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y+ cationic amino acid transport of arginine in packed red blood cells

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ABSTRACT

Background: Transfusion of packed red blood cells (PRBCs) is associated with morbidity and mortality. The mechanisms are not fully understood. Packed red blood cells deplete extracellular arginine and possess transporters for arginine, an amino acid essential for normal immunity. We hypothesize that the membrane y+ amino acid transporter contributes to arginine depletion in PRBCs.

Materials and methods: We titrated PRBCs to a 10% hematocrit with phosphate-buffered saline, blocked PRBC y+ transporters using *n*-ethylmaleimide (0.2 mM), and measured arginine and ornithine levels using liquid chromatography–mass spectroscopy. We added radiolabeled L-arginine [4,5-^{3}H] (10 μmol/L) added to similar culture conditions and measured arginine uptake in counts per minute (CPM). We examined storage periods of 6–9 d, 1–4 wk, and 6 wk, and correlated donor demographics with arginine uptake.

Results: n-Ethylmaleimide blockade of y+ transporters impaired PRBC arginine depletion from culture media (117.6 ± 8.6 μM *versus* 76.9 ± 5.8 μM; $P < 0.001$) and reduced intracellular L-arginine (7,574 ± 955 CPM *versus* 18,192 ± 1,376 CPM; $P < 0.01$). Arginine depletion increased with storage duration (1 wk *versus* 6 wk; $P < 0.002$). With *n*-ethylmaleimide treatment, 6-wk-old PRBCs preserved more culture arginine ($P < 0.008$) than at shorter durations. Nine-day storage duration increased L-arginine uptake compared with 6- to 8-day storage ($n = 77$, $R = 0.225$, $P < 0.05$). Extracellular arginine depletion and extracellular ornithine synthesis varied among donors and correlated inversely ($R = -0.5$, $P = 0.01$).

Conclusions: Membrane y+ transporters are responsible for arginine depletion by PRBCs. Membrane y+ activity increases with storage duration. Arginine uptake varies among donors. Membrane biology of RBCs may have a role in the negative clinical effects associated with PRBC transfusion.

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1. Introduction

Packed red blood cell (PRBC) transfusion suppresses immunity and increases mortality, ventilator-acquired pneumonia, and length of hospital stay [1,2]. These clinical outcomes have been collectively termed "transfusion-related immune modulation" (TRIM) [3]. Transfusion-related immune modulation was first ascribed to passenger leukocytes [4]. Recent studies have reported that ever since implementation of routine leukoreduction, negative clinical effects of PRBC transfusion persist [5,6]. Therefore, mechanisms other than passenger leukocytes must be responsible for TRIM.

Red blood cells deplete extracellular arginine, a conditionally essential amino acid necessary for nitric oxide synthesis, wound healing, bacterial killing [7], and T-cell growth and differentiation [8]. Red blood cells may deplete arginine via intracellular arginase enzyme or by arginase bound to the intact RBC membranes or fragments that develop during storage [8]. Red blood cell's nitric oxide synthetase can also deplete arginine [9–12].

Arginine uptake by intact RBCs occurs through transport channels. Red blood cells contain cationic amino acid transporters (CATs), which transport arginine in addition to other amino acids [13]. Cationic amino acid transporter activity is known to be accelerated in certain disease states [14] and could be responsible for RBC arginine depletion. Transport of arginine by CATs is concentration-dependent and occurs through the dominant y+ (high-capacity, low-affinity) transporter and y+L (low-capacity, high-affinity) transporter with less contribution [14]. Arginine diffusion through RBC membranes (independent of CATs) requires supra-physiologic concentrations of arginine, and thus is not clinically significant.

Mechanisms by which unprocessed, *ex vivo* human RBCs transport arginine have been described [15–17], but these mechanisms have not been studied in stored PRBCs. Storage of PRBCs is known to alter RBC morphology and physiology [18]. We hypothesize that the membrane y+ amino acid transporter contributes to arginine depletion in stored PRBCs.

2. Materials and methods

We purchased reagents from Sigma Aldrich (St. Louis, MO) and Perkin-Elmer (Waltham, MA). Through an institutional board–exempted protocol, we prepared leukoreduced PRBC units at an American Association of Blood Banks–accredited blood center and stored them at 4°C. Using a microhematocrit centrifuge, we titrated PRBCs to a 10% hematocrit (HCT), using phosphate-buffered saline (PBS) or Roswell Park Memorial Institute-1640 (RPMI) culture media with arginine concentration controlled, as shown in figure legends.

2.1. y+ CAT blockade

We incubated PRBCs (4 wk old) at 37°C for 3 h to achieve zero-trans conditions (amino acid transporter equilibrium). We then added *n*-ethylmaleimide (NEM), a selective, irreversible y+ transporter blocker (0.2 mM), or vehicle (PBS) to PRBCs and incubated them for 15 min, as previously described [19]. We added treated PRBCs to culture media and measured arginine by liquid chromatography–mass spectroscopy (LCMS) [20].

2.2. Extracellular L-arginine uptake

To determine whether the y+ transporter contributes to PRBC depletion of environmental arginine, we added arginine (150 μmol/L) to the solution of NEM- (0.2 mM) or PBS-treated PRBCs and incubated them at 37°C for 24 h. Uptake was halted by placing on ice for 5 min and centrifuging at 300*g* for 10 min. Supernatant was removed and analyzed for arginine by LCMS.

2.3. Radiolabeled L-arginine uptake

To determine whether the depletion of extracellular arginine by the y+ transporter was due to intracellular uptake, we prepared PRBCs (4 wk old) as described above, and added NEM (0.2 mM) or vehicle at 37°C for 15 min to inhibit the y+ transporter. We added radiolabeled L-arginine [4,5-^{3}H] (10 μmol/L) and incubated it at 37°C for 5 min. Uptake was halted by centrifugation at 10,000 revolutions per minute for 1 min, and the cell pellet was washed twice with ice-cold PBS and lysed with 0.1% Triton X-100 (Sigma Aldrich, St. Louis, MO). We placed lysates into scintillation fluid and analyzed them on a Beckman LS6500 (Brea, CA) scintillation counter; results are expressed as counts per minute (CPM).

2.4. L-Arginine extracellular depletion among donors at the same duration of storage

We then determined whether RBC arginine use varied among donors. We controlled storage duration at 6–9 d ($n = 77$) using the culture conditions described above. After centrifugation at 300*g*, 4°C for 10 min, supernatant was frozen at −80°C until analysis. We measured arginine and ornithine by LCMS. Arginine uptake was simultaneously determined using radiolabeled arginine, employing methods described above. We stratified results by donor characteristics: age, gender, race, and aspirin use.

We performed statistical analysis using t-tests after verifying equal variance using Levene's test. We assessed correlations using Spearman's Rho, and determined variability across donors using analysis of variance. All statistical tests used $P < 0.05$ as the threshold for significance. We performed statistics using SPSS Statistics, version 19 (IBM Corp., New York, NY).

3. Results

Extracellular media arginine was depleted by PRBCs (76.9 ± 5.8 μ M *versus* 150 μM control). The NEM blockade of y+ reduced arginine depletion resulting in higher culture arginine levels compared with vehicle-treated cells, but did not completely abrogate arginine depletion (117.6 ± 8.6 μM *versus* 76.9 ± 5.8 μM; $P < 0.001$) (Fig. 1). *n*-Ethylmaleimide blockade significantly reduced intracellular L-arginine [4,5-^{3}H] uptake compared with