Functional characterization of tyrosine transport in fibroblast cells from healthy controls

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Abstract

Human fibroblast cells are an advantageous model to study the transport of amino acids across cell membranes, since one can control for environmental factors. A major problem in all earlier studies is the lack of precise and detailed knowledge regarding the expression and functionality of tyrosine transporters in human fibroblasts. This motivated us to perform a systematic functional characterization of the tyrosine transport in fibroblast cells with respect to the isoforms of system-L (LAT1, LAT2, LAT3, LAT4), which are the major transporter of tyrosine.

Ten (n=10) fibroblast cell lines from healthy volunteers were included in the study. Uptake of L- [U-14C] tyrosine in fibroblasts was measured using the cluster tray method in the presence and absence of excess concentrations of various combinations of inhibitors.

This study demonstrated that LAT1 is involved in 90% of total uptake of tyrosine and also around 51% of alanine. Not more than 10% can be accounted for by LAT2, LAT3 and LAT4 isoforms. LAT2 seems to be functionally weak in uptake of tyrosine while LAT3 and LAT4 contributed around 7%. 10 % could be contributed by system-A (ATA2 isoform). Alanine consequently inhibited the tyrosine transport by up to 60%. Tyrosine transport through the LAT1 isoform has a higher affinity compared to system-L.

In conclusion, the LAT1 isoform is the major transporter of tyrosine in human fibroblast cells. Competition between tyrosine and alanine for transport is shown to exist, probably between LAT1 and LAT2 isoforms. This study established fibroblast cells as a suitable experimental model for studying amino acid transport defects in humans.

Keywords: Human fibroblasts; Tyrosine and alanine transport, LAT1, LAT2, LAT3, LAT4

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Introduction

Amino acids are transported via specific transporter systems i.e. facilitated transport to the brain through the blood brain barrier (BBB) [18]. Tyrosine, an essential amino acid for the brain [18] is of special interest in schizophrenia, bipolar disorder and drug addiction research, since it is a precursor for dopamine [20], Dopamine plays an important role in the pathophysiology of these disorders [1, 9, 39]. Furthermore, limitation of access of tyrosine to the brain could play a role in disturbed dopaminergic neurotransmission in many neuropsychiatric disorders [4].

The relationship between tyrosine and altered dopaminergic neurotransmission in schizophrenia gained support from experimental studies showing a relationship between low availability of tyrosine and reduced dopamine concentration in the human striatum [23] Moreover, studies showing impaired cognitive functioning by dietary depletion of tyrosine in humans [14], and association of low affinity constant (K_m) with poorer cognitive function in schizophrenic patients, added further support to the hypothesis [37].

Decreased tyrosine transport across fibroblast membranes of schizophrenic patients is a repeated finding, including the blood-brain barrier [35, 36]. Our group and Ramchand et al., 1996 [26] have demonstrated decreased transport of tyrosine across fibroblast membranes of schizophrenic patients in different studies [12, 13, 26, 38]. This decrease in tyrosine transport is indicated by lower maximal transport capacity *(V_{max})* [12, 13, 26, 38] and lower affinity constant *(K_m)* [12, 26]. Recent studies by our group have demonstrated increased V_{max} for alanine and lower affinity constant (K_m) for tyrosine in fibroblasts from children with autism. These results have indicated a probable elevated access of alanine and less availability of tyrosine resulting in a lower dopaminergic behavior in the brain of autistic kids [11]. A similar condition might exist in schizophrenic patients with elevated CSF (Cerebrospinal fluid) levels of alanine [27].

Transport of tyrosine across the membranes including the blood brain barrier takes place through the system–L and the system-A [18]. System-L is named for its functionality in transporting large, neutral, aromatic and branched amino acids. System-L consists of at least four isoforms LAT1 [25], LAT2 [28], LAT3 [3] and LAT4 [5]. LAT1 and LAT2 are members of SLC (solute carrier) 7 family and these proteins form heteromeric complexes via a disulphide bond with the heavy chain of 4F2 antigen (4F2hc SLC3A2), a single transmembrane domain protein essential for the functional expression of LAT1 and LAT2 at the plasma membrane [5]. LAT3 and LAT4 are the members of (SLC43) family. LAT3 and LAT4 does not require co-expression with 4F2hc to carry out transport activity at plasma membranes [3, 5]. LAT1 expression is restricted to certain organs such as BBB, placenta, testis and fibroblasts [8, 21]. LAT1 has a

specific substrate specificity and transports large neutral amino acids with high affinity [8, 22, 24]. LAT2 is distributed widely in the body and it has broader substrate specificity for both large and small neutral amino acids [3, 21, 24]. LAT3 is highly expressed in pancreas, liver, skeletal muscle, and fetal liver [3]. LAT4 is expressed in placenta kidney and peripheral blood leukocytes [5]. Knowledge about the expression of LAT3 and LAT4 in fibroblasts is not available.

System-A, with high affinity for non-metabolized methyl aminoisobutyric acid (MeAIB) transports short-chain neutral amino acids [17]. It consists of three isoforms namely ATA1 [34], ATA2 [17] and ATA3 [30]. ATA1 is predominantly expressed in neurons [29]. ATA2 is widely expressed at the blood brain barrier and in fibroblasts [17, 31] and ATA3 is expressed in liver [15]. ATA2 recognizes many substrates but with varying affinities. High affinity substrates include MeAIB and alanine, tyrosine is considered to be a low affinity substrate for ATA2 [31].

Previous analysis of tyrosine kinetics in fibroblast cells obtained from both patients with neuropsychiatric disorders and controls has only studied total tyrosine kinetics without characterizing the isoforms of transport system-L and A. New knowledge regarding amino acid transporters, their roles in tyrosine transport and their substrates [11, 24], motivated us to further characterize the tyrosine transport in fibroblast cells with respect to the isoforms of system-L, which is the major transporter of tyrosine. The specific aims of this study are to investigate and elucidate the tyrosine transport in fibroblasts from human healthy controls regarding system-L with reference to the functionality of its isoforms.

Material and Methods

Cell lines

This study included 10 fibroblast cell lines from healthy volunteers. All cell lines were derived from forearm skin biopsies and were obtained from a Biobank [12]. From these 10 cell lines, 5 were used for kinetics experiments. The same fibroblast cell lines were included in previous studies [12, 24]. The study was approved by the Ethical Committee at Karolinska Institute.

Chemicals

Cell culturing media, antibiotics, media supplements and fetal bovine serum (FBS) were obtained from Gibco Invitrogen (Sweden). L- [U-^{14}C] tyrosine, specific activity 391 mCi/mmol was purchased from Moravek Biochemicals, Inc (California, USA). All chemicals and inhibitors were purchased from Sigma-Aldrich (Sweden). Cell culturing flasks and 24 multi-well trays were purchased from Costar Europe Ltd. All solutions of amino acids and inhibitors were made with phosphate buffered saline (PBS) (pH 7.35-7.4).

Cell culturing

Fibroblasts were cultured in minimal essential medium containing 10% FBS, L-glutamine (2 mmol/l), penicillin (100 mg/ml), streptomycin (100 mg/ml) and Amnio-Max™. Cells were maintained in a humidified atmosphere at 37°C with 5% CO_2 in air. Cells were grown in tissue culture flasks to confluence and were seeded in $2cm^2$ multiwell plates before the measurement of amino acid transport. Cells in the multiplates were grown to confluence for approximately 5 days. Cell lines between 7 and 20 passages (number of splitting) were used for the experiments.

Tyrosine transport assay

Fibroblasts cultured in multiwell plates were washed twice with PBS. The cells were incubated for one hour at 37°C with PBS containing 1% D-glucose to deplete the endogenous amino acid pools. The pre-incubation medium was removed, and the cells were incubated at 37^0 C with 0.1 mmol/l of L- [U-^{14}C] tyrosine (0.2 µCi) in combination with 5 mmol/l of different inhibitors or combinations of inhibitors (Table 1) for 5 minutes at 37^0 C. The reaction was terminated by rapidly washing the cells with ice cold PBS twice [24]. The cells were then solubilized in 200µl of 0.2 mmol/l NaOH for 30 minutes. From this 200µl of cell lysate, 150 µl is mixed with scintillation cocktail and radioactivity was assayed by liquid scintillation counter and the remainder was used for protein determination using the Bradford method [6], using Bovine Serum Albumin as a standard.

Single and combinations of inhibitors

An excess of 5mmol/l of inhibitors (competing substances) Methyl-aminoisobutyric acid (MeAIB) [16], 2-aminobicyclo heptane-2-carboxylic acid (BCH) [3], *N*-ethyl maleimide (NEM) [3, 5], alanine [7, 24] and D-methionine [22] or combinations of the above were used to block system A and/or L and/or their specific isoforms to analyze the tyrosine transport ability of uninhibited amino acid transporters. Single and combinations of inhibitors and their specificities for transport systems are described in detail in Table 1.

LAT1 Tyrosine kinetics

Tyrosine kinetics for the LAT1 isoform was determined by using excess concentrations (5 mmol/l) of inhibitors MeAIB to block system-A, and NEM to block LAT2, LAT3 and LAT4. Cells were incubated for 1 minute with a constant amount of L- [U-^{14}C] tyrosine (0.2 µCurie) and 12 different concentrations (varying between 0.003 and 1.0 mmol/l) of unlabelled tyrosine in the presence or absence of 5 mmol/l of inhibitors. Termination and analysis of the transport was performed as described above.

Calculations

For uptake studies, the values of uptake and inhibition of tyrosine are presented as percentage (%) of tyrosine uptake, correlated to total percentage of tyrosine uptake in the absence of inhibitors. Each uptake experiment was performed in triplicate at same time point.

The tyrosine kinetic parameters for system-L and its LAT1 isoform were calculated from the Lineweaver-Burke plot equation $[1/V_0=(K_m/ V_{max}[S])+(1/V_{max})]$, using computerized software as described previously [12]. V_0 is the initial transport velocity and [S] is the transport substrate concentration, V_{max} is the maximal uptake rate for the carrier-mediated process (nmol/min x mg protein) and K_m is the affinity constant (the concentration at half-saturation; µmol/l). Each experiment was performed in duplicate at same time point.

Statistical analysis

All values are as means ± standard deviation (S.D). For inhibition studies, the values of uptake and inhibition of tyrosine are presented as percentage (%) of tyrosine uptake. Wilcoxon signed ranked test was used to calculate the significant differences between percentage of tyrosine uptake in presence and absence of inhibitors. Kinetic parameter values (V_{max} and K_m) for both LAT1 isoform and System-L were normally distributed when tested by Shapiro-Wilcoxon test. Paired sample student's *t*-test was used to compare kinetic parameters between LAT1 isoform and system-L assuming unequal variance. A *p*-value below 0.05 was considered to denote statistical significance.

Results

Tyrosine uptake in the presence of single inhibitors

ATA2, defined as the MeAIB sensitive uptake, accounted for 7.3% of tyrosine uptake. Tyrosine was taken up mainly through the sodium-independent system-L, defined as the BCH sensitive sodium-independent active uptake. This transport accounted for 90.2% of the total tyrosine uptake. NEM, the LAT2, LAT3 and LAT4 isoforms specific substrate, inhibited only 4.4% of the tyrosine uptake, and therefore 95.6% of tyrosine is transported through LAT1 and ATA2 isoforms. Alanine, the system-A and LAT2 isoform specific amino acid, inhibited tyrosine uptake by 60%. D-Methionine, the LAT1 specific amino acid, inhibited 91.6% of tyrosine uptake (Table 1).

Tyrosine uptake in the presence of combinations of inhibitors

MeAIB and NEM, specific substrates for system-A and LAT2, LAT3, LAT4 isoforms inhibited tyrosine uptake by 9.2%. This implies that LAT1 isoform is responsible for 90.8% of the total tyrosine uptake through system-L. MeAIB and BCH specific for ATA2 and system-L, inhibited 92% of tyrosine uptake. MeAIB and D-methionine, specific for ATA2 and LAT1 isoform inhibited 90% of tyrosine uptake and not more than 10% of tyrosine is transported through LAT2, LAT3 and LAT4 isoforms. Alanine and NEM, specific for system-A, LAT2, LAT3 and LAT4 isoforms inhibited up to 51% of tyrosine uptake. Alanine and BCH, specific for system-A, LAT2 isoform and system-L inhibited 93.5% of tyrosine uptake. Alanine and D-methionine, specific for system-A, LAT1 and LAT2 isoforms inhibited 93% of tyrosine uptake and not more than 7% of tyrosine is transported through LAT3 and LAT4 isoforms (Table 1).

Kinetic parameters (K_m and V_{max}) of LAT1

Kinetic parameters (V_{max} and K_m) of tyrosine transport by the LAT1 isoform in comparison to kinetic parameters of system-L are presented in Table 2.

The V_{max} for tyrosine did not differ significantly between the LAT1 isoform and system-L, but the LAT1 isoform had a significantly lower K_m values [p= 0.013, n=5, df=4] as compared to the system-L.

Table 1. Inhibitors, combinations of inhibitors, their specificities to the isoforms of both L and A amino acid transport systems and their effects on tyrosine uptake in fibroblast cell lines from ten (n=10) healthy volunteers.

Inhibitors	Specificity of Inhibitors	Functional transport systems after inhibition	Tyrosine uptake[1]	Inhibition of tyrosine uptake[1]
MeAIB	System-A (ATA2)	**L- system**	92.7 ± 6.1**	7.3
BCH	System-L	**ATA2**	9.7 ± 4.3**	90.3
NEM	LAT2, LAT3, LAT4	**ATA2, LAT1**	95.6 ± 15.9^{NS}	4.4
Alanine	ATA2, LAT2	**LAT1, LAT3, LAT4**	40.1 ± 9.4**	59.9
D-Methionine	LAT1	**ATA2, LAT2, LAT3, LAT4**	8.4 ± 1.7**	91.6
MeAIB + NEM	ATA2 + LAT2, LAT3, LAT4	**LAT1**	90.9 ± 10.6*	9.1
MeAIB + BCH	ATA2 + Total 'L' system	**Other[2]**	7.9 ± 2.6**	92.1
MeAIB + D-Methionine	ATA2 + LAT1	**LAT2, LAT3, LAT4**	10.2 ± 3.9**	89.8
Alanine + NEM	ATA2, LAT2 + LAT2, LAT3, LAT4	**LAT1**	49.2 ± 14.5**	50.8
Alanine + BCH	ATA2, LAT2 + Total 'L' system	**Other[2]**	6.4 ± 1.6**	93.6
Alanine + D-Methionine	ATA2, LAT2 + LAT1	**LAT3, LAT4**	6.8 ± 2.5**	93.2

*[1]The values of tyrosine uptake are presented as percentage (means ± SD) of tyrosine uptake, correlated to total percentage of tyrosine uptake in the absence of inhibitors. ** p=0.005, * p=0.028, NS: Not significant. LAT1, LAT2, LAT3 and LAT4: Isoforms of system-L, ATA2: Isoform of system-A*
[2] Other: Undefined transport systems. MeAIB: Methyl-aminoisobutyric acid, BCH: 2-aminobicyclo heptane-2-carboxylic acid, NEM: N-ethyl maleimide

Table 2. Comparison of kinetic parameters (V_{max} and K_m) of tyrosine transport between system-L and its LAT1 isoform in fibroblast from healthy volunteers

Kinetic parameters	LAT1 isoform	L-system	p-Value
V_{max}*	8.4 ± 2.2 (5)	10.4 ± 1.3 (5)	0.115
K_m**	16.4 ± 4.4 (5)	27.0± 5.3 (5)	0.013

Values are presented as means ± SD; number of cell lines in parenthesis.
** V_{max}, indicates maximal transport capacity (nmol/min x mg. protein)*
*** K_m, affinity of binding sites for a specific amino acid (µmol/l)*

Discussion

Previous analyses of tyrosine kinetics have shown that system-L is the major transporter responsible for tyrosine uptake in fibroblast cells [12, 24, 26, 38] (from both patients and controls). These studies were carried out before there was any knowledge of the different transport systems and their isoforms.

In our recent studies [24] we have demonstrated that in fibroblasts tyrosine was taken up mainly through the Na^+-independent system-L that accounted for 90%, defined as the BCH sensitive Na^+-independent active uptake and 10% could be contributed by Na^+-dependent system-A.

In the present study we have validated the previous findings and have further demonstrated that the *major transporter of tyrosine in human fibroblasts is the LAT1 isoform*. Fibroblasts exhibited 91,6% of blockage of tyrosine transport when LAT1 specific inhibiter D-Methionine [22] is used and exhibited 9.1% blockage of tyrosine transport when system-A and LAT2, LAT3, LAT4 isoforms were inhibited with excess concentrations of MeAIB and NEM. This indicates that LAT1 isoform is a Na^+-independent transport system, involved in transport of large neutral amino acids and responsible for around 90% of the total tyrosine uptake through system-L. The results of this study showing the tyrosine uptake through LAT1 isoform are in agreement with similar studies made on human brain micro capillary endothelial (hBME) cells [10, 33], human astrocytomas [2], human placenta [25], and also in rat brain [22].

Both tyrosine and alanine have affinity for the LAT2 isoform [7, 19, 32]. Combination of alanine and NEM, specific inhibitors for system-A, LAT2, LAT3 and LAT4 isoforms inhibited the tyrosine uptake by only 51% (Table 1), indicating that LAT1 isoform is also involved in transport of alanine in equivalent percentage in human fibroblasts. Recent studies conducted with animal brain-astrocytes have mentioned that, the LAT2 isoform as a candidate mediator of Na^+- independent alanine uptake, and the role of LAT1 in alanine transport was not conclusively determined [7]. The results of this study indicate that the *LAT1 isoform is also probably involved in transport of alanine, approximately 51%* in human fibroblasts. *The domination of alanine over tyrosine at the BBB may be the limiting factor for tyrosine influx into the brain that may lead to decreased dopamine synthesis at dopaminergic neurons in various psychiatric disorders.*

In our recent studies [11] we demonstrated an aberrant transport of tyrosine and alanine in fibroblasts obtained from children with autism, and speculated a probable competition between tyrosine and alanine for the LAT2 isoform. Results of the present study propose that aberrant transport of tyrosine and alanine demonstrated by Fernell et al., 2007 [11] across the cell membrane may be due to involvement of the LAT1-Isoform and very little of LAT2 isoform. LAT2 seems to be functionally very weak in uptake of tyrosine in human fibroblasts (calculated about 3%), therefore tyrosine and alanine competition at LAT2 seems to be of minor importance when compared to LAT1. However, additional

investigations are needed to verify these findings. Furthermore, we demonstrated that LAT1 isoform has a higher affinity (lower K_m values, p=0.013, n=5, df=4) for tyrosine transport in comparison to system-L (Table 2).

The existence of competition between alanine and tyrosine at system-L in fibroblasts is demonstrated in this study by using alanine as an inhibitor. Alanine is specific for ATA2 and LAT2, it inhibited around 60% of tyrosine transport. When LAT2 and ATA2 were inhibited with inhibitors (MeAIB and NEM) other than alanine, it did not resulted in inhibition of tyrosine uptake. The 60% inhibition of tyrosine uptake by alanine, is in agreement with our previous studies [24], and with similar studies carried out on human astrocytomas [2].

All the evidence indicates the existence of competition between tyrosine and alanine. Our group has found increased V_{max} of alanine over V_{max} of tyrosine in fibroblasts of autistic children in comparison with the fibroblasts obtained from controls and speculated that there might be a corresponding disturbance of amino acid transport across the blood brain barrier [11].

System-A, with three isoforms ATA1, ATA2 and ATA3, only ATA2 isoform has tyrosine specificity. System-A specific substrate MeAIB, when used as a competitive substance resulted in only 7.3% inhibition of tyrosine transport. As alanine is the specific substrate for system-A, there could also be a probability of competition of alanine with the tyrosine transport to a minor extent at ATA2 isoform.

NEM, which is highly specific for LAT3 and LAT4 and slightly specific for LAT2, when used, as an inhibitor (competitive substance) did not resulted in significant inhibition of tyrosine transport. LAT3 and LAT4 isoforms seems to be functionally weak transporters of tyrosine (in comparison to LAT1). LAT3 and LAT4 together, corresponds to about 7 % of total tyrosine uptake in human fibroblasts through system-L (Table 1).

Due to the limitations involved in obtaining and working with brain cells, there is a need for good experimental models to analyze the amino acid transport properties in various psychiatric disorders. The present study confirms the presence of system-L with its isoform LAT1 as a major transporter of tyrosine in fibroblasts. As the LAT1 is also present at human brain micro capillary endothelial (hBME) cells [10, 33] the findings of this study gives further importance to fibroblast cells as an experimental human model, useful for studying amino acid transport defects at the BBB in various neuropsychiatric disorders.

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