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Functional activity of a large neutral amino acid transporter (LAT) in rabbit retina: A study involving the in vivo retinal uptake and vitreal pharmacokinetics of L-phenyl alanine

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Abstract

Purpose: The purpose of this study is to elucidate the functional activity of large neutral amino acid transporter (LAT) in rabbit retina and to delineate its role in the retinal uptake and intravitreal pharmacokinetics of L-phenylalanine (L-Phe).
Methods: In vivo retinal uptake of L-Phe and L-alanine (L-Ala) was determined in the presence and absence of specific transport inhibitors following intravitreal administration. L and D isomers of amino acids were employed as inhibitors to determine the stereo-selectivity of LAT. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out for LAT isoforms (LAT1 and LAT2). Vitreal disposition of L-Phe following administration in rabbit vitreous was studied in the presence of an other competing LAT substrate D-methionine using microdialysis.
Results: Retinal uptake of L-Phe was significantly inhibited in presence of a specific LAT inhibitor, 2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid (BCH), L isomers of large neutral amino acids and LAT1 specific but not by LAT2 specific and charged amino acids. No significant inhibition of L-Ala retinal uptake was observed with LAT substrates. LAT isoforms (LAT1 and LAT2) were identified by RT-PCR in rabbit retina. The mean residence time (MRT) and area under curve (AUC) values of L-Phe following intravitreal adminstration were significantly increased in the presence of D-methionine, a LAT substrate.
Conclusions: This study demonstrates the functional activity and molecular expression of large neutral amino acid transporter in the rabbit retina. Furthermore, based on these studies it can be concluded that LAT is involved in the retinal uptake and intravitreal elimination of L-Phe.
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Keywords: Amino acids; Large neutral amino acid transporter; Microdialysis; Retina

1. Introduction

Amino acids play an important role in essential metabolic pathways and in performing specialized functions. In retina, amino acids are primarily utilized as neurotransmitters (glutamate, GABA and glycine) and as precursors of neurotransmitters including tyrosine, tryptophan and arginine (Neal, 1976; Pourcho, 1996; Thoreson and Witkovsky, 1999). Tyrosine is a precursor in the synthesis of dopamine and its local levels influence the rate of synthesis of dopamine (Fernstrom et al., 1984). Tryptophan is involved in the formation of serotonin and kynurenic acid; whereas, arginine is a substrate for the formation of nitric oxide. Amino acid transporters play a pivotal role in regulating the local levels of amino acids in the retina. Thus, so far, amino acid transport systems for glycine (Jones, 1995) (Glyt-1), glutamate (Pow and Barnett, 1999; Pow and Crook, 1997; Rauen et al., 1996) (EAAT1, EAAT2, EAAT3, EAAT5) and taurine (Militante and Lombardini, 1999) (Taut 1 and 2) were reported to be expressed by retina. However, very little information is available regarding the expression of large neutral amino acid transport system in the retina.

The presence of tryptophan, a large neutral amino acid has been revealed by immunohistochemical studies in the retinal glial cells and photoreceptors (Pow and Cook, 1997). Since the mammals are unable to synthesize tryptophan, it must have been accumulated into the retinal cells via the large neutral amino acid transport system (Pow, 2001). Moreover, branched chain neutral amino acids have an important role in the synthesis of

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excitatory neurotransmitter glutamate in the retina (Hutson et al., 2001; LaNoue et al., 2001; Lieth et al., 2001). LaNoue et al. (2001) has reported that BCH, an inhibitor of large neutral amino acid transport system inhibits the transport of endogenous branched chain amino acids and thereby inhibits the de novo synthesis of glutamate. However, there are no reports to date on either in vivo characterization of functional activity or molecular expression of large neutral amino acid transporter system in retina.

System L mediates the transport of large neutral amino acids with branched or aromatic side chains in a Na^+-independent manner and is characterized using BCH, a selective non-metabolizable analog (Christensen, 1990). LAT is involved in transport of large neutral amino acids in small intestine (Hidalgo and Borchardt, 1990), renal proximal tubules (Christensen, 1990), cornea (Jain-Vakkalagadda et al., 2003), placenta (Kudo and Boyd, 2001) and blood–brain barrier (Killian and Chehalis, 2001). Due to its broad substrate specificity, system L also transports compounds such as L-dopa, melphalan and gabapentin that are structurally similar to amino acids (Uchino et al., 2002). Two isoforms of large neutral amino acid transporter, LAT1 and LAT2 (L-type amino acid transporter 1 and 2, respectively), are isolated, cloned and expressed in *Xenopus* oocytes (Kanai et al., 1998; Pineda et al., 1999; Prasad et al., 1999; Segawa et al., 1999). System L is a heterodimer composed of 4F2hc heavy chain with LAT1 and LAT2 light chains (Boado et al., 1999). LAT1 is predominantly expressed in brain, placenta and testis (Wagner et al., 2001). It is also highly expressed in cultured cells and in malignant tumors (Kim do et al., 2002). However, LAT2 is ubiquitously expressed and differs from LAT1 with respect to its substrate selectivity and affinity (Pineda et al., 1999; Rossier et al., 1999; Segawa et al., 1999). LAT1 preferentially transports large neutral amino acids; whereas, LAT2 has much broader substrate selectivity and interacts with short chain neutral amino acids with comparable affinity.

The objective of the present study is to examine the functional activity and molecular expression of LAT1 and LAT2 in the rabbit retina. Vitreal pharmacokinetics of L-Phe following intravitreal administration was studied with ocular microdialysis technique. Intravitreal elimination of L-Phe was studied to provide an insight into the elimination pattern of the substrates of large neutral amino acid transporter. It will also help in further understanding/predicting the elimination of drug moieties that are substrates to LAT following intravitreal administration. This information can aid in designing drug delivery systems such as prodrugs targeting the amino acid transporters on retina and in developing optimal intravitreal drug therapy.

2. Materials and methods

2.1. Materials

Concentric CMA/20 microdialysis (polycarbonate and 10 mm membrane window) probes, used for sampling of vitreous were obtained from CMA/Microdialysis (Acton, MA, USA). The linear microdialysis probes (polyacrylonitrile and 10 mm membrane window) for the sampling of aqueous humor were purchased from bioanalytical systems (West Lafayette, IN, USA). Microinjection pump (CMA/100) employed for perfusing isotonic phosphate buffer saline (IPBS) through the probes, was procured from CMA/Microdialysis. Ketamine, HCl and xylazine were obtained from Fort Dodge Animal Health and Bayer Animal Health, respectively. Nembutal sodium was obtained from Abbott laboratories (Abbott Park, IL, USA). Tropicamide was supplied by Bausch & Lomb. New Zealand albino male rabbits weighing between 2 and 2.5 kg were purchased from Myrtle's Rabbitry (Thompson Station, TN, USA). $[^{14}C]$-L-Phenylalanine (460 mCi $mmol^{-1}$) and $[^{3}H]$-L-alanine (66 Ci $mmol^{-1}$) were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA) and Perkin-Elmer Life Sciences Inc. (Boston, MA, USA), respectively. Amino acids, BCH, glycylsarcosine (GlySar) and NMAIB (*N*-methylaminoisobutyric acid) were procured from Sigma Chemicals (St. Louis, MO, USA). Gene Amp® RNA-PCR kit was supplied by Applied Biosystems.

2.2. Animal studies

Anesthetized New Zealand white male rabbits, weighing 2–2.5 kg, were employed in this study. Ketamine hydrochloride (35 mg kg^{-1}) and xylazine (3.5 mg kg^{-1}) were administered intramuscularly to maintain anesthesia throughout the experiment. On completion of an experiment, animals were euthanized with an intravenous injection of sodium nembutal (50 mg kg^{-1}) through the marginal ear vein. All animal experiments were performed according to the guidelines and protocols established by the Institutional Animal Care and Use Committee of the University of Missouri-Kansas City.

2.3. In vivo retinal uptake studies

Rabbits were anesthetized as stated above. A specially designed canula with a 30-gauge needle was inserted in the vitreous body at about 3 mm below the corneal scleral limbus at 45° angle such that the canula resides in the mid vitreous. One hundred microliters of $[^{14}C]$-L-Phe (0.25 μCi/100 μL) was injected into vitreous chamber in the absence and presence of various inhibitors. Similarly $[^{3}H]$-L-alanine (0.66 μCi/100 μL) retinal uptake was performed in the presence of and absence of different inhibitors. Various competitive inhibitors including amino acids, amino acid type congeners and specific inhibitors at a concentration of 15 mM were utilized for the study. Although, 15 mM concentration of the inhibitor was administered, the effective concentration of the inhibitor in vitreous humor would be approximately 1 mM. One hundred microliters of the 15 mM solution is administered in the vitreous, which results in 1.5 μmol of the inhibitor in 1.5 mL of vitreous. Assuming, uniform distribution of the inhibitor, the final concentration in vitreous humor would be 1 mM. L and D isomers of amino acids were employed as inhibitors to determine the stereo-selectivity of the carrier system involved. After 30 min the animals were euthanized and the eyes were enucleated quickly.

The eyeball was cut open and the retina was dissected out with a fine forceps following an incision made at 2 mm below the scleral limbus junction. Care was taken to prevent contamination of the retina with vitreous humor (Lopez-Cortes et al., 2001). Retinal tissue was digested overnight with 1 mL of 0.1% Triton-X solution in 0.3N NaOH and then transferred to scintillation vials containing 5 mL of scintillation cocktail. Radioactivity in the tissue was measured using a scintillation counter (Beckman Instruments Inc., Model LS-6500). Protein content in the retina was determined by the method of Bradford using bovine serum albumin as the standard (Bio-Rad protein estimation kit, Hercules, CA, USA). Uptake of [^{14}C]-L-Phe and [^{3}H]-L-Ala into retina following intravitreal administration is expressed as picomol/mg protein/min.

2.4. Experimental setup for microdialysis

2.4.1. Probe implantation

The animals were anesthetized prior to the surgery as mentioned above. Pupils were dilated by topical instillation of 1% tropicamide prior to probe implantation. The linear and concentric probes were placed in the aqueous and vitreous humor, respectively, as described in our previous report (Atluri and Mitra, 2003). The concentric probe was placed in the vitreous body with the aid of a 22G needle, which was inserted into the mid vitreous at about 3 mm below the corneal scleral limbus at 45° angle preventing any damage to retina and lens. The probe was placed in the mid vitreous chamber immediately after removing the needle with aid of visual inspection. The linear probe was placed in the anterior chamber with a 25G needle. It was inserted across the cornea preventing any damage to iris–ciliary body and the outlet of linear probe was placed into the needle at bevel edge. Then the needle was slowly withdrawn such that the probe remained fixed in the anterior chamber. The microdialysis probes were perfused with isotonic phosphate buffer saline at a flow rate of 2 μL min^{-1} by a microinjection pump.

2.5. Intravitreal administration

Intravitreal injection of [^{14}C]-L-Phe (4 μCi/100 μL) was made after 2 h of recovery period following probe implantation. One hundred microliters volume of [^{14}C]-L-Phe in the presence and absence of 15 and 100 mM D-Met was administered into the vitreous body using a cannula to which 30G needle was attached. Simultaneously, D-Met was continuously perfused through the probe during the entire experimental period to maintain adequate and constant inhibitor concentration. The dialysate was collected every 20 min over a period of 10 h. Samples were analyzed by scintillation counting.

2.6. In vitro, probe calibration

In vitro probe calibration was performed by placing the probe in IPBS solution, pH 7.4, containing [^{14}C]-L-Phe. The probe was perfused at a flow rate of 2 μL min^{-1} with IPBS and the dialysate was collected every 20 min. Relative recovery of [^{14}C]-L-Phe is calculated according to Eq. (1).

$$\text{Recovery}_{\text{in vitro}} = \frac{C_\text{d}}{C_\text{s}} \quad (1)$$

C_d is the dialysate concentration and C_s is the standard concentration of [^{14}C]-L-Phe in IPBS. Concentrations of [^{14}C]-L-Phe in vitreous were calculated by dividing the dialysate concentration with in vitro recovery number. The values of in vitro recovery fraction obtained for L-Phe ranged from 0.18 to 0.25.

2.7. Data analysis

Vitreous concentrations time data of an individual rabbit were analyzed using pharmacokinetic software package Win Nonlin, v2.1 (Pharsight, CA, USA). Pharmacokinetic parameters were determined employing non-compartmental analysis, which is based on a model independent approach (Gibaldi, 1982). Area under the vitreous concentration time curve (AUC$_\text{VH}$) was estimated by the linear trapezoidal method with extrapolation to infinite time. Slope of the terminal phase of vitreous profile was determined by log-linear regression and the terminal rate constant (λ_z) was derived from the slope. Terminal vitreous half-lives ($t_{1/2}$) were calculated from the rate constants by using the formula, 0.693/λ_z. The terminal vitreous half-lives indicate the time required for the drug concentration to decrease by one-half in the vitreous body. Other parameters calculated were: clearance (Cl) = dose/AUC$_\text{VH}$; mean residence time (MRT) extrapolated to infinity = AUMC/AUC, where AUMC stands for area under the first moment curve and volume of distribution at steady state (V_ss) = MRT × Cl. Clearance is defined as the volume of fluid cleared of drug from the vitreous per unit time or it considers that certain percent of the distribution volume is cleared of drug over a given period of time. MRT represents the time for 63.2% of the administered dose to be eliminated. Volume of distribution at steady state relates the amount of drug in the body to the drug concentration in the vitreous at steady state. It can also be considered as the apparent volume in which the drug is dissolved and it does not have true physiological meaning in terms of an anatomic space.

2.8. RT-PCR studies for expression of LAT1 and LAT2 in rabbit retina

Reverse transcription-PCR was performed based on the method reported by Sugawara et al. (2000) with slight modifications. Total RNA was isolated from freshly excised rabbit retina by acid gaunidium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). The sense and antisense primers for rabbit LAT1 were 5′-GCC ATC ACC TTT GCC AAC TA-3′ and 5′-AAT AGG CCA CAT TGG TCA AG-3′, respectively. These primers correspond to the nucleotide positions 415–435 and 834–854, respectively, in rat LAT1 cDNA and were selected from two highly conserved regions among rat LAT1, mouse LAT1 and human LAT1 sequences. The resultant PCR product obtained was ∼440 bp. The forward and reverse primers for rabbit LAT2 were 5′-TAG CCC TGA AGA AAG AGA TCG G-3′ and 5′-ATT GCA GTG ATA TAC GCG

ACA T-3′. These primers correspond to the nucleotide positions 101–123 and 851–873 in rat LAT2 cDNA generating a ∼772 bp product. RT-PCR was carried out with the GeneAmpR RNA PCR kit from applied biosystems. The conditions for reverse transcription were as follows: denaturation of the template RNA for 10 min at 70 °C; reverse transcription for 60 min at 42 °C. The conditions for PCR amplification were as follows: denaturation for 1 min at 94 °C; annealing for 1 min at 58 °C and extension for 1 min at 72 °C: 37 cycles; final extension for 10 min at 72 °C. Samples were subsequently separated in a 0.8% agarose gel electrophoresis at 80 V for ∼1 h, stained with ethidium bromide and visualized with a UV illuminator. GeneRuler (1 kbp ladder) was employed to determine the size of the cDNAs. The resultant PCR products were sequenced by SeqWright using an automated Perkin-Elmer Applied Biosystems 3730 × 1 Prism™ DNA sequencer.

2.9. Statistical analysis

Unless otherwise indicated, all data points have been reported as mean ± S.D. Differences in mean values were reported to be significant when $p \leq 0.05$. Statistical significance of difference between experimental groups means was determined using unpaired, Student's t test.

3. Results

3.1. Retinal uptake of [^{14}C]-L-Phe following intravitreal administration

Uptake of [^{14}C]-L-Phe (8.54 nmol) into rabbit retina following intravitreal administration was studied to seek functional evidence for the presence of LAT, and to further characterize the LAT isoform involved in the retinal uptake. L-Phe is a substrate for both LAT1 and LAT2, and hence, was chosen as a model substrate. Uptake of [^{14}C]-L-Phe was carried out in the absence and presence of L and D isomers of amino acids, amino acid-like drugs and specific inhibitors to characterize the stereoselectivity and substrate specificity (Fig. 1). The concentration of inhibitors administered was 15 mM, but the effective concentration of inhibitors attained in the vitreous body is calculated as ∼1 mM based on the assumption that vitreous volume is 1.5 mL. The retinal uptake of [^{14}C]-L-Phe at 30 min following intravitreal administration is 3.0 ± 1.2 pmol/mg protein/min.

L-system specific inhibitor BCH significantly inhibited the [^{14}C]-L-Phe retinal uptake, indicating that the transport may be mediated by system L. Furthermore [^{14}C]-L-Phe uptake was significantly diminished by L-isomers of large neutral amino acids including L-Phe (87%) and L-Trp (63%), but not by glycine, acidic (L-Glut) and basic (L-Arg) amino acids. L-DOPA, a structurally similar drug to amino acid also significantly reduced (82%) the uptake of L-Phe. To investigate the possible involvement of multiple transport systems in the uptake process, the experiment was performed in the presence of NMAIB, a specific inhibitor for the alanine (A) preferring system. NMAIB did not inhibit L-Phe uptake into retina ruling out the possible

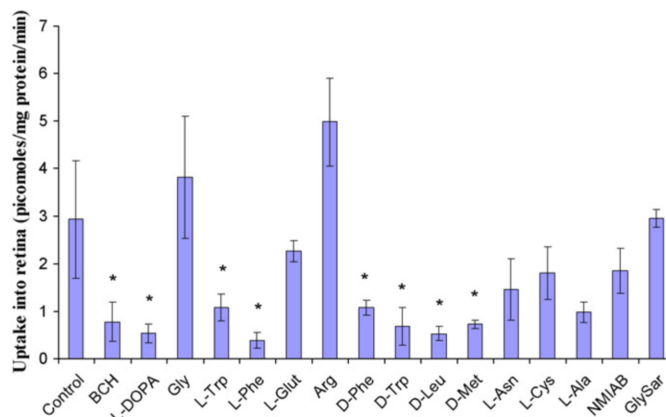

Fig. 1. Competitive inhibition of [^{14}C]-L-Phe retinal uptake by specific inhibitors, L- and D-isomers of different amino acids and amino acid type drugs (15 mM) following intravitreal administration. Retinal uptake is expressed as picomol/mg protein/min and is represented by the mean ± S.D. (n = 3–4). Values are significant at $^{*}p \leq 0.05$ level.

involvement of multiple transport systems. To further determine the LAT isoform involved in [^{14}C]-L-Phe uptake, inhibition with specific substrates for LAT1 (D isomers of Phe, Trp, Leu and Met) and LAT2 (L-Ala, L-Cys and L-Asp) was studied. Retinal uptake of L-Phe was significantly inhibited by LAT1 specific substrates but not by LAT2 specific substrates (Fig. 1).

Surendran et al. (1999) have reported that overlapping substrate specificity exists between large neutral amino acid and peptide transporters. To exclude the role of peptide transporter (PepT) in L-Phe retinal uptake, its uptake was carried out in the presence of GlySar. However, GlySar did not cause significant inhibition of L-Phe retinal uptake, thus eliminating the role of peptide transporter.

3.2. Retinal uptake of [^{3}H]-L-Ala following intravitreal administration

To investigate LAT2 functional expression in the retina [^{3}H]-L-Ala was administered intravitreally alone or with 15 mM of either L-Phe, LAT1 specific (D-Met) or LAT2 specific (L-Asp, L-Ala) substrates. Uptake of [^{3}H]-L-Ala was 7.5×10^{-3} pmol/mg protein/min. It was unaltered in presence of 15 mM of L-Asp, D-Met and L-Phe (Fig. 2). However, there

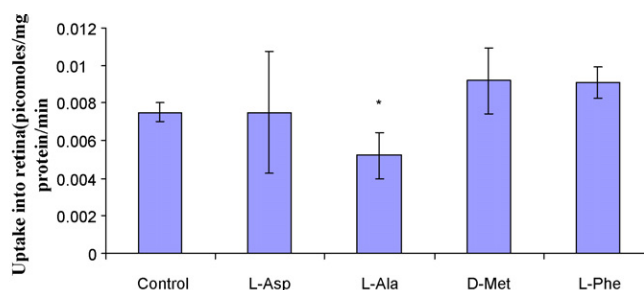

Fig. 2. Competitive inhibition of [^{3}H]-L-Ala retinal uptake following intravitreal administration of specific LAT1 and LAT2 (15 mM) inhibitors. Results are expressed as retinal uptake in picomol/mg protein/min and is represented by mean ± S.D. (n = 3–4). Values are significant at $^{*}p \leq 0.05$ level.

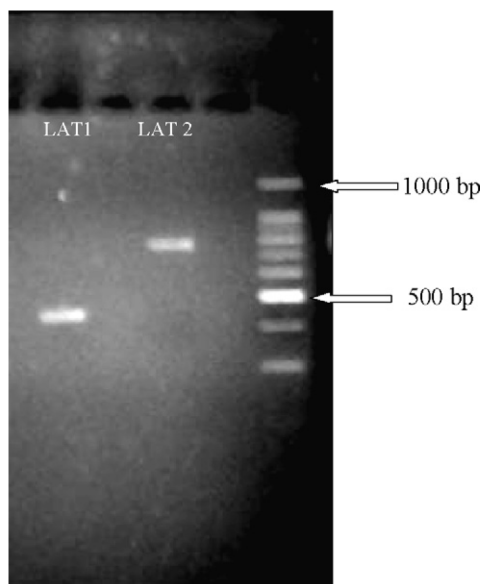

Fig. 3. RT-PCR studies for rabbit LAT1 (lane 1), LAT2 (lane 2) on rabbit retina. A 1 kbp DNA ladder is shown on the right.

was slight inhibition of [^{3}H]-L-Ala uptake in the presence of L-Ala.

3.3. RT-PCR studies (expression of LAT1 and LAT2 in rabbit retina)

The expression of LAT1 and LAT2 was studied in rabbit retina by RT-PCR studies. The PCR products obtained were analyzed by gel electrophoresis on 0.8% agarose. cDNA generated from total RNA isolated from the rabbit retina was PCR amplified with the primers specific for rat LAT1 and LAT2 sequence (primers were designed based on multiple sequence alignment between rat, mouse and human homologues of LAT1 and LAT2). A ∼440 and ∼772 bp product for LAT1 and LAT2, respectively, was obtained as shown in Fig. 3. The sequence alignment using BLAST (NCBI) search showed that the sequence of the PCR product has 100% homology to LAT.

3.4. Intravitreal kinetics of [^{14}C]-L-Phe alone and in the presence of D-Met

Vitreous disposition of [^{14}C]-L-Phe was studied in the presence and absence of D-Met to delineate the mechanism of L-Phe elimination from the vitreous body following intravitreal administration. Intravitreal concentration time profiles of control and [^{14}C]-L-Phe in the presence of 15 and 100 mM concentration of D-Met are depicted in Fig. 4. L-Phe exhibited initial vitreous diffusive equilibration followed by elimination phase from the vitreous body. Detectable levels of [^{14}C]-L-Phe were not observed in the anterior chamber throughout the experimental period in both control and study groups. The variability of initial vitreous levels observed in Fig. 4 could be due to the differences in the placement of probe and the site of injection between the animals. The initial distribution phase is governed by the diffusion of the molecule following intravitreal administration. When

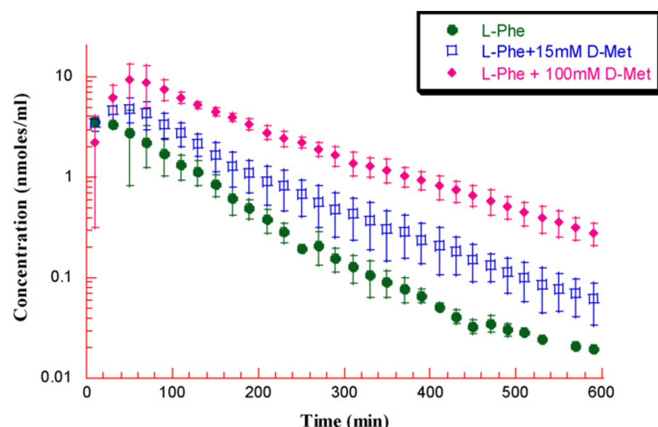

Fig. 4. Intravitreal concentration time profile of [^{14}C]-L-Phe alone and in the presence of 15 and 100 mM concentration of D-Met.

L-Phe is injected slightly farther from the probe, there would lower initial concentrations since the molecules have to diffuse to the probe. To the contrary, if the site of injection is closer to the probe, high initial concentrations would result. From animal to animal there would be slight variation in the site of injection relative to the placement of the probe. However, after initial distribution phase, homogenous concentrations of L-Phe in the vitreous body will occur. A non-compartmental model was used to analyze the vitreous concentration time profile of [^{14}C]-L-Phe. Vitreous pharmacokinetic parameters (λ_z, $t_{1/2}$, Cl, V_{ss}, MRT and AUC_{VH}) are summarized in Table 1. The pharmacokinetic parameters of [^{14}C]-L-Phe in the presence of 15 mM D-Met were not significantly different from that of the control. However, MRT and AUC_{VH} values of [^{14}C]-L-Phe were significantly higher and vitreal clearance was significantly lower than that of the control in the presence of 100 mM D-Met.

4. Discussion

Functional activity and molecular expression of LAT in the retina (in vivo) has been documented in this report. L-Phe being a substrate for both the LAT isoforms (LAT1 and LAT2) was chosen as a model substrate for in vivo retinal uptake and intravitreal elimination studies. The in vivo L-Phe retinal uptake studies in presence of specific inhibitors have demonstrated the functional activity of LAT in the rabbit retina. The functional studies were corroborated by RT-PCR using the primers specific for LAT. The retinal uptake of L-Phe was significantly inhibited by L-system specific inhibitor, BCH indicating that L-Phe is predominantly taken up into the retina via LAT. Similarly, an amino acid analogue, L-DOPA, a substrate for LAT (Kageyama et al., 2000) inhibited the retinal uptake of L-Phe. Furthermore, L-Phe retinal uptake was inhibited by L isomers of large neutral amino acids (L-Phe and L-Trp), but not by glycine, acidic (L-Glut) and basic (L-Arg) amino acids. Previous reports have suggested cross-substrate reactivity of large neutral amino acid transporter with PepT and alanine (A) preferring system. L-Phe uptake was studied in presence of NMAIB and GlySar (specific substrates for alanine (A) preferring system and PepT, respectively) to check the involvement of those transporter systems in

Table 1
Vitreous pharmacokinetic parameters of [^{14}C]-L-Phe following intravitreal administration

	λ_z ($\times 10^{-3}$, min^{-1})	$t_{1/2}$ (min)	V_{ss} (mL)	Cl ($\times 10^{-2}$, mL min^{-1})	MRT (min)	AUC$_{VH}$ (nmol min mL^{-1})
L-Phe	7.6 ± 2.0	95.5 ± 25.4	2.0 ± 0.8	2.1 ± 0.7	88.0 ± 12.0	53.4 ± 24.2
L-Phe + D-Met (15 mM)	7.1 ± 0.1	96.9 ± 2.0	1.3 ± 0.5	1.1 ± 0.4	113.0 ± 5.2	94.0 ± 30.5
L-Phe + D-Met (100 mM)	6.0 ± 0.3	115.5 ± 6.0	0.7 ± 0.2	0.4 ± 0.1*	147.4 ± 28.0*	233.0 ± 51.0*

All values are mean ± S.D. (n = 3). Values are significant at $^*p \leq 0.05$ level.

it's retinal uptake. Although, PepT is a proton dependent transporter, the uptake was carried at physiological pH 7.4 as there would be significant activity of PepT at that pH. Uptake of L-Phe was unaltered both by NMAIB and GlySar demonstrating the lack of involvement of above-mentioned transporters.

Retinal uptake of L-Phe was carried out in the presence of specific LAT1 (D-Phe, D-Trp, D-Leu and D-Met) and LAT2 (L-Asp, L-Cys and L-Ala) substrates to further delineate the exact isoform of LAT involved in L-Phe uptake. LAT1 specific substrates substantially inhibited the uptake of L-Phe; whereas, specific substrates to LAT2 did not. However, L-Ala and L-Asp exhibited weak inhibition of L-Phe uptake. These results might be either due to either their minimal affinity towards LAT1 or weak LAT2 functional activity in the retina. Mastroberardino et al. (1998) have reported that L-Ala exhibited weak affinity (apparent K_m > 10 mM) towards LAT1 expressed in *Xenopus laevis*. The relative affinities of the substrates towards LAT1 as observed by the competition of L-Phe uptake are as follows (Fig. 1): L-Phe > D-Leu > L-DOPA > D-Trp > D-Met > BCH > L-Ala ≥ L-Trp ≥ D-Phe ≥ L-Asp.

To further confirm the predominant functionality of LAT1 in retina, uptake of [^{3}H]-L-Ala (substrate for LAT2 as well as alanine (A) preferring system) was conducted in presence of LAT1 (L-Phe and D-Met) and LAT2 (L-Asp and L-Ala) substrates. No significant inhibition of L-Ala uptake was observed in presence of LAT substrates (L-Phe, L-Asp and D-Met). However, L-Ala significantly diminished the uptake of [^{3}H]-L-Ala, which is also a substrate for alanine (A) preferring system. These results indicate that L-Ala uptake is presumably not mediated by LAT2 but by system A in the rabbit retina. This data is consistent with lack of significant competition for [^{14}C]-L-Phe retinal uptake by LAT2 specific substrates such as L-Ala, L-Asp and L-Cys. These results indicate the specific role of LAT1 in uptake of L-Phe into the retinal cells from vitreous humor. RT-PCR studies were carried out to investigate molecular expression of LAT in the rabbit retina and to corroborate uptake results that demonstrated the functional activity of LAT. Primers for LAT1 and LAT2 were designed based on multiple sequence alignment between rat, mouse and human homologues of LAT1 and LAT2. A ∼440 bp product corresponding to rabbit LAT1 and ∼772 bp product corresponding to rabbit LAT2 were obtained. Therefore, RT-PCR provides molecular evidence for the presence of LAT1and LAT2 in the rabbit retina. Although, rabbit retina expresses both the LAT isoforms, L-Phe uptake was predominantly mediated by LAT1, which may be due to weak functional activity of LAT2 in rabbit retina inspite of its molecular expression. However, further immunohistochemistry studies should be performed to identify the cellular localization of LAT isoforms in the retina. Also, specific studies should be performed in retinal cell culture systems to further understand the functionality of LAT isoforms and their physiological role in the retina.

Retinal uptake studies carried out for shorter duration (30 min) have indicated that L-Phe can be actively taken up into the retina from vitreous humor by LAT. In order to further delineate the role of LAT in the disposition of [^{14}C]-L-Phe from the vitreous body, pharmacokinetics of L-Phe were studied using microdialysis technique. In this study [^{14}C]-L-Phe was administered intravitreally either alone or with LAT substrate, D-Met, at a concentration of 15 and 100 mM. Intravitreal elimination half-life of L-Phe was observed to be 95 min. Also, no detectable levels of radioactivity were observed in the anterior chamber. Major drug elimination from vitreous chamber may occur through the anterior hyaloid membrane into the posterior chamber followed by aqueous drainage and/or across the retinal surface (Maurice, 1984). Large and hydrophilic molecules, which cannot easily traverse the blood retinal barrier (BRB), are predominantly eliminated via the aqueous drainage route. Small, lipophilic molecules and compounds known to be substrates for the transporters/receptors may eliminate primarily across the retina. Compounds eliminating predominantly through the aqueous pathway tend to exhibit longer half-lives (>8–10 h); whereas, substances with either lipophilic properties or substrates for transporters exhibit shorter half-lives in the vitreous body (<5–6 h). Since L-Phe is a zwitterionic and hydrophilic moiety, it cannot easily traverse the blood retinal barrier unless it is a substrate for amino acid transport system present on blood retinal barrier. Therefore, it could be inferred that L-Phe is eliminating predominantly through the retina and probably by utilizing system L present on the BRB. Retinal uptake could possibly play a role in elimination but the major mechanism of the elimination could be transport across retinal pigment epithelium and/or endothelium of retinal blood vessels constituting blood retinal barrier. Reddy et al. (1977) have reported earlier that cycloleucine is removed from the vitreous humor into blood by an active mechanism located on the pigment epithelium of the retina. The pharmacokinetic parameters of L-Phe in the presence of 15 mM D-Met were not significantly different from the control. However, in presence of 100 mM D-Met, the AUC and MRT values of L-Phe were significantly higher and the vitreal clearance was significantly lower with respect to control. With 15 mM concentration of D-Met, the final concentration that can be achieved in the vitreous humor is approximately 1 mM. This concentration was assumed to completely saturate the transport process based on in vitro studies. But in the in vivo studies no significant inhibition was observed with this concentration. The D-Met could have been eliminated from the vitreous and

sustained higher concentrations were not maintained for longer duration of the experiment. However, with 100 mM concentration of D-Met, higher sustained concentrations could have been achieved in the vitreous humor. These results further corroborate the involvement of LAT in the elimination of L-Phe from the vitreous body.

In conclusion, molecular expression of LAT1 and LAT2 in retina has been demonstrated. LAT1 is predominantly functionally active in retina and is responsible for the uptake of large neutral amino acids administered in the vitreous body. The transporter identified here can be utilized to deliver hydrophilic drugs to the retina following intravitreal administration. For example, the uptake of GCV the drug of choice for CMV retinitis into retinal cells after intravitreal injection is limited due to its hydrophilic nature. Therefore, strategies to increase the uptake of ganciclovir into retina would play a critical role in improving the efficacy of CMV retinitis treatment. Targeting drug molecules to the transporters expressed on the retina via prodrug derivatization following intravitreal administration may present a promising approach to enhance retinal concentrations of the parent drug. Also, the present study would have implications in further understanding the role of this transporter in efflux mechanism from the vitreous and uptake of amino acids into the retinal cells after intravitreal administration.

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