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Erythrocytes L-Arginine y+ Transporter Inhibition by N-Ethylmaleimide in Ice-bath

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Abstract Erythrocytes L-arginine uptake is conveyed by y+ and y+L membrane transport systems. Pre-incubation with N-ethylmaleimide for 10 min at 37°C inhibits the y+ system. The aim of this study was to determine the ideal pre-incubation temperature in evaluating y+ and y+L systems. Cells were pre-incubated with or without N-ethylmaleimide for 10 min at 4°C and 37°C. L-Arginine uptake was quantified by radioisotope and standard erythrocytes membrane flux methodology. Results demonstrate that erythrocytes L-arginine content is depleted by pre-incubation at 37°C for 10 min, thus changing the V_{max} measurement. The inhibitory effect of N-ethylmaleimide pre-incubation was temperature independent and already complete after 1 min of incubation. No significant difference in kinetic parameters was detected between cells pre-incubated at 37°C or 4°C, under *zero-trans* conditions. In conclusion, we suggest that measurement of erythrocytes L-arginine uptake by y+ and y+L systems could be carried out without N-ethylmaleimide pre-incubation at 37°C.

Keywords Membrane transporters · Nitric oxide · Cationic amino acid transporters · Carrier · Erythrocytes

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Introduction

Amino acid transport across the plasma membrane is mediated by a variety of carriers [1]. Modifications of carrier-mediated membrane transport have been described in pregnancy [2, 3], uremia [4–10], hypertension [11], pregnancy-induced hypertension [12–14], and inherited diseases [1]. The hypothesis of this study is that cell incubation at physiological temperature (37°C) depletes the existing intracellular arginine pool and alters membrane transporters kinetic parameters. The current data demonstrate that a mischaracterization of the L-arginine transporters activity might result if cells were kept at 37°C during the pre-incubation with N-ethylmaleimide (NEM). Additionally, 1 min ice-bath NEM pre-incubation inhibits erythrocyte y+ activity, making pre-incubation at 37°C unnecessary.

Regulation of blood pressure involves the L-arginine-nitric oxide pathway where the single precursor of nitric oxide—a potent endothelium-derived relaxing factor—is L-arginine [15, 16]. L-Arginine influx into cells occurs through two cationic amino acids transporters-system y+ [17] and the high affinity system y+L [18]. The transport systems also exhibit affinity to other amino acids such as lysine, ornithine, and arginine analogs [18–20]. Levels of endogenous L-arginine analogs are modified by diverse pathological conditions, leading to decreased nitric oxide production, secondary to L-arginine uptake blockade, depletion of intracellular L-arginine and inhibition of nitric oxide synthase [21–23].

Cells are usually pre-incubated in NEM [24] for 10 min at 37°C—a condition known to selectively inhibit the y+ system [25, 26]—to separate y+ from y+L activity. However, it has been known that previous cells exposition at physiological temperature (37°C) depletes the basal

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arginine intracellular pool and changes membrane transporters kinetic parameters [27]. As consequence, the maximal transport capacity (V_{max}) and half-saturation constant (K_m) estimation may be modified, resulting in erroneous evaluations.

The aim of the current study was to determine the most favorable inhibitor pre-incubation temperature, when evaluating y+ and y+L systems transport function separately in erythrocytes.

Materials and Methods

The study protocol was approved by the Pontifícia Universidade Católica do Rio Grande do Sul Ethics Committee. Erythrocytes for the experiments were obtained from the Hospital São Lucas Blood Bank–PUC-RS, Porto Alegre, RS, Brazil.

L-Arginine uptake was measured using established protocols for erythrocytes transmembrane flux experiments [28]. After an initial blood aliquot spinning (3000g, 10 min), erythrocytes were separated, washed, and precipitated by centrifugation (3000g, 2 min) three times in ice-cold saline: glucose 10 mmol/l; KCl 5 mmol/l; NaCl 140 mmol/l; 4-morpholinepropanesulphonic acid 10 mmol/l; pH 7.4. Cells were resuspended in ice-cold saline to achieve a hematocrit between 8 and 11% (spectrophotometrically determined) and stored at 4°C. Cell suspension aliquots of 0.225 ml were added to 0.05 ml of ice-cold saline or NEM (Sigma Chemical Co., St Louis, MO, USA) stock solution (8 µM). Duplicate tubes, with and without NEM, were maintained at 4°C or 37°C for 10 min, and transferred to an ice-bath for at least 3 min. Cold saline and L-arginine (Sigma Chemical Co., St Louis, MO, USA) (1111 µM) containing trace amounts of [14C]L-arginine (50 µCi/mL, Amersham Biosciences, Little Chanfolt, UK) were added to the tubes in order to achieve 8.8, 15.5, 24.5, 51, 100, 300, and 500 µM L-arginine final concentration, in a final volume of 500 µl. Cells were then incubated at 37°C for 3 min, and the transmembrane flux was stopped on ice. Following, erythrocytes were washed free of the extracellular radioactivity by three fast spin/washes (15,000g, 5 s) in ice-cold saline. The final cell pellet containing intracellular beta-emitters was lysed with 0.25 ml 0.1% (v/v) Triton X-100 (Sigma Chemical Co., St Louis, MO, USA), and, subsequently, deproteinized by addition of 0.25 ml 5% (w/v) trichloroacetic acid. Proteins were precipitated by centrifugation (15,000g, 5 min) and the supernatant was added to vials containing scintillation fluid for radiation counting (LS6500, Beckman Counter, Inc., Brea, CA. USA). The initial influx rate of L-arginine was expressed in µmol/l of cells/h. L-Arginine uptake in saline represents the total influx—through y+ and y+L systems. Additionally, L-arginine uptake in tubes pre-incubated with

NEM (the y+ inhibitor) represents flux mediated by the y+L system. The difference between total and y+L influx at each point-concentration stands for the y+transport value. An Enzfitter (Biosoft, Cambridge, UK) software was used to fit data into Michaelis-Menten kinetics. L-Arginine V_{max} and K_m were computed for each transport system.

A majority of experiments were performed immediately after erythrocytes preparation. Some experiments were performed under zero-trans conditions by pre-incubating cells (at a 1% hematocrit) in saline at 37°C for 3 h with constant shaking, before flux measurements, to totally deplete the intracellular substrate pool.

Data are expressed as mean ± standard deviation (SD). Two-tailed paired Student's t-tests and ANOVA were employed in statistical analyses. A P-value ≤0.05 was considered significant.

Results

Preliminary experiments had shown that total L-arginine flux was linear when cells were incubated at 37°C for 3 min ($n = 6$)—depicted in Fig. 1. That condition was thus chosen to perform erythrocyte L-arginine transport at 37°C.

Table 1 shows erythrocytes total L-arginine uptake and y+ fluxes, measured at two different NEM pre-incubation temperatures (37 and 4°C). The total L-arginine uptake V_{max} was higher with pre-incubation at 4°C, in comparison with pre-incubation at 37°C, suggesting that depletion of intracellular substrates occurred at physiological temperature. No significant difference was demonstrated in the transporter affinity (K_m).

Table 2 depicts L-arginine uptake (V_{max}), measured with and without NEM pre-incubation for different time periods, under both experimental temperatures. Total V_{max} decreased with incubation time, when NEM was used at 37°C. It also shows that 1 min pre-incubation in NEM suffices to inhibit y+ carrier.

Figure 2 illustrates L-arginine uptake at different erythrocytes incubation times in saline, showing that 3 h was adequate in achieving depletion of the intracellular content
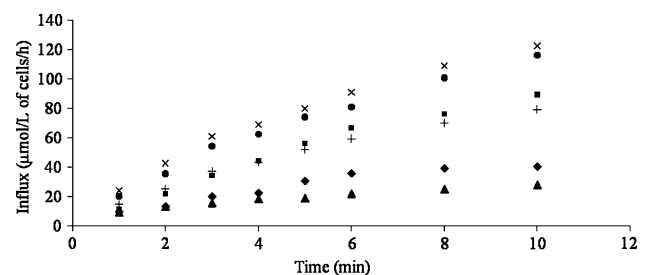

Fig. 1 Time course of L-arginine influx in erythrocytes (extracellular arginine concentration of 500 µM)

Table 1 L-Arginine uptake kinetics (mean ± SD) at different NEM pre-incubation temperatures

Transport systems	Total influx			y+			y+L		
NEM pre-incubation temperature:	37°C	4°C	P	37°C	4°C	P	37°C	4°C	P
Kinetic parameters									
V_{max}	913.2 ± 608.3	1213.7 ± 827.9	0.027	579.5 ± 337.7	779.5 ± 431.1	0.010	148.8 ± 78.3	149.0 ± 77.9	0.975
K_m	47.4 ± 28.2	55.1 ± 30.8	0.213	72.9 ± 26.6	74.8 ± 28.1	0.864	9.9 ± 5.6	8.6 ± 6.0	0.396

* $P < 0.05$ Student's t-test for paired samples: 4 and 37°C total fluxes; V_{max}: μmol/l of cells/h and K_m: μmol/l

Table 2 L-Arginine uptake (μmol/l of cells/h) at different NEM incubation time under both experimental conditions (37°C and 4°C)

NEM pre-incubation temperature (10 min)	Transport systems	Time (min)				
		1	5	10	20	30
37°C	y+ and y+L*	574.8 ± 215.4	527.1 ± 189.2	439.7 ± 143.7	368.6 ± 115.4	373.3 ± 122.0
	y+*	439.1 ± 202.4	385.9 ± 173.0	295.8 ± 138.9	231.0 ± 104.4	235.5 ± 108.6
	y+L	135.7 ± 30.8	141.2 ± 33.8	143.9 ± 27.5	137.6 ± 31.8	137.8 ± 38.2
4°C	y+ and y+L	522.3 ± 209.3	596.3 ± 217.3	569.5 ± 206.2	556.9 ± 217.3	588.0 ± 226.9
	y+	323.3 ± 232.4	401.5 ± 263.6	379.1 ± 240.0	366.1 ± 245.5	393.7 ± 257.5
	y+L	145.3 ± 26.7	129.3 ± 17.6	128.9 ± 21.1	132.9 ± 26.8	133.7 ± 19.0

Data are expressed as means ± SD ($n = 6$); influx in μmol/L of cells/h; * ANOVA $P < 0.05$

($n = 5$). Table 3 depicts total and y+L L-arginine uptakes under zero-trans conditions. Both transport systems kinetic parameters (V_{max} and K_m) were very similar, independent of the pre-incubation temperatures.

Discussion

It has been known that intra and extracellular exchanges occur in cells kept at 37°C, before flux measurements [27]. The current experiments demonstrated that mischaracterization of L-arginine membrane transporters activity might result with cells kept at 37°C, during the pre-incubation
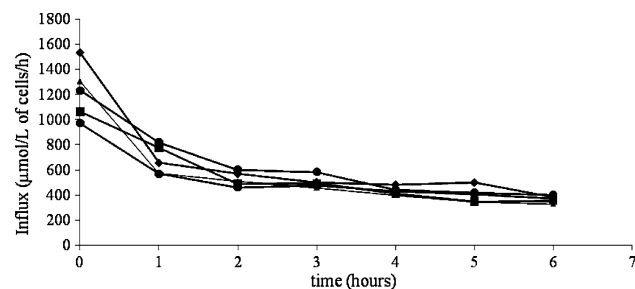

Fig. 2 L-Arginine influx at 500 μM extracellular concentration. Fluxes were performed with immediately washed cells (time 0) and at every hour after the cells had been incubated at a hematocrit <1% at 37°C in L-arginine-free saline. Influx rates reached steady-state condition after 2–3 h

time with NEM. Additionally, the ice-bath NEM pre-incubation inhibits erythrocyte y+ activity within 1 min. The current results suggest that a small, but important, modification should be introduced in protocols used for erythrocytes uptake measurements. Cells should be kept at 4°C—as in the evaluation of total uptake—when performing experiments that use NEM to block y+ system. The 10 min NEM pre-incubation time at 37°C, as usually carried out, seems unnecessary.

Studies that compare normal with sick cells could be particularly affected. In diseases with increased intracellular amino acids content [12, 29–31], trans-stimulation would be higher, making loss of intracellular content greater than in control cells. Under such conditions, a 10 min pause to prepare the cells would cause a difference between case and control kinetic parameters. Alternatively, a long incubation time in conditions with decreased intracellular amino acids pool would underestimate kinetics parameters, compared with normal cells.

Changes in erythrocytes maximal transport capacity was the only change observed between the two different protocols. Affinity was unaffected by pre-incubation temperature, either in fresh cells or in zero-trans conditions. In fact, previous amino acids cell depletion may represent a more favorable condition to investigate carrier-mediated transport. However, obtaining data on the trans-stimulated status in critical in such case as levels of analogs may change under different conditions [21, 25, 26].

Table 3 L-Arginine uptake kinetics (mean ± SD) at different NEM pre-incubation temperature in zero-trans conditions

Transport systems	Total influx			y+			y+L		
NEM pre-incubation temperature:	37°C	4°C	P	37°C	4°C	P	37°C	4°C	P
Kinetic parameters									
V_{max}	175.5 ± 118.6	170.9 ± 106.4	0.950	100.5 ± 66.4	122.9 ± 107.1	0.727	36.1 ± 7.9	39.3 ± 10.6	0.627
K_m	33.2 ± 13.8	29.0 ± 7.7	0.570	62.4 ± 44.7	38.2 ± 14.9	0.364	10.2 ± 3.7	7.4 ± 3.5	0.281

No statistic difference were detected by Student's t-test for paired samples; V_{max}: μmol/l of cells/h and K_m: μmol/l

Previous studies have evaluated the L-arginine/nitric oxide pathway [22, 23, 25, 26, 32], and an L-arginine paradox has been often referred [33, 34] to—exogenous L-arginine causing NO-mediated biological effects, despite nitric oxide synthases theoretical saturation with substrate [33]. Yet y+L L-arginine activity up-regulation [29] could explain such paradox. Since y+ is a high capacity and low affinity system, it may allow the intracellular accumulation of other substrates [4], such as L-arginine analogs, thereby causing inhibition of nitric oxide synthase. The cell membrane holds a caveolar complex between y+ L-arginine and the endothelial nitric oxide synthase [35], suggesting a mechanism of direct substrate delivery to the enzyme. Also, direct transfer of extracellular L-arginine to membrane-bound nitric oxide synthase may account for the arginine paradox, explaining why caveolar location of nitric oxide synthase is required for optimal endothelial nitric oxide production [36]. Data presented in this study suggest that y+ transporter activity could be underestimated in cells incubated at 37°C, so that activity of y+L system in relation to total influx would also be misinterpreted.

The current data allow proposing a modification to the assay protocol for measuring erythrocyte y+ and y+L membrane transporters independently—canceling the 10 min pre-incubation at 37°C with NEM. The message is simple, but a relevant modification in refining erythrocytes L-arginine transport evaluation technique. Incubation at high temperature, previous to the influx, depletes the intracellular L-arginine content, while low temperature incubation does not. A more dependable estimate of erythrocytes y+ and y+L transport is achieved by the suggested modification.

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