics and expressions of enzymes and growth factor receptors (Hidalgo et al. 1989; Delie and Rubas 1997), the present study has limitations. Since Caco-2 cell is originally derived from colon cancer, there might be differences in amino acid metabolisms in the model from those in actual epithelial cells in small intestines, a major absorption site for amino acids. Further study using intestinal epithelial monolayer model of other cell lines and primary enterocytes will be required in future.

In conclusion, the present study demonstrated that the Caco-2 intestinal epithelial monolayer metabolized almost all the apical glutamate and limit the apical-to-basolateral transport of glutamate. Glutamate concentrations in the circulation are maintained despite the dynamic changes in dietary input of glutamate. The present study suggests that the intestinal epithelial cells contribute to the maintenance of glutamate homeostasis in the systemic circulation at least partially.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants performed by any of the authors. This article does not contain any studies with animals performed by any of the authors.

Informed consent This article does not contain any studies with human participants performed by any of the authors.

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