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Modifications of L-Glutamine and L-Leucine Transport in Proliferating Lymphocytes

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Abstract

Lymphocytes respond to mitogens that stimulate proliferation by increasing theirs metabolic activity. In this study we investigate L-Glutamine and L-Leucine uptake as markers of cell response to Concavalina A (ConcaA) stimulation, using a high-resolution flow technique. We found that lymphocytes induced to blast transformation enhanced rate and efficiency of amino acid uptake during cell proliferation. Considering that increases in transport is the first quantifiable response of cells during malignant transformation, amino acid uptake could also be useful as an early marker of malignancy.

Keywords

Cell Membrane Transport, Lymphocytes, L-Glutamine, L-Leucine, Malignancy Biomarkers

1. Introduction

Substrate uptake across the cell membrane is the first step in many of the principal metabolic pathways. For this reason mitogens that stimulate cell proliferation, such as Concanavalin A (ConcaA), produce both a general increase in cellular metabolism and in membrane transport. In this regard, certain increases in cell membrane transport are early predictors of cell proliferation [1]. The non-essential amino acid Glutamine and the essential amino acid Leucine are both necessary for lymphocyte growth. Glutamine is transported into lymphocytes by N system which is Na^+, temperature and pH dependent [2]. On the other hand, Leucine, is transported into these cells by L system, and his influx through cell membrane can be a good indicator of protein synthesis rate [3].

It has been shown that ConcaA induces an increase in L-Gln and L-Leu uptake by lymphocytes. In addition, ConcaA accelerates the rate of enzymatic processes implicated in L-Gln and L-Leu metabolism [4]. Further, it is known that kinetic parameters of amino acids transport in proliferating lymphocytes are markedly different from those of resting lymphocytes. However, there is little direct experimental information on the sequence of steps that mediate these changes during the blast transformation process.

Considering that increases in transport is the first quantifiable response of cells during malignant transformation, amino acid uptake could also be useful as an early marker of malignancy. In this work we analyze the

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transport's kinetics of L-Gln and L-Leu during the different phases of blast transformation of lymphocytes after ConcaA induction *in vitro*. The primary objective was to determine amino acid uptake parameters during blast transformation. Transport kinetics were measured using a flow system combined with dilution analyses of paired-tracers. This method has higher time resolution than traditional bath systems and allows studying the earlier phases of the transport process. Moreover, live cells can also be recovered for biochemical, morphologic and viability studies after experiments.

2. Material and Methods

2.1. Lymphocyte Isolation, Culture and ConcaA Stimulation

Venous blood was obtained from 20 healthy blood donors (9 female and 11 male) of ages ranging from 18 to 40 years at Carlos Haya Hospital, Málaga, Spain, according to institutional guidelines. All donors had serum glucose, creatinine, transaminases and thyroid hormones within normal ranges. Red and white blood cell counts were also within normal ranges. Lymphocytes were separated by density gradient centrifugation. Lymphocytes (2736×10^3 cells/µl) were incubated in M-199 medium (Sigma, USA) supplemented with 10% of foetal bovine serum (Gibco BRL, USA) and 1% of antibiotics at 37˚C in a 5% CO_2 incubator. Concanavalin A [ConcaA] (Sigma, USA) was added to the cultures at a final concentration of 5 mg/ml and the incubation times where 0, 3, 9, 18, 24, 48 and 72 hours.

2.2. Amino Acid Uptake

Amino acid uptake was measured using a rapid paired-tracer dilution technique [5]. Briefly, cells were packed in a dialysis column (Minifiltro™ Hemofiltro Amicon Corporation, SA) and perfused with Hanks balanced salt solution (Sigma) at 37˚C and pH 7. Lymphocytes (1 × 10) were packed in the "vascular" compartment of the column (made of a bundle of 100 semipermeable Teflon tubes with 20 µm pore diameter). Cells were perfused via an "extravascular" compartment through an "arterial" input and a "venous output". The perfusate was delivered by a peristaltic pump (p3, Pharmacia Biotech, Sweden) at constant flow rate of 0.6 ml/min and a pressure 15 - 20 mm·Hg. Amino acid uptake was measured by comparing output concentrations profiles of [^{14}C]D-mannitol (extracellular tracer), with either [^{3}H]L-Gln or [^{3}H]L-Leu, following an intrainput bolus of 400 µl perfusate containing 14 µl of [^{14}C]D-mannitol and 7 µl of [^{3}H]L-Gln or L-Leu (1 pmol of [^{3}H]L-Gln or [^{3}H]L-Leu and 0.95 pmol of [^{14}C]D-mannitol). Fractional amino acid uptake (%U) was calculated, for each one of 30 sequential samples, according to the equation: %U = (1 − [3H]/[14C]) × 100). Substrate saturation was investigated by measuring unidirectional flow rates over a wide range of Glutamine and Leucine concentrations (0.05 to 5 mM). Cell viability was assessed by trypan blue at the end of each experiment.

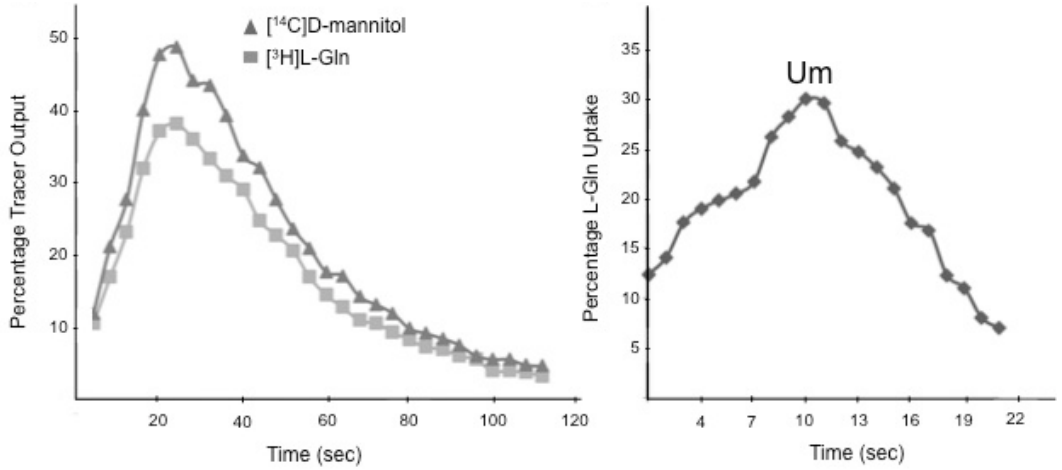
3. Results

3.1. Glutamine and Leucine Uptake of Resting Lymphocytes

Amino acid uptake was measured in resting lymphocytes. A representative curve showing sequential collection of [^{14}C]D-mannitol and [^{3}H]L-Gln outputs is shown in **Figure 1(a)**. Fractional amino acid uptake (%U) was then estimated by comparing concentrations profiles of [^{14}C]D-mannitol (extracellular tracer), with either [^{3}H]L-Leu or [^{3}H]L-Gln. **Figure 1(b)** shows a illustrative curve for L-Gln uptake, where unidirectional uptake, represented by the ascendant region of the curve, reached a plateau value within 10 - 15 seconds. Maximal uptake (Um) estimated from average %U at the plateau was followed by a decline, suggesting cellular efflux of the transported amino acid.

L-Gln and L-Leu maximal uptake was estimated from three independent experiments, results showed that Um for L-Gln was 29.8% ± 3.5% and 32.5% ± 3.3% for L-Leu. Non-specific uptake of Glutamine and Leucine due to adsorption to the tubing in the dialysis apparatus was investigated in control experiments conducted in the absence of cells. Under these conditions uptake was below 5%. High uptake was maintained for prolonged intervals under perfused conditions. Lymphocyte uptake of both amino acids was 93% of initial values after one hour of perfusion. Cell viability at the end of each experiment was higher than 90%.

L-Gln and L-Leu uptake characteristics were analyzed subjecting the lymphocytes to variances of pH and temperature, in absence of Na^+ and in presence of unlabeled amino acids. **Table 1** summarized the results



(a) (b)

Figure 1. Representative L-Gln uptake curve.

Table 1. L-Gln and L-Leu maximal uptake under different culture conditions.

	Culture conditions					
	Basal	Na^+ Free	pH 6.5	25˚C	10 mM L-Gln	10 mM L-Leu
L-Gln (%Um)	29.8%	19.5%	1.2%	26.7%	2.1%	7.2%
L-Leu (%Um)	32.48%	30.2%	1.0%	26.4%	24.8%	3.47%

obtained, the transport of L-Gln was proved to be Na^+, pH and temperature dependent and inhibited by unlabeled L-Gln and L-Leu. On the other hand, the transport of L-Leu was Na^+-independent but pH and temperature dependent.

3.2. Glutamine and Leucine Uptake by Lymphocytes Exposed to ConcaA

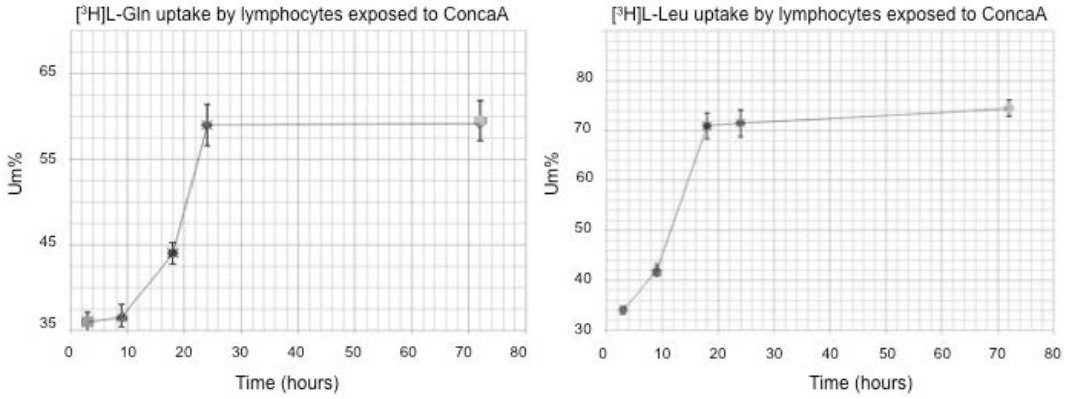
Lymphocytes were exposed to ConcaA for intervals of 0, 3, 6, 9, 18, 24, 36 and 72 hours and uptake was estimated after each interval. Uptake of L-Gln and L-Leu increased with time after ConcaA stimulation (**Figure 2**). Kinetic parameters of L-Gln and L-Leu were determined after exposing the cells to ConcaA for 3, 9, 18, 24 and 72 hours (**Table 2** and **Table 3**). For L-Gln, V_{max} increased 10 fold while L-Leu, V_{max} increased by 19 folds. For both amino acids the K_m decreased by 3.5 and 3.4 fold, for L-Gln and L-Leu, respectively.

3.3. Substrate Specificity of the Glutamine and Leucine Transport Systems

Substrate specificity of the L-Glutamine and L-Leucine transport systems was investigated with various amino acids that use System N and System L. As shown **Figure 3(a)**, L-Gln, L-Asn and L-His, substrates of System N, inhibited the Um of Glutamine by 93.2% ± 2%, 57.3% ± 5% and 49.5% ± 6%, respectively. In addition to specific inhibition we found a non-specific inhibition by the System L-preferring amino acids L-Met (10 mM) and L-Leu (10 mM), which inhibited 73.6% ± 3% and 83.2% ± 1.5% Glutamine Um, respectively. The substrates of system L, as shown **Figure 3(b)**, L-Leu, L-Val, L-Phe and L-Met inhibited the Um of Leucine by 89.3% ± 2%, 42.4% ± 4.5%, 60.5% ± 2.7% and 75.2% ± 2.3% respectively. The non-specific substrates of L-system, L-His and L-Asn, inhibited L-Leu Um by 17.3% ± 7% and 23.6% ± 3% respectively.

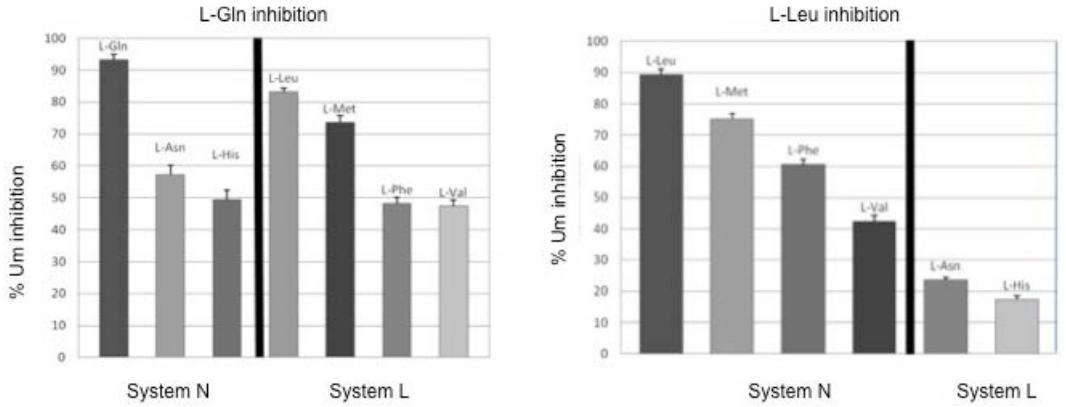
4. Discussion

Lymphocytes respond to antigenic stimulus by increasing theirs metabolic activity [6]. Lymphocyte activation and proliferation is associated to a wide range of changes in cell metabolism, notably an increase in the rate of protein synthesis [7]. In this study we investigate L-Glutamine and L-Leucine uptake as markers of cell response



(a) (b)

Figure 2. Uptake of L-Gln and L-Leu after ConcaA stimulation.



(a) (b)

Figure 3. Substrate specificity of the Glutamine and Leucine transport systems.

Table 2. Kinetic parameters of L-Gln uptake after ConA exposition.

L-Gln	Time					
	0 h	3 h	9 h	18 h	24 h	72 h
V_{max}	5.25	15.2	15.8	41.3	53.8	54.6
K_m	417.3	375.1	360.3	253.8	120.2	118.6

Table 3. Kinetic parameters of L-Leu uptake after ConA exposition.

L-Leu	Time					
	0 h	3 h	9 h	18 h	24 h	72 h
V_{max}	1.08	6.3	5.1	13.2	21.4	20.7
K_m	114.8	60.7	65.2	37.8	33.8	33.5

to ConcaA stimulation, using a high resolution flow technique, in order to detect the initial modifications of lymphocytes during blast transformation for a possible application to the diagnostic of tumoral process.

Previous studies have demonstrated that peripheral human lymphocytes contain a high affinity amino acid carrier for Glutamine, which is shared by serine, asparagine and histidine, which efficiency greatly increased by ConcaA induction to cover the demand of metabolic pathways [8].

Here, kinetic parameters of L-Gln and L-Leu transport V_{max} and K_m, were determined in perfused lymphocytes under basal conditions and after incubation with ConcaA. In addition, specific and non-specific inhibitions for both amino acids, during each of the proposed phases of experimental blast transformation were achieved. We observed an increase in the V_{max} and a decrease in K_m for the two amino acids during the blast transformation. These changes in L-Gln and L-Leu transport kinetics during the blastic modification of lymphocytes show an increased of transport efficiency of both amino acids. It is important to show that the stimulation in amino acids uptake was not a transitory phenomenon but was sustained during the observation period. Incorporation of L-Gln in the Iymphocytes was not very specific. The amino acid L-Leu was a non-specific inhibitor and L-met, which prefer the system L, inhibited L-Gln transport too partially his specificity. Inhibition of [^{3}H]L-Gln uptake by an excess of cold L-Gln decreased from 90% to 60% and inhibition of [^{3}H]L-Leu for unlabeled L-Leu, from 84% to 34%. The other unlabeled substrates used in competition experiments, both for specific and non-specific inhibition transport, also lost the ability to inhibit L-Gln transport.

The stimulation of lymphocytes with ConcaA seems to lead a conformational change in L-Gln and L-Leu transport systems directed towards increase it capacity and decrease it specificity. The increase of L-Gln and L-Leu uptake by the lymphocytes was correlated with the increase of tritiated Thymidine ([^{3}H]Thymidine) uptake during the first 24 hours of ConcaA stimulation (data not shown). This fact could represent metabolic adaptation to nutrient during cell proliferation [9] [10], although discrepancies have also emerged during leukemic process [11].

We concluded that the enhanced rate and efficiency of amino acid uptake during cell proliferation could be used to detect earlier changes in the functionality of the lymphocyte membrane that alert about a malignant process. Because the stimulated lymphocytes maintained a high L-Gln and L-Leu uptake in contrast with the incorporation of Thymidine that decreased after 24 hours (data not shown), amino acid transport could be used as an indicator of the cellular metabolic activity.

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