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QUARTERLY

Is the glutathione conjugate of *trans*-4-hydroxy-2-nonenal transported by the multispecific organic anion transporting-ATPase of human erythrocytes?*°

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***Trans*-4-hydroxy-2-nonenal (4-HNE), a cytotoxic end product of lipid peroxidation, is present in normal human blood plasma at concentrations of 0.1–1.0 μM. It can be, however, further metabolized within a cell, and one of the main products is 4-HNE glutathione conjugate (HNE-SG). In human erythrocyte membrane the system for active extrusion of glutathione (GSH) conjugates of various endo- and xenobiotics has been described; it exhibits either a low (K_m at submillimolar concentration range) or a high (K_m at low micromolar range) affinity for the transported substrates, such as for example *S*-(2,4-dinitrophenyl)glutathione (Dnp-SG). In the present study it has been shown that the high affinity transport system for Dnp-SG is competitively inhibited by HNE-SG with K_i of 0.2 μM, while 4-HNE inhibits non-competitively the activity of the transport system for Dnp-SG with K_i of 220 μM. These observations point to the possibility that HNE-SG shares the same transport system with GSH conjugates of other endo- and xenobiotics in erythrocytes. This may be of importance for overall detoxification of the organism under oxidative stress.**

Free oxygen radicals react with membrane lipids to form lipid hydroperoxides. Degradation of these molecules generates several aldehydic compounds, among them 4-HNE [1]. Aldehydes are chemically stable, and may diffuse from the site of their origin across relatively long distances, affecting multiple targets in the organism [2]. In the plasma of human venous blood the steady-state concentrations of 4-HNE are moderate (0.1–1 μM) but they can increase tenfold as a result of oxidative stress [1]. In erythrocytes, the main role in protection of the cell against oxidative stress is played by GSH [3]. 4-HNE, while

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Abbreviations: Dnp-SG, *S*-(2,4-dinitrophenyl)glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; 4-HNE, *trans*-4-hydroxy-2-nonenal; HNE-SG, glutathione conjugate of 4-HNE; GSH, reduced glutathione; MOAT, multispecific organic anion transporter.

spontaneously reacting with GSH, diminishes the GSH pool in the cell [4]. In addition, it also reacts with -SH groups and histidyl residues of enzymes, which may result in inhibition of their enzymatic activities [5]. The main products of 4-HNE metabolism in the cells are 4-hydroxynonenoic acid, 1,4-dihydroxynonene and HNE-SG [6–7]. HNE-SG is further degraded to mercapturic acid conjugates [8].

The GSH-conjugates of various compounds are transported from the cell by an energy-dependent mechanism involving a multispecific organic anion transporter (MOAT) [9] and a multidrug resistance-associated protein [10], which exists also in erythrocytes [11–12]. The aim of our work was to answer the question whether HNE-SG is actively transported in human erythrocytes and, if so, whether this transport is catalyzed by MOAT.

MATERIALS AND METHODS

Chemicals. Hydroxynonenal diethylacetal was a generous gift from Professor Herman Esterbauer and Dr. Marianne Hayn of University of Graz (Austria). [Glycine-2-^{3}H]glutathione (44.8 Ci/mmol) was obtained from New England Nuclear (U.S.A.). ATP, creatine phosphate, creatine kinase, heparin, glutathione *S*-transferase from equine liver, and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma (U.S.A.). Silica gel 60 plates were from Merck (Germany). Akwascynt from Bio-Care (Poland) was used as a scintillation cocktail. All other chemicals were of the highest purity commercially available.

Preparation of human erythrocyte ghosts. Erythrocyte ghosts were prepared and sealed by the procedure described by Steck & Kant [13], and stored at 1–2 mg protein/ml in 10 mM Tris/HCl, pH 7.4, 250 mM sucrose at -80°C.

Synthesis of Dnp-SG and HNE-SG. [^{3}H]Dnp-SG was synthesized in 250 μl of 10 mM phosphate buffer, pH 6.9, containing 1.5 mM CDNB, 1 mM [^{3}H]GSH (spec. act. 56 mCi/mmol) and 2 units of glutathione *S*-transferase for 3 h at 37°C, according to Awasthi *et al.* [14]. [^{3}H]Dnp-SG was purified by thin-layer chromatography on silica gel 60 plates developed in acetonitrile/H_2O (7:2, v/v). Non-radiolabeled Dnp-SG was synthesized as described above.

The stock solution of 4-HNE was prepared by treatment of hydroxynonenal diethylacetal with 1 mM HCl for 1 h at 37°C. The concentration of 4-HNE was determined spectrophotometrically at 224 nm using extinction coefficient of 13750 $M^{-1} \times cm^{-1}$. Spontaneous reaction between 4-HNE and GSH at their equimolar concentrations was run in 50 mM phosphate buffer, pH 6.5, at 30°C for 1 to 3 h.

Transport of [^{3}H]Dnp-SG. The assay mixture contained in a total volume of 150 μl: 0.08–0.1 mg protein/ml, 10 mM Tris/HCl, pH 7.4, and 250 mM sucrose, 1 mM ATP or 3 mM NaCl (to determine ATP-independent uptake), 10 mM $MgCl_2$, 10 mM creatine phosphate, and 12 units of creatine kinase, as described by Saxena & Henderson [15] or as otherwise indicated in the text. The reaction was started by the addition of [^{3}H]Dnp-SG (spec. act. 21–29.7 mCi/mmol) to desired concentrations. Samples were incubated at 37°C for the time indicated, and the reaction was stopped by the addition of 1 ml of ice-cold 10 mM Tris/HCl, pH 7.4, 250 mM sucrose, and 100 mM NaCl, and rapid filtration through Millipore HAWP 0.45 μm filters. Then, the filters were washed with four 1-ml portions of the same ice-cold buffer, and analyzed for associated radioactivity. The difference in uptake between samples incubated in the presence and absence of ATP was taken as a measure of energy-dependent transport of GSH conjugates.

Other procedures. Protein concentration was determined according to Bradford with bovine serum albumin as a standard [16]. The number of -SH groups in erythrocyte ghosts was measured in Ellmann reaction [17]. Phospholipids were extracted from the membranes according to Bligh & Dyer [18] and separated

by one dimensional thin-layer chromatography in chloroform/ethanol/water/triethylamine (30:34:8:35, by vol.) [19]. Phospholipid phosphorus was determined according to Rouser *et al.* [20].

RESULTS

The effect of 4-HNE on active transport of Dnp-SG in human erythrocyte vesicles

The ATP-dependent uptake of [^{3}H]Dnp-SG into vesicles from human red blood cells was linear up to 60 min at 37°C in the presence of 20 μM substrate. Alternatively, measurements of Dnp-SG uptake at low substrate concentration range (< 2 μM), close to the K_m value determined for MOAT3 ([15], see also Table 1), were performed for 3 min to ensure that the uptake did not exceed 10% of the substrate concentration in the assay medium. Double-reciprocal plots of transport of [^{3}H]Dnp-SG *versus* its concentration over a range from 0.07 to 10 μM exhibited a biphasic character. Analysis of these data revealed the presence of two transport components with K_m of 0.19 μM and 4.1 μM and V_{max} of 17 or 36 pmol/min per mg protein, respectively (Table 1) [15, 21, 22].

Interaction of 4-HNE with erythrocyte ghosts leads to concentration-dependent inhibition of uptake of Dnp-SG at 1.2 μM concentration of the conjugate in the assay medium (Fig. 1). No inhibition was observed up to 50 μM 4-HNE and the K_i value amounted to 220 μM 4-HNE. Analysis of inhibition by Dixon plot (not shown) suggested non-competitive inhibition of the transport system for GSH conjugates by the aldehyde under our experimental conditions (i.e. 1.2 μM Dnp-SG).

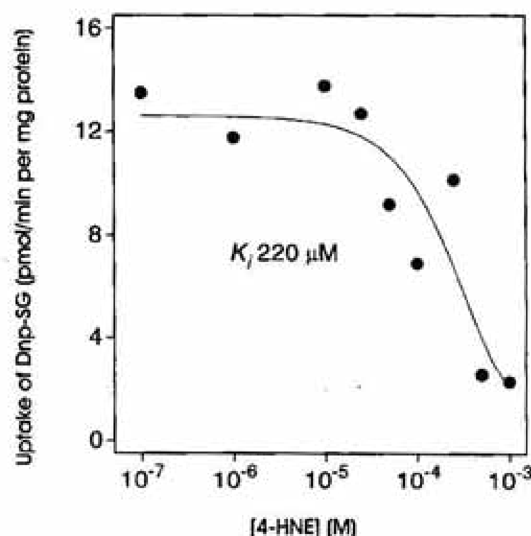

Figure 1. The effect of 4-HNE on uptake of [^{3}H]Dnp-SG transport in erythrocyte ghosts.

Dnp-SG uptake was measured as described in Materials and Methods, in the presence of 1.2 μM of [^{3}H]Dnp-SG and varying concentrations of 4-HNE. Transport in the absence of 4-HNE amounted to 13.5 ± 3.2 pmol/min per mg protein. The mean values for three independent ghost preparations and two to three measurements per preparation are shown.

Table 1. Kinetic parameters of Dnp-SG uptake by human erythrocyte ghosts

MOAT3		MOAT4		Reference
K_m (μM)	V_{max} (pmol/ min per mg protein)	K_m (μM)	V_{max} (pmol/ min per mg protein)	
0.18 and 0.58	22 and 16	n.d.	n.d.	[15]
2.7	111	897	1567	[21]
3.9	105	1600	2183	[22]
0.19 and 4.1	17 and 36	n.d.	n.d.	present communication

n.d., not determined.

Interaction of 4-HNE with erythrocyte ghosts

Binding of 4-HNE to erythrocyte ghosts was assessed by determination of the concentration of the aldehyde remaining in the supernatant after incubation of ghosts with 4-HNE and centrifugation, as described in the legend to Fig. 2. The incubation of erythrocyte ghosts with 4-HNE led to saturable and time-dependent binding of the aldehyde (250 nmol/mg protein per 60 min) to the membrane, reaching the half-maximal value within 10 min of incubation of ghosts with 4-HNE (Fig. 2). Since 4-HNE interferes with Ellmann reaction, it was difficult to assess the exact number of -SH groups reacting with the aldehyde. It can be concluded that 4-HNE accumulates at the hydrophobic domain of the membrane lipid bilayer rather than chemically reacts with lipids and proteins within the erythrocyte membrane. In fact, only minor changes in the composition and content of erythrocyte phospholipids after incubation with 4-HNE were observed and no effect of the aldehyde on the activity of erythrocyte Mg^{2+}-ATPase, measured in the presence of 10 mM $MgCl_2$, 1 mM ouabain and 0.1 mM EGTA was noticed. This points to the conclusion that, upon oxidative stress, erythrocyte MOAT is the potential target for 4-HNE.

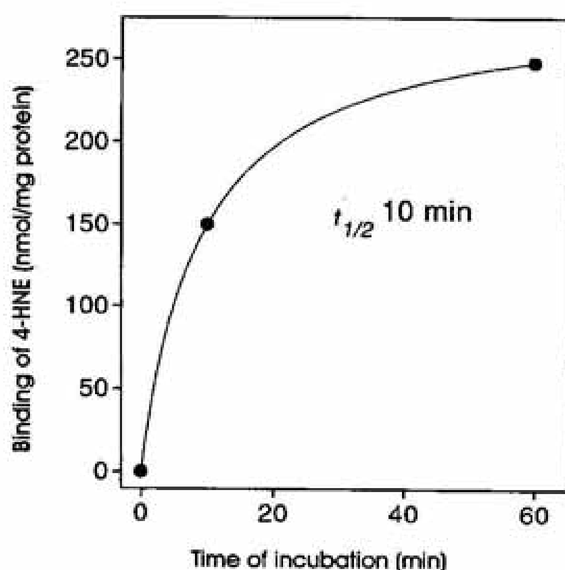

Figure 2. Binding of 4-HNE to erythrocyte ghosts.

Erythrocyte ghosts (1 mg of protein) were incubated in the absence and presence of 4-HNE in the medium consisting of 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, and 10 mM $MgCl_2$ for the time indicated. Then the samples were cooled in ice and ghosts were pelleted by centrifugation at 100000 × *g* for 30 min. Concentration of 4-HNE in supernatant was determined spectrophotometrically at 224 nm and the obtained value was subsequently subtracted from the starting concentration in the assay medium. The difference was taken as a measure of 4-HNE binding to erythrocyte ghosts. Mean values of two experiments are shown. They varied by 5–7%. $t_{1/2}$, time of incubation required to reach half-maximal binding.

Inhibition of MOAT by GSH conjugate of 4-HNE

To further study the effect of 4-HNE on erythrocyte MOAT activity we synthesized GSH conjugate of 4-HNE and examined its effect on active transport of Dnp-SG to erythrocyte membrane vesicles. The activity of the transport system in the presence of 1 μM Dnp-SG was inhibited by HNE-SG with K_i for HNE-SG of 0.2 μM (Fig. 3), i.e. by four orders of magnitude lower than that determined for 4-HNE. Analysis of these results by Dixon plot (not shown) pointed to a competitive mechanism of the inhibition. These results suggest that HNE-SG may be a physiological substrate for erythrocyte MOAT3 (Table 1).

DISCUSSION

4-Hydroxynonenal is a very toxic aldehyde. Fortunately, it is metabolized in the cells and detoxified, mainly by formation of *S*-conjugate with GSH [23], and transported out of the cell. Transport of GSH-conjugates from erythrocytes is energy-dependent and is catalyzed by MOAT [3] and a multidrug resistance-associated protein [12]. The interrelationship between these two transport systems has not been established yet. However, the existence of two MOAT transport systems referred to as

MOAT3 and MOAT4 in erythrocytes exhibiting low and high affinity for the transported substances, has been demonstrated [15, 20, 21]. It has been suggested that the high affinity transport is catalyzed by MOAT3, the activity of which is regulated by phosphorylation at tyrosine residues of the transporter [15]. The data of the present communication confirm the existence in erythrocyte plasma membrane of an ATP-dependent Dnp-SG transport system, identical with respect to kinetic parameters with MOAT3. In the course of the present study it was found that MOAT3 is non-competitively inhibited by 4-HNE only at concentrations exceeding the physiological range (Fig. 1).

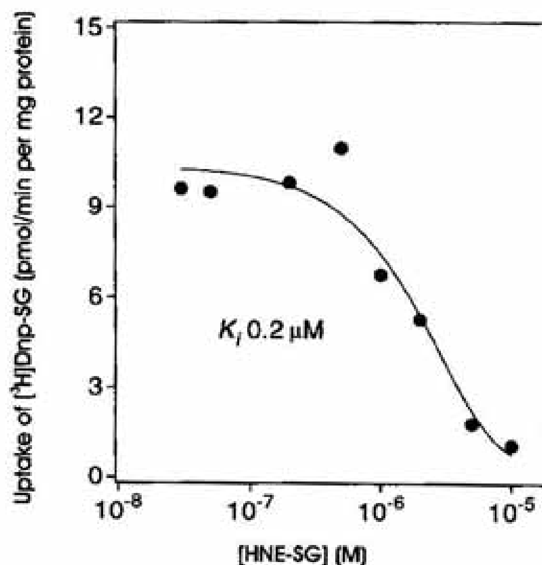

Figure 3. Inhibition of uptake of Dnp-SG by HNE-SG.

Uptake of Dnp-SG at 1 μM concentration of the conjugate in the assay medium was measured as described in Materials and Methods, in the presence of various concentrations of HNE-SG. Control activity without HNE-SG amounted to 9.6 ± 1.7 pmol/min per mg protein. The mean values for three independent ghost preparations and two to three measurements per preparation are shown.

The inhibitory effect of 4-HNE on MOAT can be ascribed to the reaction of the aldehyde with protein -SH groups, as in the case of the effect of 4-HNE on Na^+-K^+-ATPase [24] and adenine nucleotide translocase [25]. The modification of histidine and lysine residues of proteins cannot be excluded, either [26]. Most probably 4-HNE evokes changes in membrane surface charge (leading to membrane aggregation), in membrane phospholipid asymmetry, and in membrane fluidity. It has been also found that products of phospholipid peroxidation decrease the mitochondrial membrane fluidity [27].

4-HNE was shown to spontaneously react with GSH. The resulting compound competitively inhibited the high affinity transport of Dnp-SG in erythrocyte ghosts (MOAT3) (Fig. 3, Table 1). Thus it seems possible to conclude that HNE-SG shares the same high affinity transport system in erythrocytes which is involved in extrusion of various glutathione *S*-conjugates of endo- and xenobiotics out of the cell. Further studies are required to confirm directly that HNE-SG is transported in erythrocytes by MOAT3, especially under oxidative stress.

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