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OXIDATIVE STRESSES INDUCED THE CYSTINE TRANSPORT ACTIVITY IN HUMAN ERYTHROCYTES

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SUMMARY: Cystine was transported into human erythrocytes in the presence of tertiary-butyl hydroperoxide (t-BH) or 1-chloro-2,4-dinitrobenzene (CDNB). The transport rate of cystine was dependent on the extracellular concentration of t-BH or CDNB, and on the incubation time. According to Dowex-1 column chromatography, the transported cystine was incorporated into fractions of glutathione disulfide (GSSG) and glutathione-S (GSH-S) conjugate. The transport of cystine was competitively inhibited by DL-homocystine and alanine. The inhibition rates by DL-homocystine and alanine were 75% and 68%, with similar Ki values of 0.7mM and 0.6mM, respectively. It is suggested that cystine transport is induced for glutathione synthesis when human erythrocytes are exposed to oxidative stresses. This transport system of cystine may serve as an emergency function in human erythrocytes.

Matured human erythrocytes have no nuclei, nor do they synthesize any protein. Thus, amino acid transport in erythrocytes is mainly for the synthesis of glutathione (GSH). Under oxidative stresses, GSH is rapidly oxidized to glutathione disulfide (GSSG) or conjugates of GSH with electrophilic agents are formed, and they are actively transported from the cells as a function of intracellular concentrations of GSSG or GSH-S conjugate (1-5). Glutamate, cysteine and glycine are transported into erythrocytes and utilized for the synthesis of GSH to restore the decreased GSH concentration. In contrast, it is reported that cystine is not transported into human erythrocytes under physiological conditions (6).

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Abbreviations: GSH, reduced form of glutathione; GSSG, glutathione disulfide; t-BH, tertiary-butyl hydroperoxide; CDNB, 1-chloro-2,4-dinitrobenzene

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We report here that the transport of cystine is induced by electrophilic agents such as t-BH and CDNB in human erythrocytes, and also describe the kinetic properties of the cystine transport system.

MATERIALS AND METHODS

Preparation of erythrocytes

Whole blood from normal subjects was collected in heparin, and leukocytes and platelets were removed by passage through a column of α-cellulose and microcrystalline cellulose (7). Erythrocytes were washed three times with saline and suspended in TES buffer, at 20% hematocrit, consisting of 1% bovine serum albumin, 8mM glucose, 62mM NaCl, 40mM NaH_2PO_4/Na_2HPO_4 and 35mM Na-TES (N-Tris-(hydroxymethyl) methyl-2-aminomethane sulfonic acid), pH 7.4 (8).

Cystine transport study

Erythrocytes in TES buffer were incubated with 10μM L-[^{14}C]cystine (306mCi/mmol) and 0.0035-0.0105% t-BH or 0.5-2mM CDNB for 0-2h at 37℃. CDNB was dissolved in ethanol to a final concentration of 4.75%. After incubation, aliquots of the incubation mixture were drawn and washed with 20 vol. of saline three times to remove most of the residual [^{14}C]cystine. Four vol. of ice-cold water were added to the cells and proteins were precipitated by the addition of 1/2 vol. of 6% perchloric acid. After centrifugation for 10min at 3000rpm, transported cystine was determined in the supernatant using a liquid scintillation counter. Hemolysis observed during the incubation periods was less than 0.1%.

Dowex-1 column chromatography

Erythrocytes incubated with electrophiles were treated with the same volume of 10% trichloracetic acid (TCA) to precipitate proteins. TCA in the supernatant was removed by mixing with diethyl ether; then the supernatant was retreated with 0.3% H_2O_2 and applied to a column of Dowex-1 (1x10cm). After washing with 100ml water, the column was eluted with a 60ml linear gradient of 0-4N formic acid (8). The radioactivity of each fraction was estimated and the concentration of GSSG was assayed as described by Owens and Belcher (9).

Reconstitution of erythrocyte ghosts

Erythrocyte ghosts were prepared by a modification of the method of Prchal et al. (10). Erythrocytes were mixed at 0℃ with approximately 3 vol. of ice-cold water, containing 1.2-4.6mM GSSG. Following 5min at 0℃, sufficient sodium chloride was added to restore isotonicity. The mixture was incubated at 37℃ for 50min to permit resealing of the ghosts. Resealed erythrocytes were washed twice with saline and resuspended in TES buffer and the transport of cytine was examined.

RESULTS

Cystine transport Cystine was transported into erythrocytes when the cells were incubated with t-BH. The transport rate of cystine was dependent on the extracellular concentration of t-BH and the transport was linear over the initial 2h of incubation (Fig. 1). Cystine was also transported into erythrocytes when the cells were treated with CDNB. The transport rate of cystine was dependent on the extracellular concentration of CDNB and on the incubation time (Fig. 2). The cystine transport increased linearly up to 2h.

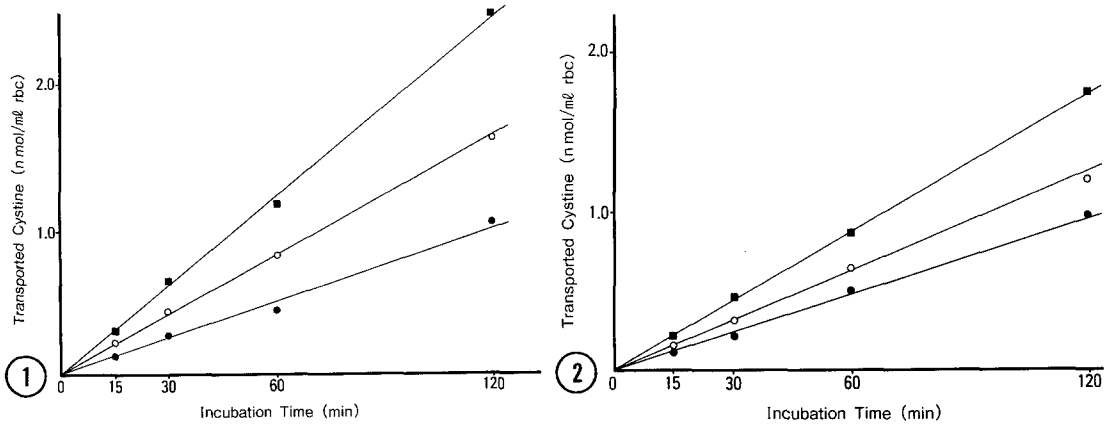

Fig. 1. Effect of t-BH on the transport of cystine in human erythrocytes. Erythrocytes were incubated with 10μM L-[^{14}C]cystine in the presence of 0.0035% (●), 0.007% (○) or 0.0105% (■) t-BH at the indicated times. Transported cystine was estimated as described in MATERIALS AND METHODS. Data represent means of six determinations in three separate experiments.

Fig. 2. Effect of CDNB on the transport of cystine in human erythrocytes. Erythrocytes were incubated with 10μM L-[^{14}C]cystine in the presence of 0.5mM (●), 1mM (○) or 2mM (■) CDNB at the indicated times. Transported cystine was estimated as described in MATERIALS AND METHODS. Data are shown as means of six determinations in three separate experiments.

The elution profiles of Dowex-1 column chromatography of erythrocytes treated with t-BH or CDNB are shown in Fig. 3. When the cells were treated with t-BH, a single peak fraction of radioactivity that was identical to that of GSSG was observed (A). Upon incubation with CDNB, two peak fractions, which corresponded to GSSG and GSH-S conjugate were observed (B).

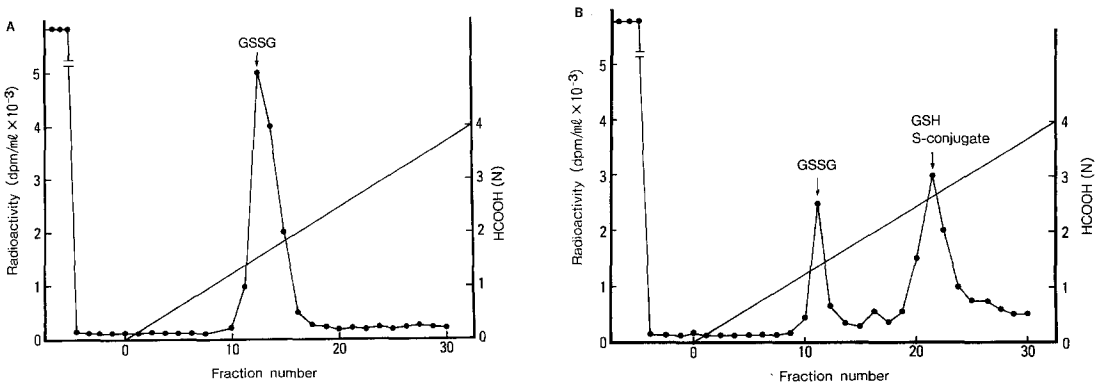

Fig. 3. Dowex-1 column chromatography elution profile. Arrows indicate the peak fractions of GSSG and GSH-S conjugates. Erythrocytes were incubated with 10μM L-[^{14}C]cystine in the presence of 0.0035% t-BH (A) or 2mM CDNB (B) for 1hr at 37℃. Then the cells were hemolysed, treated with TCA and applied to a column of Dowex-1 as described in MATERIALS AND METHODS.

Table 1. Effect of intracellular concentration of GSSG on the transport of L-cystine

Intracellular Concentration of GSSG (mM)	Transported Cystine (nmol/h/ml erythrocytes)
1.2	320
2.3	400
4.6	550

Reconstituted erythrocyte ghosts loaded with various concentrations of GSSG were incubated with 10μM L-[^{14}C]cystine for 1h at 37℃. After being washed, the cells were treated with perchloric acid and centrifuged for 10min at 3000rpm. Radioactivity in the supernatant was determined using a liquid scintillation counter. Values are means of triplicate estimates.

To determine the effect of the intracellular concentration of GSSG on the transport of cystine, we used reconstituted erythrocyte ghosts loaded with GSSG. The transport rate of cystine was dependent on the intracellular concentrations of GSSG (Table 1). In contrast, the transport of cysteine into the reconstituted cells did not increase under the same conditions (data not shown).

Effects of Na^+ and NEM In order to learn whether the transport of cystine is mediated by a Na^+-dependent system or not, Na^+ was replaced by 0.154M choline chloride. The induction of cystine transport by CDNB was inhibited by 78% in the absence of Na^+. The transport of cystine was also inhibited by 59% in the presence of 1mM NEM (Table 2).

Kinetic properties of cystine transport The results in Table 3 show the inhibitory effects of various amino acids on the cystine transport in the presence of 2mM CDNB. Neutral amino acids such as DL-homocystine and L-alanine potently inhibited the transport of cystine by 75% and 68%, respectively. L-serine, L-threonine and L-asparagine also inhibited the transport of cystine.

Table 2. Effect of N-ethylmaleimide (NEM) and Na^+ on the L-cystine transport induced by CDNB

Condition	% Inhibition
0.154M Na^+	0
0.154M Na^+, 1mM NEM	59
0.154M choline chloride	78

Erythrocytes in TES buffer were incubated with 10μM L-[^{14}C]cystine and 2mM CDNB with or without 1mM NEM for 1h at 37℃. After being washed, the cells were treated with perchloric acid. Transported cystine in the supernatant was determined using a liquid scintillation counter. Incubation in which Na^+ was replaced by choline was also performed. Values are means of triplicate estimates.

Table 3. Effect of various amino acids on the transport of L-cystine into human erythrocytes

Amino Acid (2.5 mM)	Transport of L-Cystine (% inhibition)
DL-Homocystine	75
L-Alanine	68
L-Serine	67
L-Threonine	66
L-Leucine	60
L-Valine	56
L-Proline	47
L-Phenylalanine	44
Glycine	28
L-Asparagine	65
DL-α-aminoadipate	58
L-Glutamine	51
DL-α-aminopimelate	35
L-Aspartate	32
D-Glutamate	26
L-Glutamate	13
L-Lysine	32
L-Arginine	31

Erythrocytes were incubated with 10μM L-[^{14}C]cystine, 2mM CDNB and various amino acids for 1h at 37℃. After being washed, the cells were treated with perchloric acid. Radioactivity in the supernatant was counted. Each value represents means of three to four determinations.

Fig. 4 shows double-reciprocal plots of cystine transport in the presence of DL-homocystine or L-alanine. An apparent Km value for cystine was 0.15mM. Both amino acids inhibited cystine transport competitively with similar Ki values of 0.7mM and 0.6mM, respectively.

DISCUSSION

Glutamate and cysteine are substrates for γ-glutamyl-cysteine synthetase which is rate-limiting for GSH synthesis (11). In the enzyme reaction, cysteine is considered to be a rate-limiting substrate *in vivo* because the Km for cysteine seems to be higher than the intracellular concentration of cysteine (12,13). Thus, the transport of cysteine is the most important step to synthesize GSH. In normal erythrocytes, cystine is not transported into the cells instead of cysteine (6). In order to determine if the transport of cystine is induced under oxidative stresses, we incubated erythrocytes with electrophilic agents such as t-BH or CDNB. Cystine tranport was observed and the transported cystine was involved in the glutathione fraction (Fig. 1-3). This cystine transport in erythrocytes was not thought to be brought about by the destruction of the surface membrane

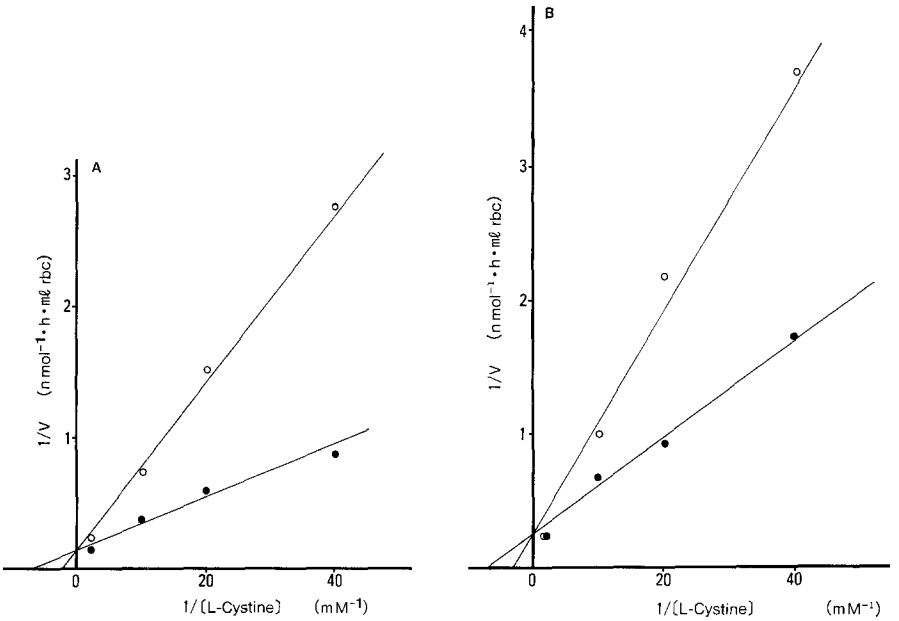

Fig. 4. Double reciprocal plots of the transport of cystine. Erythrocytes were incubated with L-[^{14}C]cystine for 1h at 37℃ in the presence (○) or absence (●) of 1mM amino acids (A; DL-homocystine B; L-alanine). Then the transport rates of cystine at various concentrations were estimated. Data are means of duplicate determinations from one experiment typical of three similar experiments. Nanomoles of cystine transported per h per ml erythrocytes are abbreviated as V.

since not only was no hemolysis observed during incubation periods, but an intracellular high concentration of GSSG also induced cystine transport in reconstituted erythrocyte ghosts (Table 1).

Induction of cystine transport activity was recently reported by Bannai et al. using human fibroblasts and isolated rat hepatocytes (13-15). The transport of cystine induced by some electrophilic agents was mediated by a Na^+-independent system called x_c^-, and inhibited by extracellular glutamate and DL-homocysteate. Young et al. (16) reported that normal human erythrocytes have three discrete amino acid transport systems, designated L, Ly^+ and ASC. Leucine is a good substrate for the Na^+-independent L system and the L system shows low sensitivity to N-ethylmaleimide (17). The Ly^+ system is specific for dibasic amino acids, including lysine and arginine. The ASC system is Na^+-dependent and selective for neutral amino acids of intermediate size such as alanine. Cysteine is transported mainly via this ASC system (18). According to Christensen (18), the transport of amino acids is, so to speak, a transport of ions. The ASC system is for dipolar

ions but cystine does not exist as a dipolar ion in normal conditions; therefore, the transport of cystine in human erythrocytes may not be mediated by the ASC system. Our results show that the transport of cystine in human erythrocytes is Na^+-dependent and inhibited by NEM (Table 2), and that the transport is competitively inhibited by L-alanine and DL-homocystine (Fig. 4). From these data, it is suggested that the characteristics of the cystine transport system induced by electrophilic agents are similar to those of the ASC system. Further experiments are required to elucidate the system of cystine transport in human erythrocytes.

In conclusion, cystine is transported into human erythrocytes under oxidative stresses and utilized for glutathione synthesis. This transport system of cystine may serve as an emergency function in human erythrocytes.

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