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L-Cysteine influx and efflux: A possible role for red blood cells in regulation of redox status of the plasma

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Abstract

The objective of this study was to investigate if erythrocytes play a role in the maintenance of redox homeostasis of the plasma. Thus, we studied L-cysteine efflux and influx *in vitro* in human erythrocytes. In the present study, we exposed the erythrocytes to different concentrations of L-cysteine and then measured the intracellular free –SH concentrations. Erythrocytes treated in the same manner were later utilized for the cysteine efflux studies. The effect of temperature on the influx and the efflux processes were also evaluated. Change in the free –SH content of the buffer was evaluated as a measure for the presence of an efflux process. The effects of free –SH depletion on L-cysteine transport is also investigated. We also determined the rate of L-cysteine efflux in the presence and absence of buthionine sulfoximine (BSO) in erythrocytes that are pretreated with 1-chloro-2,4-dinitro benzene, a glutathione (GSH) depletory. Our L-cysteine influx studies demonstrated that erythrocytes can respond to increases in L-cysteine concentration in the extracellular media and influx L-cysteine in a concentration-dependent manner. Free –SH concentrations in erythrocytes treated with 1 mM L-cysteine reached to 1.64 ± 0.06 mM in 1 h whereas this concentration reached to 4.30 ± 0.01 mM in 10 mM L-cysteine treated erythrocytes. The L-cysteine efflux is also determined to be time-and concentration-dependent. Erythrocytes that are pretreated with higher L-cysteine concentrations displayed a higher efflux process. Outside concentration of free –SH in 1 mM L-cysteine pretreated erythrocytes reached to 0.200 ± 0.005 mM in 1 h whereas this concentration reached to 1.014 ± 0.002 with 10 mM L-cysteine pretreated erythrocytes. Our results also indicate that the rate of inward and outward transport of L-cysteine is affected by the oxidative status of the erythrocytes. When GSH is depleted and GSH synthesis is blocked, the L-cysteine uptake and the efflux processes are significantly decreased. Depending on our results, it could be concluded that erythrocytes play a role in the regulation of the plasma redox status and intracellular level of GSH determines the rate of the L-cysteine efflux.

Keywords: *Erythrocytes, cysteine efflux, redox homeostasis, plasma*

Introduction

L-Cysteine is the only amino acid that has an important functional free –SH group. This amino acid is required for erythrocytes mainly in glutathione (GSH) synthesis [1]. Erythrocytes cannot synthesize protein since they do not have a functional protein synthesizing system. Thus, L-cysteine in erythrocytes is not incorporated into proteins. However, this does not make L-cysteine less significant for erythrocytes. On the contrary, it is a strictly required amino acid for the erythrocyte integrity. L-Cysteine is mainly utilized in GSH synthesis in erythrocytes. Except being incorporated into the soluble antioxidant, GSH, L-cysteine itself plays a role in the maintenance of a proper intracellular or extracellular redox status. GSH is a tripeptide, which is synthesized from three amino acids, glutamic acid, cysteine and glycine. Although three amino acids are required for GSH synthesis, the rate of GSH synthesis is determined only by L-cysteine availability [1]. The functional –SH group which gives GSH its critical role is provided by the amino acid L-cysteine. GSH functions as a soluble antioxidant and protects

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the cells against free radicals and lipid peroxidation [2,3]. It is also involved in detoxification of several xenobiotics [3–5]. GSH reacts with toxic xenobiotics in a glutathione S-transferase catalyzed reaction and forms GSH conjugates that are then transported out from the cells in an ATP dependent manner [6–11]. Although GSH is actively synthesized in erythrocytes, the synthesized GSH is not effluxed from the erythrocytes [12]. Thus in contrast to liver, erythrocytes do not provide the plasma with GSH [13].

In this study, we investigated if erythrocytes provide the blood plasma, therefore, the other cells with L-cysteine which is the rate limiting amino acid in GSH synthesis. The study also questioned if erythrocytes function in regulation of L-cysteine concentrations in the blood plasma, by uptaking when its concentration is raised, and carrying and releasing it at distal tissues where its concentration is relatively low. Recent investigations point that L-cysteine may contribute to the redox homeostasis in the blood plasma and in the periplasm of some bacteria also. Thus L-cysteine availability in the blood plasma may influence the oxidized/reduced state of several other metabolites normally found to occur in the plasma. L-Cysteine influx into erythrocytes has been extensively studied. It has been shown that the L-cysteine transport into erythrocytes and other cells is mainly mediated by Na-dependent and Na-independent systems [14]. However, to the best of our knowledge, L-cysteine efflux from erythrocytes has not been studied.

Materials and methods

Materials

L-Cysteine, L-alanine, L-serine, buthionine sulfoximine (BSO), and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained form Sigma Chemical Co. (St Louis, Missouri, MO, USA). 5,5′-Dithiobis(-nitro benzoate) (DTNB) was obtained from Fluka BioChemica, (Switzerland). Blood was obtained from the blood bank of SSK Hospital, (Antakya, Turkey) as 300 ml units derived from people with no prerecorded medical conditions.

Preparation of erythrocytes

Plasma was separated by centrifugation at 2000 g for 5 min. The plasma and the buffy coat were then removed and discarded. The resulting erythrocyte pellet was washed twice with two volumes of phosphate buffered saline (PBS) (9 parts of 0.15 M NaCl and 1 part of 0.1 M potassium phosphate buffer, pH 7.4) and was further used in the experiments at 20% hematocrit [15]. PBS-glucose contained 8 mM of glucose in the PBS.

L-Cysteine influx studies

A total of 0.25 ml of washed erythrocytes were suspended in 1 ml of PBS-glucose containing 1.4 and 10 mM concentrations of L-cysteine and incubated for 10, 30, 60 and 120 min at 37°C in a water bath. At the end of incubation erythrocytes were removed, centrifuged and the supernatants were discarded. The free –SH concentrations in erythrocytes were then determined as described by Sedlak [16]. Briefly 100 μl of erythrocytes were lysed in 100 μl of 10% TCA prepared in sodium phosphate–EDTA buffer (0.01 M sodium phosphate/0.005 M EDTA). The erythrocyte lysates were then centrifuged at 12,000 g for 5 min. At the end of centrifugation 100 μl of the supernatant was mixed with 1.9 ml of Tris–EDTA buffer containing 0.6 μM/ml DTNB (262 mM Tris base, 13 mM EDTA, pH 8.9). Samples were allowed to stand for 5 min to develop color. The absorbances of the samples were then measured at 412 nm and the concentrations of free –SH were calculated by using the mM extinction coefficient of 13.6. Uptake rate where indicated was calculated from the following equation.

$$\text{Influx rate} = \text{Free} -\text{SH concentration obtained following treatment with L} - \text{cysteine} - \text{Control Free} -\text{SH}.$$

L-Cysteine efflux studies

A total of 0.25 ml of washed erythrocytes were resuspended in 1 ml of PBS-glucose in the presence of different concentrations of L-cysteine. Erythrocytes were incubated at 37°C in a water bath for 1 h to allow the uptake process. At the end of incubation erythrocytes were centrifuged and the supernatants were discarded. The erythrocytes were then resuspended in 1 ml of fresh PBS-glucose and incubated at 37°C for indicated times to allow the efflux process. At the end of incubation erythrocytes were centrifuged and the supernatants were transferred to fresh tubes. The free –SH concentrations in the supernatant was then measured as described above.

Statistical analysis

One-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparison tests were applied to process the data statistically. All tests were performed on triplicate samples. Results were expressed as mean ± SD $p < 0.05$ values were considered to be significant.

Results

Table I shows the result of the time and the concentration dependent L-cysteine influx by erythrocytes. As seen, erythrocytes accumulated L-cysteine

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Table I. Time course L-cysteine influx in erythrocytes: washed erythrocytes were resuspended in PBS-glucose containing the indicated concentrations of L-cysteine and incubated for 10, 30, 60, and 120 min. At the end of incubation free –SH concentration in the erythrocytes were determined. Results are the mean ± SD of three separate experiments.

	Free –SH concentrations in erythrocytes (mM)			
	10 min	30 min	60 min	120 min
Control	0.94 ± 0.04	0.91 ± 0.03	0.94 ± 0.03	0.90 ± 0.03
1 mM Cysteine	1.20 ± 0.02*	1.24 ± 0.01*	1.64 ± 0.06*	1.63 ± 0.06*
4 mM Cysteine	1.5 ± 0.03*	1.91 ± 0.02*	2.13 ± 0.04*	2.14 ± 0.03*
10 mM Cysteine	2.48 ± 0.09*	3.42 ± 0.02*	4.30 ± 0.01*	4.33 ± 0.04*

* Significantly different from the control. $p < 0.05$.

inside cell from the media in an efficient way. The influx process is increased when L-cysteine concentration in the media is increased. The influx process is continued to increase for a 1 h time period. Beyond this time, further incubation did not increase the L-cysteine influx. Table II displays the results of the L-cysteine efflux studies. Our results demonstrated that erythrocytes may transport the influxed L-cysteine back into the media from the cytoplasm when L-cysteine is absent. The rate of the L-cysteine efflux is dependent on time and on the intracellular levels of L-cysteine. Incubation of L-cysteine pretreated erythrocytes in an L-cysteine free media resulted in an efflux of the amino acid back into the media. The efflux rate increased for up to 1 h and further incubation did not result in a more efflux. Table III shows the effect of temperature changes on the L-cysteine influx process. Erythrocytes do not influx L-cysteine efficiently from the media when incubated at 4°C. When the temperature is increased, the transport process restarts and increases as the temperature is increased. Table IV shows the effect of temperature on the L-cysteine efflux process in erythrocytes. As seen this process is also temperature dependent and efflux process increases as the temperature is increased. Figures 1 and 2 show the effects of GSH depletion by CDNB on the L-cysteine influx and efflux processes, respectively. As seen in the figures, both the influx and the efflux process are significantly decreased when GSH is predepleted by CDNB. The decreases in the influx and efflux processes are time depended and become more efficient as the exposure time to CDNB is increased. In the last step, we investigated the effect of inhibition GSH synthesis by BSO following depletion by CDNB. Figure 3 shows the results of this experiment. It is shown that inhibition of GSH synthesis following its depletion further decreases the amount of L-cysteine effluxed from the erythrocytes.

Discussion

As shown in the table, erythrocytes take up L-cysteine from the media effectively in a time and concentration dependent manner. The influx process is continued in an increased manner as the L-cysteine concentration is increased. These results indicate that erythrocytes are equipped with highly efficient L-cysteine uptake systems. The L-cysteine uptake in erythrocytes is mainly mediated by Na- and ATP-dependent ASC system and Na-independent systems [17–19]. However, these described systems are all indicated to operate at lower L-cysteine concentrations than we used. Therefore, we suggest that erythrocytes may have carrier systems that can respond to the higher concentrations of the amino acid. The concentration of L-cysteine in the blood plasma is usually low [20]. Thus, it is difficult to ascribe a certain role for the presence of such efficient uptake systems in erythrocytes. However, it could be suggested that erythrocytes are equipped as such to function as a regulatory mechanism against L-cysteine increase in the blood plasma. High concentration of the amino acid in the plasma is known to be toxic [21]. This possibility also

Table II. Time course L-cysteine efflux from erythrocytes preincubated with L-cysteine: 0.25 ml of washed erythrocytes were first preincubated in 1 ml of PBS glucose containing 1.4 and 10 mM of L-cysteine for 1 h. At the end of incubation erythrocytes were centrifuged, the supernatants were discarded and the resulting erythrocyte pellets were washed. The washed erythrocytes were then resuspended in 1 ml of fresh PBS-glucose and incubated further for 10, 30, 60, and 120 min to allow the efflux process. At the end of incubation free –SH levels in the supernatants were measured. Results are the mean ± SD of three separate experiments.

	Free–SH concentrations in the media (mM)			
	10 min	30 min	60 min	120 min
Control	0.008 ± 0.0032	0.008 ± 0.007	0.006 ± 0.004	0.005 ± 0.004
1 mM Cysteine	0.096 ± 0.004*	0.146 ± 0.007*	0.200 ± 0.005*	0.200 ± 0.02*
4 mM Cysteine	0.245 ± 0.006*	0.385 ± 0.006*	0.501 ± 0.01*	0.504 ± 0.01*
10 mM Cysteine	0.673 ± 0.02*	0.849 ± 0.01*	1.014 ± 0.002*	1.019 ± 0.03*

* Significantly different from the control. $p < 0.05$.

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Table III. The effect of temperature on L-cysteine influx: washed erythrocytes were resuspended in PBS-glucose containing the indicated concentrations of L-cysteine and incubated for 1 h at different temperatures. At the end of incubation free −SH concentrations in erythrocytes were determined. Results are the mean ± SD of three separate experiments.

	Free−SH concentrations in erythrocytes (mM)			
	4°C	15°C	25°C	37°C
Control	1.210 ± 0.004	1.276 ± 0.004	1.254 ± 0.003	1.242 ± 0.02
1 mM Cysteine	1.278 ± 0.003*	1.621 ± 0.03*	1.650 ± 0.004*	1.640 ± 0.06*
4 mM Cysteine	1.254 ± 0.002*	1.892 ± 0.06*	1.919 ± 0.005*	2.130 ± 0.04*
10 mM Cysteine	1.253 ± 0.03*	2.484 ± 0.15*	2.985 ± 0.16*	4.300 ± 0.01*

* Significantly different from the control and from the values obtained at other temperatures. $p < 0.05$.

predicts that erythrocytes may function in the modulation of L-cysteine concentrations in the blood plasma. In the next step of our study, we tested this possibility and thus we studied L-cysteine efflux in erythrocytes pretreated with different concentrations of the amino acid. Our results showed that erythrocytes efflux L-cysteine in a concentration dependent manner. The efflux rate is found to be higher in erythrocytes treated with higher concentrations of L-cysteine. Therefore, we suggest that erythrocytes may influx L-cysteine when its concentration is elevated, especially as a result of food intake rich in L-cysteine or cysteine supplementation, and release it to distal tissues where its concentration is lower. In order to assess if the bidirectional L-cysteine transport in erythrocytes is a metabolically active and carrier mediated process we evaluated the influx and the efflux processes at different temperatures. The influx process is significantly inhibited at 4°C at all of the concentrations tested. Increasing of the temperature later recovered the influx process. Similar results were obtained with the efflux studies. Based on these results we conclude that both the influx and the efflux processes are metabolically active and carrier mediated. Additional evidence came from the GSH depletion experiments. Exposure of erythrocytes to CDNB resulted in a decrease in both the influx and the efflux processes in a time-dependent manner. CDNB-induced depletion of GSH in erythrocytes may result in a more oxidized membrane lipid and protein thiol. This in turn may induce structural or conformational changes in the membrane and/or in the amino acid carrier that finally results in a decrease in the bi-directional L-cysteine transport. Another possibility may be that the influxed L-cysteine is rapidly consumed in GSH predepleted and oxidatively stressed erythrocytes. However, later experiment strongly support our prediction that L-cysteine transport may require a properly reduced membrane lipid and protein thiol. Inhibition of GSH synthesis by BSO in CDNB treated erythrocytes resulted in an additional inhibition of L-cysteine efflux. This result also suggests that L-cysteine efflux is regulated by intracellular GSH concentrations. This regulation may involve reduction of some membrane proteins that are involved in the transport process. The efflux of amino acids is usually investigated in cells in small intestine. These cells uptake the ingested amino acids from the intestinal lumen and efflux the taken amino acids into the circulation. It has also been shown that *Escherichia coli* efflux cysteine from the cytoplasm to the periplasm by CydDC transporters to provide a balance in redox status [22]. Mutants in cydDC have been shown to cause a disturbance in redox status in the periplasm. In addition, lysosomes have been shown to have a system that is used to efflux the amino acids derived from enzymaticaly digested proteins [23]. This system called LYAAT-1 is an amino acid transporter involved in the efflux of L-proline, L-alanine, or glycine, from the organelle lumen to the cytosol. Similar or the same efflux systems may also function in erythrocytes. In these respects, the L-cysteine uptake and efflux processes in erythrocytes may posses different functions. One function may be

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Table IV. The effect of temperature on L-cysteine efflux: 0.25 ml of washed erythrocytes were first preincubated in 1 ml of PBS-glucose containing 1.4 and 10 mM of L-cysteine for 1 h at 37°C. At the end of incubation erythrocytes were centrifuged, the supernatants were discarded and the resulting erythrocyte pellets were washed. The washed erythrocytes were then resuspended in 1 ml of fresh PBS-glucose and incubated further for 60 min at different temperatures to allow the efflux process. At the end of incubation free−SH levels in the supernatants were measured. Results are the mean ± SD of three separate experiments.

	Free−SH concentrations in the media (mM)			
	4°C	15°C	25°C	37°C
Control	0.006 ± 0.001	0.009 ± 0.002	0.007 ± 0.003	0.009 ± 0004
1 mM Cysteine	0.025 ± 0.004*	0.096 ± 0.04*	0.187 ± 0.004*	0.200 ± 0.001*
4 mM Cysteine	0.111 ± 0.005*	0.313 ± 0.01*	0.388 ± 0.006*	0.501 ± 0.010*
10 mM Cysteine	0.143 ± 0.02*	0.595 ± 0.002*	0.873 ± 0.018*	1.014 ± 0.002*

* Significantly different from control and from the values obtained at other temperatures. $p < 0.05$.

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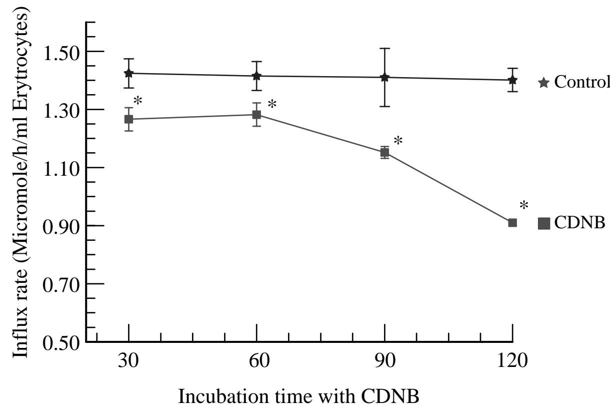

Figure 1. The effect of CDNB on L-cysteine influx. The control group was incubated with 4 mM L-cysteine for 1 h and then the rate of the influx was determined. The CDNB group was first incubated with 1.2 mM CDNB for the indicated time periods. The washed erythrocytes were treated with 4 mM of L-cysteine for 1 h. Then the rate of influx was determined in washed erythrocytes. Results are the mean, SD of three separate experiments. $p < 0.05$. *Statistically different from the control.

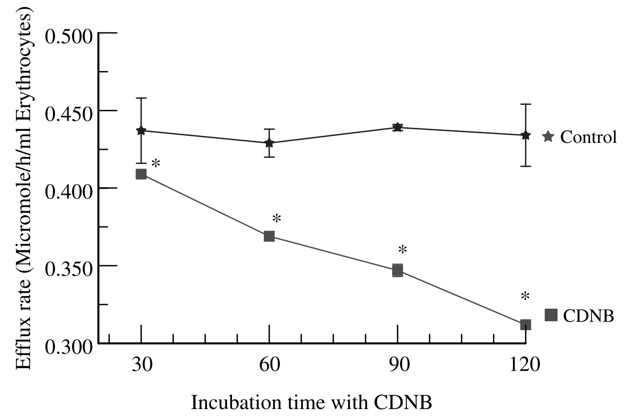

Figure 2. The effect of CDNB on L-cysteine efflux. The control group was incubated with 4 mM L-cysteine for 1 h and then the washed erythrocytes were transferred to fresh media and incubated for an additional 1 h. The efflux rate was then measured in the media. The CDNB group was first incubated with 1.2 mM CDNB for the indicated time periods. Washed erythrocytes were treated with 4 mM L-cysteine and incubated for 1 h. At the end of incubation, the erythrocytes were washed and transferred to fresh media and incubated for 1 h. The rate of efflux was then determined in the media. Results are the mean, $p < 0.05$. *Statistically different from the control.

the modulation of the L-cysteine concentrations in the plasma by lowering when its high and by increasing it when its concentration is low thus providing a homeostasis in the blood. A similar function is displayed by intestinal cell membranes [24]. Glucose transporters that function on the apical membrane influx glucose from the intestinal lumen into intestinal cells when glucose is increased especially after a carbohydrate rich diet. This process is reversed on the basolateral membrane of intestinal cells and in this case GLUT2 exports the concentrated glucose from the intestinal cells to the plasma. The liver is also known to display a similar function influxing glucose when its concentration in the plasma is high and effluxing glucose when the concentration of glucose in the plasma is low. This type of a function in erythrocytes becomes of significant importance especially when feeding with cysteine rich diets or cysteine supplementation therapies. Cysteine supplementation has been used for different purposes in children and in adults [25,26]. Cysteine supplements or formulations of cysteine rich diets seem to become popular since it has been understood that it is the rate limiting amino acid in GSH synthesis. GSH functions in scavenging of free radicals that are known to participate in development of several life threatening diseases [27]. Thus another function may be related to redox regulation in erythrocytes and in the plasma. Efficient influx and the following efflux of L-cysteine by erythrocytes may also contribute to the redox status of the blood plasma. L-Cysteine is known to be oxidized easily when it is outside of the cells. Thus formation of cystine form L-cysteine would be limited in the blood plasma due to high uptake. On the other hand, plasma thiol levels have been shown to be implicated in vascular diseases. It has been shown that

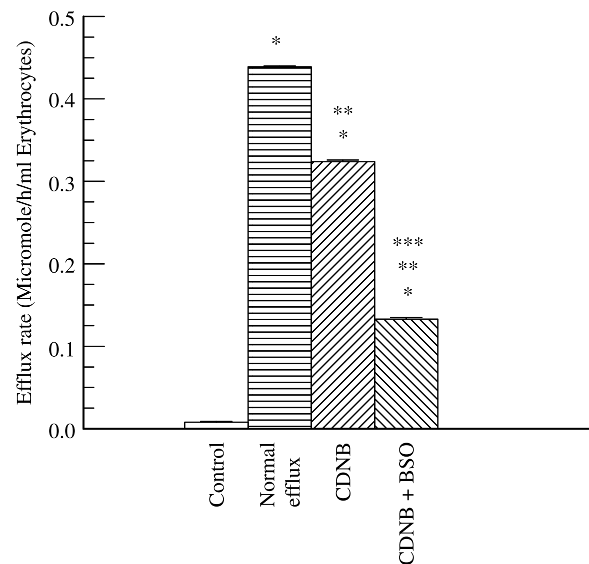

Figure 3. The effect of BSO on L-cysteine efflux. The control group was incubated with only PBS-glucose. The normal efflux group was incubated with 4 mM of L-cysteine for 1 h and then transferred to fresh media and the rate of efflux was determined for 1 h in the media. The CDNB group was preincubated with 1.2 mM CDNB and for 90 min. The washed erythrocytes were then treated with 4 mM L-cysteine for 1 h and then erythrocytes were transferred to fresh media and incubated further for 1 h. The efflux rate was determined in the media. The CDNB + BSO group was first preincubated with 1.2 mM CDNB for 90 min. The washed erythrocytes were then treated with 4 mM L-cysteine and 5 mM BSO for 1 h. Washed erythrocytes were then transferred to fresh media containing 5 mM BSO and incubated further for 1 h. The efflux rate was determined in the media. Results are the mean, SD of three separate experiments. *Significantly different from the control. **Significantly different from the normal efflux group. ***Significantly different from the CDNB group. $p < 0.05$.

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reduced cysteine is correlated with the amount of reduced homocysteine preventing its oxidation to disulfide form [28]. This function was correlated with total blood cysteine concentration. Thus it was concluded that reduced cysteine plays a role in the maintenance of plasma redox status. In this sense it could be suggested that L-cysteine efflux plays a role in the maintenance of a proper redox status in the blood plasma. Another function may be the fast and safe transport of cysteine from sites its concentration is higher to distal tissues. Thus, erythrocytes may function, except being hemoglobin bags, in the transport of several other metabolites, in addition to L-cysteine, in the blood plasma.

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