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Homocysteine Influx and Efflux: Participation of Erythrocytes in Homocysteine Homeostasis of the Plasma

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ABSTRACT

One of the main objective of the present study was to determine if erythrocytes play a role in regulation of plasma homocysteine concentration. Another objective was to investigate if erythrocytes convert homocystine into homocysteine. In the present study, we exposed erytrocytes to different concentrations of homocysteine and then measured the nonprotein sulfhydryl (NPSH) concentrations. Erythrocytes treated in the same manner were later utilized for the homocysteine efflux studies. The effect of temperature on the influx and the efflux processes were also evaluated. We also determined the rate of homocysteine influx in the presence of different amino acids. The homocysteine influx studies demonstrated that erythrocytes can respond to increases in homocysteine concentration in the extracellular media and influx homocysteine in a concentration-dependent manner. NPSH concentrations in erythrocytes treated with 1 mM homocysteine reached to 1.47 ± 0.01 μmol/ml erythrocyte in 1 h whereas this concentration reached to 2.01 ± 0.1 μmol/ml erythrocyte in 3 mM homocysteine treated erytrocytes. The homocysteine efflux is also determined to be time-and concentration -dependent. Extracellular concentration of NPSH in 1 mM homocysteine pre-treated erythrocytes reached to 0.266 ± 0.02 μmol/ml erythrocyte in 1 h whereas this concentration reached to 0.64 ± 0.01 μmol/ml erythrocyte with 3 mM homocysteine pre-treated erythrocytes. Our results also indicate that erythrocytes convert extracellulary applied homocystine into homocysteine. Depending on our results, it could be concluded that eryhtrocytes may play a significant role in the regulation of plasma homocysteine homeostasis.

Key words: Homocysteine, Homocystine, Erythrocytes, Membrane transport

1. INTRODUCTION

Homocysteine is a free-SH containing amino acid which is not utilized in protein synthesis. Homocysteine is essentially originated from methionine metabolism as a by product [1, 2]. It has been shown that once homocysteine is produced there will be three different metabolic pathways that it can take [3]. One of the metabolic pathways homocysteine can take is remethylation to methionine. The second is the transsulfuration pathway to give cysteine and the last pathway is homocysteine transport out of the cells.

Once the methyl group of the S-adenosylmethionine is transferred to an acceptor the remaining compound is S-adenosylhomocysteine. The enzymatic hydrolysis of S-adenosylhomocysteine gives adenosine and homocysteine [4]. Normally, thus formed homocysteine can be remethylated to methionine by the methionine synthase in the methyl cycle [5]. Methionine synthase is an enzyme that requires the presence of vit B12 as a cofactor for the activity. Another metabolic pathway that S-adenosylhomocysteine can take is as mentioned transsulfuration pathway [5]. In this pathway, homocysteine is condensed with serine to form cystathionine. This reaction is catalyzed by cystathionine beta synthase which requires the presence of vit B6. In the last step of transsulfuration, cystathionine is broken down by gamma cystathionase to give cysteine. As mentioned, the third way that homocysteine can take is transport out of the cells.

Most of the harmful effects of homocysteine start when the third pathway which is transport out of cells is taken by homocysteine. Once homocysteine is transported out of the cells the concentration of homocysteine in the blood plasma starts to increase. Plasma homocysteine level is measured and is found normally to be around 5-15 μM [6]. This concentration is found to rise about to 15-25 μM in intermediate level hyperhomocysteinemia [7,8].

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However, in severe hyperhomocysteinemia the levels of homocysteine further increase to around 50-500 μM [9]. Hyperhomocysteinemia generally results from the defects in homocysteine metabolism and vitamin deficiencies. Deficiencies in folate (vit B6) and cobalamine (vit B12) have been shown to result in an increase in homocysteine level in the plasma [10]. As mentioned before these two vitamins are involved in homocysteine metabolism as cofactors for important enzymes in the pathway.

Increased homocysteine level is widely accepted as a risk factor for cardiovascular, serobrovascular and peripheral vascular diseases [1,11]. In 1975, following investigation of homocystinuri patients, the homocysteine theory of atherosclerosis was suggested [12]. According to this hypothesis one of the main causes for atherosclerosis is the increased plasma concentrations of homocysteine. It has later been shown that the atherosclerotic effect of homocysteine is alleviated by the presence of antioxidants [13-15]. These observations lead to the idea that the deteriorating effects of homocysteine on vascular systems may result from homocysteine dependent production of free radicals. In the presence of oxygen, homocysteine becomes oxidized and is converted to its disulfide form, homocystine. As a result of this reaction certain free radicals such as superoxide and hydrogen peroxide are produced and these free radicals cause damages [16, 17].

As mentioned the deleterious effects of homocysteine start following its intracellular increase and the subsequent release from the cells increasing plasma homocysteine concentrations. Increased plasma homocysteine concentration then leads to production of free radicals in the presence of oxygen by auto oxidation. Thus, factors that influence the transmembrane transport of homocysteine may become significantly important. In the present study we investigated the influx and efflux of homocysteine in erythrocytes. We also investigated if there is a homocystine-homocysteine cycle between the erythrocytes and plasma regenarating reduced homocysteine in the plasma continously following its oxidation. The principal aim was to find out if the erythrocytes play a role in the regulation of plasma concentration of homocysteine.

2. MATERIALS AND METHODS

2.1. Materials

Homocysteine, and all other amino acids were obtained form Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). 5,5'-Dithiobis(-nitrobenzoate) (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.

2.2. 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 three times 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). PBS-glucose contained 5 mM of glucose in the PBS [18]

2.3. Homocysteine influx studies

Homocysteine determination was based on detection of the nonprotein sulfhydryl (NPSH) groups in the homocysteine chemical structure. Since there were no other factors except homocysteine added to the experimental system that would influence NPSH levels, changes in NPSH levels compared to control group were directly related to homocysteine concentrations.

0. 25 ml of washed erythrocytes were suspended in 0.5 ml of Krebs Ringer Phosphate buffer (135 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 1.2 mM MgSO4, 5 mM Glucose, 10 mM NaH2PO4, pH 7.4) containing containing different concentrations of homocysteine and incubated for the indicated time periods at 37 ^{o}C in a water bath. At the end of incubation erythrocytes were removed, centrifuged and the supernatants were discarded. The NPSH concentrations in erythrocytes were then determined as described by Sedlak [19]. 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 minutes. At the end of centrifugation, 100 μl of the supernatant was mixed with 1. 9 ml of Tris-EDTA buffer containing 0.6 μmole/ml DTNB, (400 mM Tris base, 20 mM EDTA, pH 8. 9). Samples were allowed to stand for 5 minutes to develop color. The absorbances of the samples were then measured at 412 nm and the concentrations of NPSH were calculated by using the mM extinction coefficient of 13. 1.

2.4. Homocysteine efflux studies

0. 25 ml of washed erythrocytes were resuspended in 0.5 ml of Krebs Ringer Phosphate buffer in the presence of different concentrations of homocysteine. Erythrocytes were incubated at 37 ^{o}C in a water bath for 1 hour to allow the uptake process. At the end of incubation erythrocytes were centrifuged and the supernatants were discarded. The erythrocytes were then resuspended in 0.5 ml of fresh Krebs Ringer buffer in the presence or absence of indicated compounds and incubated at 37 ^{o}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 NPSH concentrations in the supernatant were then measured as described above.

2.5. 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 ± S.D. $p < 0.05$ values were considered to be significant.

3. RESULTS

Figure 1 shows the result of time and concentration dependent homocysteine influx in erythrocytes. As seen, erythrocytes accumulated homocysteine inside cells from the media. The influx process is increased when homocysteine concentration in the media is increased. The influx process seems to be saturated in 1 h time period. Beyond this time, further incubation of erythrocytes did not result in a large increase in homocysteine influx.

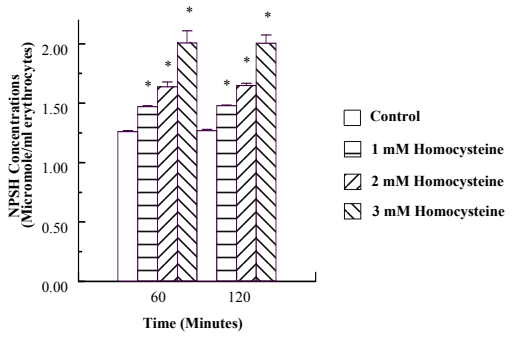

Figure 1. Homocysteine influx in erythrocytes. Results are the mean and S.D. of three separate experiments
*Signigicantly different from the control
$p<0.05$

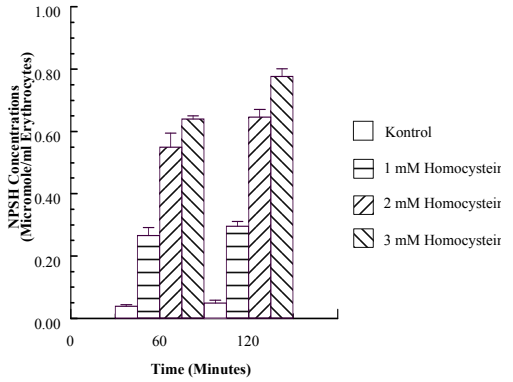

Figure 2. Homocysteine efflux from erythrocytes. Results are the mean and S.D. of three separate experiments.
*Significantly different from the control
$p<0.05$

Figure 2 displays the results of homocysteine efflux studies. Our results demonstrated that erythrocytes may transport the influxed homocysteine back into the media from the cytoplasm when homocysteine is absent in the environment. The rate of the homocysteine efflux is dependent on time and on the intracellular levels of homocysteine. Incubation of homocysteine pretreated erythrocytes in an homocysteine free media resulted in an efflux of homocysteine back into the media. The efflux rate increased for up to 1 h and additional incubation of eryhtrocytes for another hour did not result in a large extent of further efflux thoug the efflux process is continued.

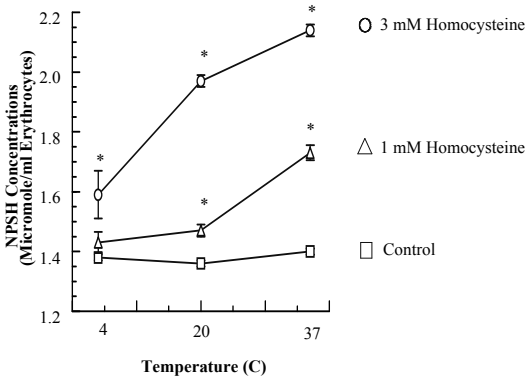

Figure 3. Temperature dependency of homocysteine influx. Results are the mean and S.D. of three separate experiments
*Signigicantly different from the control
$p<0.05$

Figure 3 shows the effect of temperature changes on the homocysteine influx process. Erythrocytes do not influx L-homocysteine efficiently from the media when incubated at 4 ^{o}C. When the temperature is increased, the transport process restarts and increases as the temperature is increased. In the next step of our experiments we tried to evaluate if certain amino acids interfere with the transmembrane flux of homocysteine

Figure 4 and Figure 5 show the results of these studies. In figure 4 it could be seen that the presence of certain amino acids significantly decreases the rate of homocysteine influx. All of the amino acids tested displayed an inhibitory effect on homocysteine influx. However homocysteine efflux process was not as much sensitive to the presence of the same amino acids. Only glutamate significantly inhibited the homocysteine efflux process from erythrocytes.

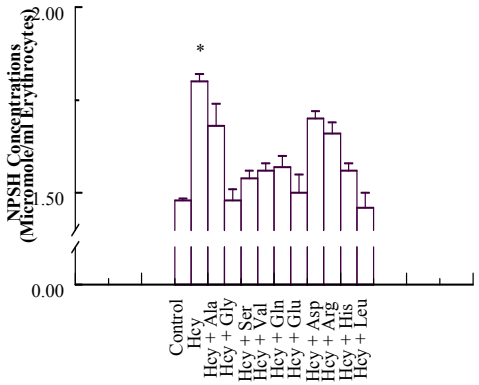

Figure 4. The effects of amino acids on homocysteine influx.

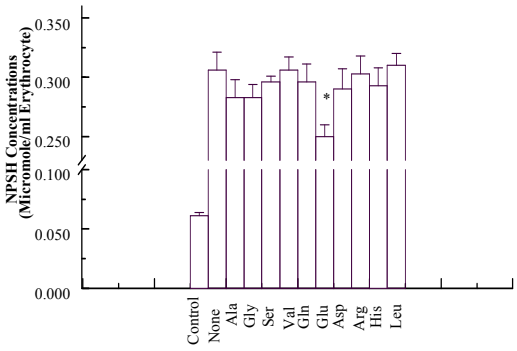

Figure 5. The effects of aminoacids on homocysteine efflux.

In the next step we investigated if erythrocytes convert extracellulary applied homocystine into homocysteine. Our results as presented in Figure 6 indicate that erythrocytes indeed can convert homocystine into homocysteine. In order to see if this conversion of homocystine into homocysteine is a metabolically active process we repeated the experiments at different temperatures. As seen in Table 1 at lower temperature the process of homocystine conversion into homocysteine was inhibited indicating that the process is biologically active. In the last step of our study we investigated if erythrocytes also convert cystine into cysteine in similar experimental system. However, erythrocytes did not convert the extracellularly applied cystine into cysteine as indicated in Figure 7.

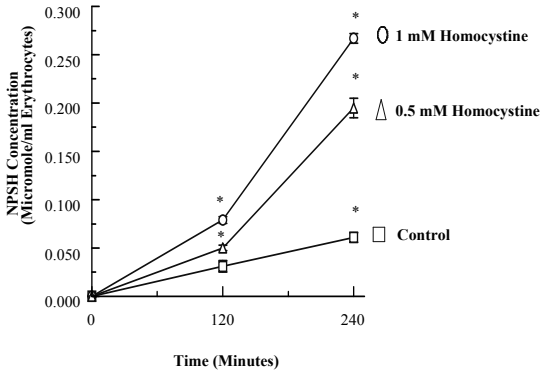

Figure 6. Homocysteine generation from homocysteine.

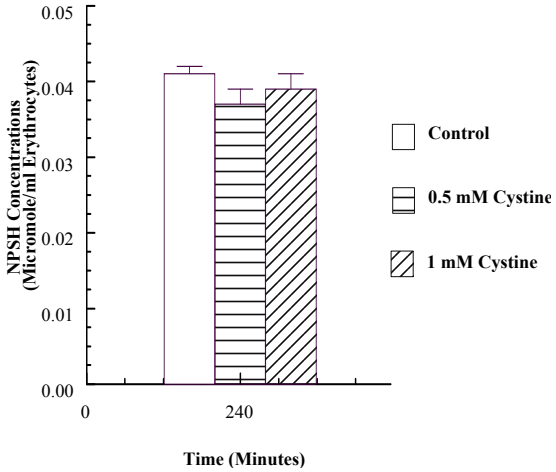

Figure 7. Generation of cysteine from cystine

Table 1. Temperature dependency of homocysteine generation from homocystine. Results are the mean and S.D. of three seperate experiments. $P < 0.05$
*Significantly different from control

	NPSH (Micromole/ml Erythrocyte)					
	120 Minutes			240 Minutes		
	4 °C	20 °C	37 °C	4 °C	20 °C	37 °C
Control	0.00	0.016 ± 0.003	0.020 ± 0.002	0.00	0.026 ± 0.002	0.034 ± 0.008
0.5 mM Homocystine	0.00	0.029* ± 0.001	0.046* ± 0.001	0.00	0.79* ± 0.004	0.184* ± 0.004
1 mM Homocystine	0.00	0.043 *± 0.002	0.086* ± 0.002	0.00	0.119* ± 0.003	0.249* ± 0.006

4. DISCUSSION

The objective of the present study was to determine if erythrocytes play a role in the regulation of plasma homocysteine concentrations. Our results suggest that erythrocytes may respond to changes in homocysteine concentrations in the plasma through bidirectional membrane flux and thus modulate transiently the concentration of homocysteine present in the plasma.

Our results also suggest that erythrocytes are able to convert the extracellularly introduced homocystine into homocysteine, an effect which may be significant in prevention of homocystine precipitation in the plasma that may cause mechanical endothelial damage.

Erythrocytes are the largest cell population among the blood cells. Erythrocytes are also directly in contact with the plasma. Thus they are the cells that are more readily exposed to changes in plasma conditions. In this respect, erythrocytes may have some regulatory functions like modulating the plasma metabolite levels by uptaking when the concentrations of certain metabolites are increased and releasing them back when the plasma concentrations of the same metabolites are decreased. In this respect, we investigated the influx and efflux of homocysteine in erythrocytes. Our results showed that erythrocytes take up homocysteine when its concentration is increased and then release it back to the environment when its concentration is lowered or homocysteine is absent outside the membrane. This observed property of erythrocytes may serve at least two different functions. One is to act as a transient reservoir for increased homocysteine preventing its excess oxidation in the plasma generating free radicals and thus alleviating the homocysteine induced cardiovascular damage. The other function may be to remove excess homocysteine from the sites where it is highly generated and further carry and release it to the sites where its metabolism and elimination is highly active such as the kidneys. The membrane carrier systems for homocysteine in erythrocytes are not investigated in detail. However, in other cellular systems it has been shown that homocysteine is transported through the membrane by systems which were initially described for the transport of cysteine such as ASC, A, L, and X(AG) [20]. The presence of system X(AG) in erythrocytes membrane has not been documented. Thus it is more probable that homocysteine is carried bidirectionally in erythrocytes by the other systems that carry cysteine. Indeed, inhibition of homocysteine influx by several different amino acids in our experimental system suggests that homocysteine utilizes the vast majority of amino acid carriers present in the erythrocytes membranes. The observed significant inhibition of homocysteine influx into erythrocytes by diverse amino acids also suggest that the concentration of other amino acids in the plasma may influence and increase the homocysteine concentrations in the plasma. A high protein diet has been shown to increase the homocysteine concentrations in the plasma [21]. However, diet induced variations in the homocysteine concentration is more generally related to the methionine content of the diet. Here we suggest that a high protein diet may increase homocysteine concentration in the plasma, in addition to the methionine content, by inhibition of homocysteine uptake and hence its metabolism due to the presence of other amino acids. When a persistently high amino acid concentration is maintained this may interfere with homocysteine reuptake by the cells and thus may cause a persistent elevation of homocysteine in the plasma.

In a previous study it was suggested that endothelial damage in hyperhomocysteinemia may result from mechanical damage by the elevated homocystine [22]. Homocystine concentration is suggested to reach almost to the threshold where homocystine precipitates. Thus any rapid fluctuation that increases the homocystine concentration in the plasma may result in homocystine precipitation. In the present study we also investigated if erythrocytes are able to convert extracellularly applied homocystine into homocysteine. In a previous study it was shown that erythrocyte membranes were impermeable to homocystine [23]. However we have showed that the NPSH levels in the supernatants gradually increase in homocystine applied erythrocytes indicating that homocystine is converted to homocysteine by the erythrocytes. There are two possible mechanism by which this process may take place. One is that the erythrocytes uptake homocystine, intracellularly reduce it to homocysteine and then release it back to the environment. The second possibility is that homocystine does not gain entry into the erythrocytes but intracellular reducing power of GSH is transduced through the membrane and homocystine becomes reduced generating homocysteine in the environment. There are direct evidences that strongly support the second possibility. In a previous study it was demonstrated that treatment of erythrocytes with a membrane impermeant disulfide DTNB lowered intracellular GSH concentration [24]. Based on this observation, it was concluded that the reducing power of GSH was transduced into the environment through membrane spanning thiol containing proteins. Another interesting study evaluated if extracellularly applied GSH increases the intracellular level of GSH in erythrocytes [25]. They found that extra cellular treatment of erythrocytes with GSH actually increases the intracellular level of GSH. Based on their observation, it was again suggested that reducing power of GSH could be transduced through the membranes by the thiol containing and membrane spanning proteins. In this respect extracellularly administered homocystine may be converted into homocysteine by the same mechanism. Interestingly axtracellularly administered cystine did not result in formation of cysteine under the same conditions. This apparent discrepancy between the homocystine and cystine may be related to their structural stability or to their ability to get involved in disulfide exchange reactions. As a result, whether homocystine is transported into the erythrocytes or the intracellular reducing power of erythrocytes is transduced through the membrane it is obvious from our results that erythrocytes convert the extracellularly administered homocystine into homocysteine. This process may be significantly important in prevention of homocystine precipitation in the plasma that may cause mechanical endothelial cell damage.

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