Determination of Intrinsic Creatine Transporter (Slc6a8) Activity and Creatine Transport Function of Leukocytes in Rats

Ayaka Taii,* Masanori Tachikawa,* Yusuke Ohta, Ken-ichi Hosoya, and Tetsuya Terasaki

*Division of Membrane Transport and Drug Targeting, Graduate School of Pharmaceutical Sciences, Tohoku University; 6–3 Aramaki, Aoba, Sendai 980–8578, Japan; b Graduate School of Biomedical Sciences, Tokushima University; 1–78–1 Shomachi, Tokushima 770–8505, Japan; and c Department of Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan.

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Creatine transporter (CRT) deficiency (CRT-D) results in a significant reduction of brain creatine levels, which causes various neurological symptoms in early childhood, and diagnosis of the severity of CRT-D based on the residual CRT transport activity in liquid biopsy samples would be beneficial for early intervention. The apparent reduction in creatine transport activity in CRT-D is thought to be due to reduced intrinsic CRT-mediated creatine transport per CRT protein and/or reduced absolute CRT protein expression on the plasma membranes. The purpose of this study was thus to determine the normal level of intrinsic CRT-mediated creatine transport activity based on absolute CRT protein quantification using rat CRT-overexpressing HEK293 cells (CRT/HEK293 cells), and to clarify creatine transport in erythrocyte- and leukocyte-enriched fractions isolated from the circulating blood of rats. The intrinsic creatine transport rate was calculated to be 0.237 µM/(min·fmol CRT) based on the initial uptake rate and the absolute CRT protein level in CRT/HEK293 cells. Taking into account Avogadro’s constant, the creatine transport activity per CRT protein is estimated to be 1190 creatine/(min·CRT molecule) in the presence of [14C]creatine at an extracellular concentration of 5 µM. Isolated leukocyte-enriched fraction exhibited mRNA expression of CRT and partially Na+/plus.K+- dependent [14C]creatine transport, whereas erythrocytes showed neither. These characteristics suggest that the leukocytes contain the CRT-mediated creatine uptake system, and are available for evaluation of residual CRT transport activity in CRT-D patients.

Key words: creatine transporter; quantitative targeted absolute proteomics; leukocyte; intrinsic transport activity; diagnosis; creatine transporter deficiency

INTRODUCTION

Creatine plays essential roles in the storage and regeneration of intracellular ATP phosphate-bound energy via creatine kinase (CK) in the brain. We have previously demonstrated that creatine is transported into the brain of mice via creatine transporter (CRT) encoded by the Xq28-chromosome-located Slc6a8 gene, which is expressed at the blood–brain barrier (BBB) and in neurons. The importance of CRT-mediated creatine transport into the brain is evidenced by the occurrence of X-linked intellectual disability in male patients with gene defects resulting in creatine transporter deficiency (CRT-D), which has a prevalence of 2.1%. CRT-D patients exhibit a significant reduction of the brain creatine level, as determined by proton magnetic resonance spectroscopy, and exhibit neurological symptoms such as mental retardation, developmental delay, intractable epilepsy, and extrapyramidal movement disorder in early childhood. Therefore, symptom- and genome-sequence-based identification of CRT-D patients, followed by diagnosis of the severity of CRT-D, is crucial for early treatment, starting during development.

Previous reports have shown that fibroblasts derived from CRT-D patients exhibit various levels of residual creatine transport activity, depending on the position of the SLC6A8 gene mutation. Most patients who show clinical improvement in response to supplementation with creatine or its biosynthetic precursors, such as arginine and glycine, have detectable brain creatine levels and are initiated on treatment before nine years of age. This may imply that patients who are treated when they still have residual CRT function could have a better outcome. Thus, evaluation of residual CRT-mediated creatine transport activity in early developmental stages should be useful for diagnosis of the severity and prediction of the outcome of treatment in CRT-D patients. In this context, the reduction in creatine transport activity due to CRT gene mutation could be due to reduced intrinsic transport activity per CRT protein and/or reduced CRT protein expression on plasma membranes. Here, in order to better guide the development of CRT-D therapeutics and treatment, we aimed to measure the intrinsic creatine transport activity based on absolute CRT protein quantification in order to establish the reason for the reduced activity.

Wada et al. have found an increased creatine-to-creatine ratio in urine in Japanese CRT-D patients, which is presumably due to a defect of CRT-mediated creatine reabsorption at brush-border membranes of renal epithelial cells. While screening of the urine creatine-to-creatine ratio provides an indirect measure of CRT-mediated creatine transport activity, direct assessment of CRT transport activity would be preferable to determine the residual transport function of CRT in the brain of the CRT-D patients. Patient-derived circulating blood cells are already used for functional diagnosis of enzymes in several genetic diseases. For example, definitive diagnosis of mucopolysaccharidosis type II is established by enzymatic assay using leukocytes and substrates specific for iduronate-2-sulfatase. Considering that Slc6a8 mRNA is present in rat

* To whom correspondence should be addressed. e-mail: tachik-dds@umin.ac.jp

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blood cells,\textsuperscript{13} it seems likely that CRT transport activity could be evaluated by means of assay using circulating leucocytes and/or erythrocytes.

Therefore, the purpose of this study was to determine the normal intrinsic CRT-mediated creatine transport activity as a standard value for comparison, based on absolute CRT protein quantification, and to measure creatine transport in erythrocyte- and leucocyte-enriched fractions isolated from the circulating blood of rats.

**MATERIALS AND METHODS**

**Animals and Reagents** Male Sprague Dawley (SD) rats at 8 weeks of age were purchased from Japan SLC (Hamamatsu, Japan) and maintained on a 12-h light/dark cycle in a temperature-controlled environment with free access to food and water. All experiments were approved by the Institutional Animal Care and Use Committee in Tohoku University, and were performed in accordance with the guidelines of Tohoku University. All reagents were commercial products of analytical grade.

| [\textsuperscript{14}C]Creatine Uptake by HEK293 Cell Line Stably Overexpressing Rat CRT (CRT/HEK293) | CRT/HEK293 cells, which we established previously,\textsuperscript{14} and mock-transfected HEK293 cells were collected on 10 cm dishes (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) at 37°C in an atmosphere of 5% CO\textsubscript{2} in air in Dulbecco’s modified Eagle’s medium, high glucose, supplemented with 100000 units/L penicillin G, 100 mg/mL streptomycin sulfate, 10% fetal bovine serum (Moregate, Bulimba, Australia), and 200 μg/mL hygromycin B. The cells were seeded on poly-D-lysine-coated 24-well plates (Asahi Techno Glass Corporation, Tokyo, Japan) at a density of 0.5 × 10\textsuperscript{5} cells/well and cultured for 72 h at 37°C. The uptake of [\textsuperscript{14}C]creatine ([4-\textsuperscript{14}C]creatine, 2.04 GBq/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO, U.S.A.) by CRT/HEK293 cells was evaluated according to the method reported previously.\textsuperscript{14} Cell-associated protein was determined using a DC protein assay kit (Bio-rad, Richmond, CA, U.S.A.) with bovine serum albumin as a standard. The uptake activity was expressed as the cell-to-medium ratio (μL/mg protein) as reported previously.\textsuperscript{14} The initial uptake rate of [\textsuperscript{14}C]creatine in CRT/HEK293 cells and mock/HEK293 cells was calculated from the slope of the time-dependent uptake in the linear region. The fitting was carried out by iterative non-linear least-squares regression analysis with the program MULTI.\textsuperscript{15}

**Isolation of Erythrocyte and Leucocyte Fractions from Circulating Blood Cells of Rats** Whole blood was collected in the presence of 1000 U/mL heparin from 8-week-old SD rats under deep isoflurane anesthesia. Erythrocyte and leucocyte fractions were separated by density gradient centrifugation with Lympholyte-Mammal (Cedarlane Laboratories, Burlington, Canada), according to the manufacturer’s instructions. The leucocyte fraction was collected from the middle layer and washed three times with phosphate-buffered saline by centrifugation at 800 × g for 15 min at 4°C. The erythrocyte fraction was collected from the underlayer and washed three times with phosphate-buffered saline by centrifugation at 800 × g for 15 min at 4°C.

**Preparation of Crude Membrane Fraction of Rat Leukocytes and Plasma Membrane Fraction of Rat Erythrocytes and CRT/HEK293 Cells** Crude membrane fraction of rat leukocytes was prepared as previously described.\textsuperscript{16} Plasma membrane fractions of rat erythrocytes and CRT/HEK293 cells were prepared as previously described.\textsuperscript{17} These fractions were stored at −80°C.

**Preparation of Brush-Border Membrane Fraction of Rat Kidney** Under deep anesthesia with intramuscular ketamine (125 mg/kg) and xylazine (1.22 mg/kg), the kidney was transcardially perfused with phosphate-buffered saline and excised. The renal cortex was dissected and homogenized with a Potter-Elvehjem glass homogenizer in an isolation buffer (300 mM D-mannitol, 5 mM ethylene glycol bis(2-aminoethyl ether)-N,N',N''-tetraacetic acid (EGTA), 1.2 mM KHPO\textsubscript{4}, 12 mM Tris–HCl, pH 7.4). After adding 12 mM MgCl\textsubscript{2}, the homogenate was left on ice for 15 min and then centrifuged at 3940 × g for 15 min at 4°C. The resultant supernatant was centrifuged at 49800 × g for 30 min at 4°C, and then the pellet was suspended in buffer A (150 mM D-mannitol, 6 mM Tris–HCl, 2.5 mM EGTA, pH 7.4) and centrifuged at 49800 × g for 30 min at 4°C. The resultant pellet was suspended in buffer B (500 mM mannitol, 100 mM K-glucanate, and 50 mM N-(2-hydroxyethyl)piperezaine-N''-2-ethanesulfonic acid (HEPES)–Tris) to obtain the brush-border membrane fraction. This fraction was aliquoted and stored at −80°C.

**Quantitative Targeted Absolute Proteomics (qTAP) Analysis** The absolute protein levels of CRT, CD29/Integrin\textsubscript{β1}, and Na\textsuperscript{+}/K\textsuperscript{+} ATPase was quantified by LC-MS/MS with multiplexed selected/multiple reaction monitoring (SRM/HRM) as described previously.\textsuperscript{18} Sample preparation with trypsin and lysylendopeptidase, LC separation of peptides, and detection/quantification of the target peptides were performed as we reported previously.\textsuperscript{17} The LC-MS/MS system consisted of an expert microLC 200 (Eksigent Technologies, Dublin, CA, U.S.A.) with a micro-LC column (HALOTM C18 2.7 μm, 90A 0.5 × 100 mm, Eksigent Technologies, Dublin, CA, U.S.A.) and a QTRAP 5500 (AB SCIEX, Framingham, MA, U.S.A.). The peptides were separated and eluted from the column at a flow rate of 10 μL/min with a linear gradient as follows (mobile phase A:B): 99:1 for 5 min after injection for sample loading, 0:100 at 35 min and up to 38.9 min, 99:1 at 39 min and up to 55 min. The protein expression levels were calculated as the amounts of trypsin-generated specific target peptides whose sequences were selected based on the in silico selection criteria reported previously.\textsuperscript{15} The SRM/HRM transitions used for the quantification of each peptide are shown in supplementary Table S1.

| [\textsuperscript{14}C]Creatine Uptake by Isolated Erythrocytes and Leukocytes | Erythrocyte and leucocyte fractions were preincubated with 200 μL ECF (extracellular fluid) buffer (122 mM NaCl, 25 mM NaHCO\textsubscript{3}, 3 mM KCl, 0.4 mM KHPO\textsubscript{4}, 10 mM D-glucose, 1.4 mM CaCl\textsubscript{2}, 1.2 mM MgSO\textsubscript{4}, 10 mM HEPES, pH 7.4, 300 ± 10 μM) in 1.5 mL tubes for 5 min at 37°C before the uptake experiment. Uptake was initiated by addition of the ECF buffer with 200 μL of the same solution containing [\textsuperscript{14}C]creatine at a final concentration of 3.6 μM, and terminated by addition of ice-cold ECF buffer after incubation for a designated time (30 min) at 4°C. The incubation time with [\textsuperscript{14}C]creatine was set to be 30 min based on a previous report\textsuperscript{10} showing that [\textsuperscript{14}C]creatine uptake by human erythrocytes continued to increase during 0 to 60 min. Erythrocyte fractions were then washed three times with ice-cold ECF buffer
by centrifugation (1000 × g for 3 min, 4°C). Leukocyte fractions were also washed three times with ice-cold ECF buffer by centrifugation (1000 × g for 5 min, 4°C). Leukocytes were then solubilized with 5 M NaOH solution and neutralized with 5 M HCl. Erythrocytes were homogenized with 800 µL MilliQ water, deproteinized with 800 µL acetone at 25°C for 1 h, and centrifuged (21000 × g, 15 min, 4°C). Radioactivity was determined with a liquid scintillation counter (LS-6500, Beckman Coulter, Fullerton, CA, U.S.A.) using Hionic-Fluor (PerkinElmer, Inc., Waltham, MA, U.S.A.). For the inhibition study, the uptake of [14C] creatine (3.6 µM) was measured in the presence or absence of unlabeled creatine (5 mM) and NaCl-free ECF buffer. Na+-free buffer was prepared by equimolar replacement of NaCl and NaHCO3 with lithium chloride and potassium bicarbonate, respectively. The uptake activity was expressed as the cell-to-medium ratio (µL/mg protein), as reported previously.14)

RT-PCR Analysis Total RNA was isolated from rat erythrocytes, leukocytes and kidney using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s protocol. The RNA was reverse-transcribed using oligo(dT) primer and ReverTra Ace (Toyobo, Osaka, Japan). PCR was conducted using DNA polymerase (TaKaRa, Shiga, Japan) with the following thermal cycle program: 40 cycles of 98°C for 10s, 52°C for 5s, 72°C for 15s, and a final elongation of 72°C for 6 min. The sequences of the specific primers were as follows: sense 5’-GAAATGTGTGCTGTCCTTTCACACGCA-3′ and antisense 5’-GTCACAATGAGCAGCTCCACAAGCGA-3′ for rat CRT (GenBank Accession number: X66494), sense 5’-ATGGCCTGCTGCTATCGTGTG-3′ and antisense 5’-AAGCCAAGGATGGAACACG-3′ for rat MCT12 (GenBank Accession number: NM_001191637). The sequences of the specific primers for β-actin were reported previously.20) The PCR products were separated by electrophoresis on agarose gel. The sequence of the product was determined by a DNA sequencer (ImageQuant LAS4000; GE Healthcare Bioscience, Piscataway, NJ, U.S.A.).

Statistical Analysis All data except for kinetic parameters are expressed as the mean ± standard error of the mean (S.E.M.). The mean ± standard deviation (S.D.) was used to present the kinetic parameters. The significance of differences between two group means was determined by an unpaired, two-tailed Student’s t-test. The statistical significance of differences among means of more than two groups was assessed by one-way ANOVA followed by the modified Fisher’s least-squares difference method.

RESULTS

Determination of Intrinsic Rat CRT-Mediated Creatine Transport Activity in Rat CRT-Overexpressing HEK293 Cells (CRT/HEK293) As shown in Fig. 1, [14C] creatine uptake by CRT/HEK293 cells exhibited a time-dependent increase with the initial transport rate of 107 ± 2 µL/(min·mg protein), which was obtained from the slope between 0.2 min and 10 min. The initial creatine transport rate in mock-transfected HEK293 cells was calculated to be 10.7 ± 0.4 µL/(min·mg protein) (Fig. 1). Thus, the initial transport rate specifically mediated by rat CRT was calculated to be 96.3 µL/(min·mg protein). The absolute rat CRT protein amount in CRT/HEK293 cells was determined to be 228 ± 8 fmol/µg plasma membrane protein, while that in rat kidney, used as a positive control, was 2.96 ± 0.12 fmol/µg brush-border membrane protein (Table 1). The yield of plasma membrane proteins from 1 mg whole cell lysates was 1.78 µg and then the absolute CRT protein expression level in whole-cell lysate of CRT/HEK293 cells was calculated to be 406 fmol/mg protein (228 fmol/µg plasma membrane protein × 1.78 µg/µg whole-cell lysate). We then calculated the intrinsic creatine transport rate per CRT protein as 0.237 µL/(min·fmol CRT); this was obtained by dividing the initial creatine transport rate specifically mediated by CRT [96.3 µL/(min·mg protein)] by the absolute CRT protein expression level in whole-cell lysate of CRT/HEK293 cells (406 fmol/mg protein).

Table 1. Absolute Protein Expression Levels in Brush-Border Membrane Fraction of Rat Kidney, and Plasma/Crude Membrane Fractions of Rat Erythrocyte and Leukocyte

| Protein name | Kidney_Brush-border membrane fraction | Erythrocyte_Plasma membrane fraction | Leukocyte_Crude membrane fraction |
|--------------|--------------------------------------|-------------------------------------|----------------------------------|
| CRT          | 2.96 ± 0.12                          | U.L.Q. (<0.0193)                    | U.L.Q. (<0.0186)                 |
| CD29/Integrinβ1 | N.D.                                | U.L.Q. (<0.280)                     | 17.7 ± 2.5                      |
| Na+/K+ ATPase | 7.63 ± 0.46                          |                                    | 6.04 ± 0.43                     |

The protein expression levels were calculated as the average of quantitative values obtained from three or four selected/multiple reaction monitoring transitions in one analysis. Each protein expression level represents the mean ± S.E.M. (n = 3–4). Molecules for which the protein expression levels were under the limit of quantification are shown as U.L.Q. (< the amount of quantification limit). N.D.; not determined.
Isolation of Rat Erythrocyte and Leukocyte Fractions
Erythrocyte- and leukocyte-enriched fractions were isolated from circulating blood cells, and the plasma membrane fraction of erythrocytes and the crude membrane fraction of leukocytes were prepared. Proper isolation of erythrocyte- and leukocyte-enriched fractions was validated by examination of microscopic images (Supplementary Fig. S1). Table 1 summarizes the protein expression levels of CRT, CD29/integrinβ1, a leukocyte marker, and Na⁺/K⁺ ATPase, a plasma membrane marker, in isolated erythrocyte and leukocyte fractions. In the plasma membrane fraction of erythrocytes, the protein level of CD29/integrinβ1 was below the detection limit, whereas the level of Na⁺/K⁺ ATPase was 7.63 ± 0.46 fmol/µg protein. In the crude membrane fraction of leukocytes, the expression levels of CD29/integrinβ1 and Na⁺/K⁺ ATPase were 17.7 ± 2.5 fmol/µg protein, and 6.04 ± 0.43 fmol/µg protein, respectively. These results confirmed that erythrocytes and leukocytes were indeed enriched in the respective fractions. The levels of CRT protein were under quantification limit in isolated erythrocyte and leukocyte fractions.

[^14C]Creatine Uptake by Isolated Rat Erythrocytes and Leukocytes
The cell-to-medium ratio of[^14C] creatine uptake by leukocytes (Fig. 2A) was 5-fold greater than that by erythrocytes (Fig. 2B).[^14C]Creatine uptake by rat leukocytes was significantly inhibited by 19 and 31% in the presence of unlabeled creatine and under extracellular Na⁺-free conditions, respectively (Fig. 2A). In contrast, the uptake of[^14C] creatine by rat erythrocytes was not inhibited in the presence of unlabeled creatine and under extracellular Na⁺-free conditions, respectively (Fig. 2B). These results indicated that leukocytes contained an Na⁺-dependent carrier-mediated creatine uptake system.

Expression of Creatine Transporters CRT and MCT12 mRNAs in Rat Erythrocytes and Leukocytes
As shown in Fig. 3, RT-PCR gave single amplified products at the expected sizes of 353 bp for CRT and 298 bp for MCT12 in rat leukocytes and in kidney (used as a positive control), but not in rat erythrocytes. The nucleotide sequences of the products were identical with those of rat CRT/Slc6a8 (GenBank accession number X66494) and rat MCT12/Slc16a12 (GenBank accession number NM_001191637), respectively. A single band corresponding to β-actin was detected in all samples. These results indicated that CRT and MCT12 mRNAs are expressed in rat leukocytes.

**DISCUSSION**

The present study is the first to determine the intrinsic CRT-mediated creatine transport activity based on absolute CRT protein quantification, and to demonstrate leukocytespecific[^14C] creatine uptake in the blood of rats. Analyses of qTAP and[^14C] creatine uptake by CRT/HEK293 cells indicated that the normal intrinsic creatine transport activity per CRT protein is 0.237 µL/(min·fmol CRT) in the presence of the physiological inward Na⁺ gradient. Moreover, since the extracellular concentration of[^14C] creatine was 5 µM in the present uptake experiment, the intrinsic creatine transport amount per CRT protein can be expressed as 1.19 pmol/(min·fmol CRT) [0.237 µL/(min·fmol CRT) × 5 µM]. Using Avogadro’s constant (6.02 × 10²³ molecules/mol), the intrinsic creatine transport activity is estimated to be 1190 creatine/(min·CRT molecule), or 19.8 creatine/(s·CRT molecule) in the presence of 5 µM extracellular[^14C] creatine. Considering that the reported Kₘ value of rat CRT and the extracellular[^14C] creatine concentration in the present study are 46.2 and 5 µM, respectively, the occupied ration of CRT by the extracellular[^14C] creatine is estimated to be approximately 10% of the maximal velocity (Vₘₚₓ) [Vₘₚₓ × 5 µM/(46.2 + 5 µM)]. In this regard, the rat CRT-mediated creatine transport amount at a maximum velocity can be estimated to be 183 creatine/(s·CRT molecule). Therefore, the present value is in line with reported
values, which are in the range of 1 × 10^2 to 1 × 10^4 molecules/s per transporter molecule.\(^{22}\)

\[^{14}C\]Creatine uptake by leukocyte-enriched fraction was significantly reduced by 31% under extracellular Na\(^+\)-depleted conditions, and was inhibited by 19% in the presence of 5 mM unlabeled creatine, which might 99% saturate CRT-mediated creatine transport, based on the \(K_m\) value of 46.2 \(\mu\)M.\(^{23}\)

Moreover, the RT-PCR results indicate that Na\(^+\)-dependent and Na\(^+\)-independent creatine transporters CRT and MCT12, respectively, are both expressed at the mRNA level in the leukocyte fraction. These results suggest that CRT contributes at least in part to Na\(^+\)-dependent carrier-mediated creatine uptake by isolated leukocytes.

It should be noted that the level of CRT protein in isolated leukocytes is under the quantification limit (0.02 fmol/mg protein) by qTAP analysis, whereas the CRT protein level in the brush-border membranes isolated from rat kidney is 2.96 fmol/mg membrane protein (Table 1). This suggests that the uptake activity of creatine in isolated leukocytes is lower than that in the kidney brush-border membrane, which is the site of creatine re-absorption. The low protein expression of CRT in isolated leukocytes is consistent with the following calculations based on the intrinsic creatine transport activity of CRT [0.237 \(\mu\)L/(min·fmol CRT protein)]. \[^{14}C\]Creatine uptake clearance by rat leukocytes is calculated to be 0.124 \(\mu\)L/min·mg whole cell lysate protein, assuming linear uptake up to 30 min, and so the CRT expression level can be estimated to be 0.523 fmol/mg whole-cell lysate protein in leukocyte fraction by dividing the \[^{14}C\]creatine leukocyte uptake clearance by the intrinsic creatine transport activity of rat CRT. Here we assume 16-fold concentrative purification of crude membrane proteins from the whole-cell lysate based on our finding that 17 \(\mu\)g of crude membrane proteins was obtained from 270 \(\mu\)g whole cell proteins in the rat leukocyte fraction. Based on this assumption, the CRT protein level would be 0.00837 fmol/mg crude membrane protein in rat leukocytes, which is 2-fold lower than the present protein quantification limit.

We also found that \[^{14}C\]creatine uptake by erythrocytes is not inhibited in the presence of 5 mM unlabeled creatine. This suggests that erythrocytes do not possess a carrier-mediated creatine transport system. On the other hand, however, previous studies have found that human erythrocytes exhibit partial concentration-dependence and inward Na\(^+\)-gradient-dependence of creatine uptake,\(^{20}\) suggesting that human erythrocytes contain an Na\(^+\)-dependent concentrative creatine uptake system. This idea is supported by the fact that the ratio of physiological creatine contents of erythrocytes and plasma is 11 in human erythrocytes and 1 in rat erythrocytes.\(^{24}\) Therefore, there seems to be a species difference in the creatine uptake ability of erythrocytes between human and rats.

In conclusion, we have determined the intrinsic CRT-mediated creatine transport activity as a standard value, and have shown that leukocytes possess CRT-mediated creatine uptake capability. This information will be helpful in the clinical context for evaluating residual CRT transport activity in patients with CRT-D.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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