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ATP stimulates the uptake of *S*-dinitrophenylglutathione by rat liver plasma membrane vesicles

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Incubation of inverted plasma membrane vesicles from rat liver with micromolar concentrations of *S*-dinitrophenylglutathione (DNP-SG) in the presence of ATP resulted in the uptake of DNP-SG into the vesicles. ATP-dependent DNP-SG accumulation was half-maximal with 9 μM DNP-SG, while the K_m for ATP was 320 μM. Glutathione disulfide (GSSG), but not reduced glutathione, inhibited the ATP-dependent accumulation of DNP-SG by the vesicles, suggesting that the same, ATP-dependent transport system is responsible for the extrusion of glutathione conjugates and GSSG from liver cells.

Glutathione; Hepatocyte; Plasma membrane; ATPase

1. INTRODUCTION

Mammalian cells contain a large pool of glutathione which is present predominantly in the reduced form (GSH), with glutathione disulfide (GSSG), mixed disulfides and thioethers constituting minor fractions [1]. GSH is critically involved in cellular defence against oxidative injury, serving as a reductant in the metabolism of hydrogen peroxide and organic hydroperoxides catalyzed by glutathione peroxidases. Increased formation of GSSG during oxidative stress is followed by its active excretion from the cells [2]. In addition, GSH plays an important role in the inactivation of electrophilic compounds by the formation of glutathione conjugates catalyzed by glutathione transferases.

Transport systems mediating the extrusion of GSSG and glutathione conjugates have been identified in the plasma membrane fraction of various cells and tissues, including erythrocytes [3,4], ocular lens [5], liver [6] and heart [7]. Studies in erythrocytes have revealed that the extrusion of GSSG and glutathione conjugates requires ATP [4,8]. Similarly, the efflux of GSSG observed in the isolated, perfused heart following exposure to oxidative stress has been found to be an ATP-dependent process [7].

We have previously reported that GSSG and glutathione conjugates at low concentrations stimulate ATPase activity in the plasma membrane fraction from rat hepatocytes and suggested that the ATPase may function in the active extrusion of GSSG and glutathione conjugates from liver cells [9,10]. The present study was designed to investigate further this hypothesis in liver plasma membrane vesicles. Our results suggest that GSSG and glutathione conjugates share a common ATP-dependent transport system in plasma membrane vesicles, which may be involved in their active extrusion from liver cells.

2. EXPERIMENTAL

A liver plasma membrane fraction, enriched in inverted vesicles, was prepared from male Wistar rats (190–250 g; allow-

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ed food and water ad libitum) by Percoll density gradient centrifugation according to Prpic et al. [11]. Membrane pellets from each preparation were resuspended in 50 mM Tris-HCl, pH 7.4, and immediately used for transport studies. The glutathione *S*-conjugate of 1-chloro-2,4-dinitrobenzene was prepared by incubating 3 mM [^{3}H]GSH and 4 mM 1-chloro-2,4-dinitrobenzene, in the presence of 1 unit glutathione *S*-transferase, in a total volume of 1 ml of 50 mM phosphate buffer (pH 6.5). The formation of *S*-dinitrophenylglutathione (DNP-SG) was monitored by recording the absorption changes at 340 nm in a Shimadzu UV-240 spectrophotometer [12]. Purification of DNP-SG was performed according to Kondo et al. [13]. To determine the uptake of labeled DNP-SG into rat liver plasma membrane vesicles, syringes (1 ml barrel, 5 mm internal diameter) were loaded with Sephadex G-50 (40–80 mesh), suspended in 150 mM NaCl, 2 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.4, placed in a test tube and centrifuged for 2 min at 800 × *g*. Thereafter, aliquots of the reaction mixture (110 μl), containing labeled DNP-SG, plasma membrane vesicles and other additions, were applied on the Sephadex-loaded syringe barrels, and centrifuged as above. The effluent was collected and assayed for radioactivity. Protein content in the effluent was determined by the method of Peterson [14].

3. RESULTS AND DISCUSSION

We have previously reported that the plasma membrane fraction from rat hepatocytes contains an ATPase whose activity is stimulated by GSSG and glutathione conjugates [9,10]. Similarities between the kinetic properties of this activity and those reported earlier for GSSG transport in erythrocytes, led us to propose that this ATPase may be involved in the active extrusion of intracellular GSSG and glutathione conjugates from liver cells. In the present study, we examined the ATP requirement for the transport of a model glutathione conjugate, DNP-SG, in liver plasma membrane vesicles. Addition of Tris-ATP to plasma membrane vesicles in the presence of DNP-SG, 1 mM EGTA, 1 mM ouabain and 2 mM $MgCl_2$, resulted in rapid accumulation of DNP-SG by the vesicles (fig.1). In the absence of ATP, addition of increasing concentrations of $MgCl_2$ did not stimulate DNP-SG uptake, which was about 20% of that observed in the presence of ATP. However, the presence of millimolar concentrations of $MgCl_2$ was required for maximal ATP-stimulated DNP-SG accumulation by the vesicles, and removal of Mg^{2+} from the incubation mixture markedly reduced the ATP-stimulated uptake of DNP-SG in a fashion similar to that observed with other ATP-stimulated transport systems [15].

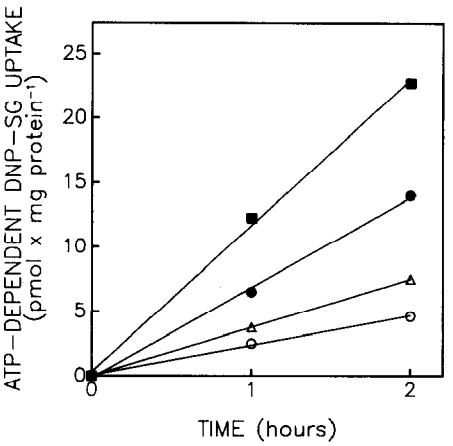

Fig.1. Time course of *S*-dinitrophenylglutathione uptake by liver plasma membrane vesicles. Plasma membrane fraction (≈70 μg protein/ml) was incubated at 37°C with 16 μM DNP-SG in Tris-HCl buffer, pH 7.4, supplemented with 1 mM EGTA, 1 mM ouabain and 180 mM sucrose. Uptake of DNP-SG was determined as described in section 2. (○) In the absence of ATP and Mg^{2+}, (△) plus 2 mM Mg^{2+}, (●) plus 2 mM Tris-ATP, (■) plus 2 mM Mg^{2+} and 2 mM Tris-ATP. One experiment typical of four.

The finding that ATP-stimulated DNP-SG uptake by the vesicles was unaffected by ouabain and EGTA, which were included in the incubation medium to inhibit the Na^+,K^+-ATPase and the Ca^{2+}-ATPase, respectively [9], indicates that the transport of DNP-SG into the vesicles under our experimental conditions was independent of the activity of the two ion pumps. A previous study has suggested that the transport of glutathione conjugates across liver membranes is driven by Na^+ gradients [16]. Although our results do not exclude the existence of an additional, Na^+-driven transport system for glutathione derivatives, a comparison between the affinity of the ATP-stimulated uptake (9 μM) and the Na^+-dependent transport (1 mM) suggests that the latter is less efficient and may be functional only under conditions of extreme intracellular accumulation of GSSG or glutathione conjugates.

To determine whether the time-dependent accumulation of radiolabeled DNP-SG by the vesicles reflected binding of the conjugate to membrane sites, the dependence of DNP-SG uptake on the osmolarity of the medium was investigated. As shown in fig.2, a decrease in vesicle-associated radioactivity was observed when the osmolarity of

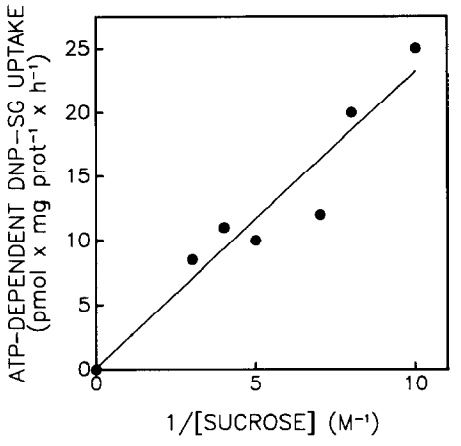

Fig.2. Effect of medium osmolarity on *S*-dinitrophenylglutathione uptake by plasma membrane vesicles. Plasma membrane fraction (≈150 μg protein/ml) was incubated for 60 min at 37°C as described in the legend to fig.1, except that increasing concentrations of sucrose were added to the medium. Tris-ATP (1 mM) was then added to the incubation medium and DNP-SG uptake was determined as described in section 2. One experiment typical of four.

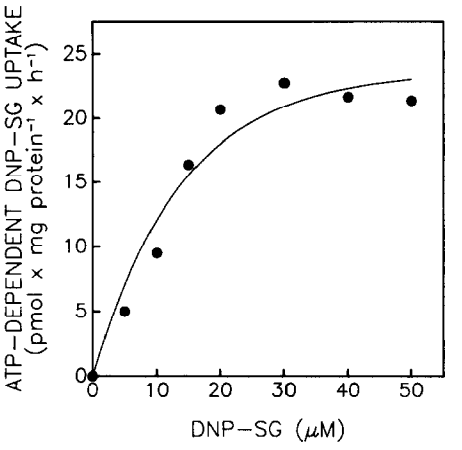

Fig.3. Effect of increasing *S*-dinitrophenylglutathione concentrations on ATP-stimulated DNP-SG uptake by liver plasma membrane vesicles. Plasma membrane fraction (≈70 μg protein/ml) was incubated at 37°C for 60 min in 50 mM Tris-HCl buffer, pH 7.4, in the presence of 3.3 mM Tris-ATP, 2 mM $MgCl_2$, 180 mM sucrose, 1 mM ouabain, 1 mM EGTA, and increasing DNP-SG concentrations. Uptake of DNP-SG was determined as described in section 2. One experiment typical of four.

the medium was increased by the addition of sucrose. Since increased osmolarity would be expected to lower intravesicular volume without affecting non-specific binding, the observed decrease in vesicle-associated radioactivity indicates that the accumulation of DNP-SG was not the result of its binding to membrane sites.

The ATP-dependent uptake of DNP-SG by the vesicles showed saturable kinetics with an apparent K_m of 9 μM and V_{max} of 21 pmol·mg protein^{-1}·h^{-1} (fig.3 and table 1). The ATP requirement was analyzed with increasing concentrations of ATP from 10 μM to 1 mM; transport of DNP-SG was half-maximal with 320 μM ATP. This value is similar to that previously reported for the GSSG-stimulated ATPase activity (≈300 μM) [9], suggesting that the active transport of glutathione conjugates is coupled with the GSSG-stimulated ATP hydrolysis.

As shown in fig.4, uptake of DNP-SG by the vesicles was inhibited when GSSG was present in the incubation medium. In the presence of 50 μM GSSG and a saturating concentration of DNP-SG, transport of the conjugate into the vesicles was decreased to 38% of that observed in the absence of GSSG; 100 μM GSSG inhibited the uptake of DNP-SG completely. In contrast, concentrations of GSH up to 1 mM did not affect DNP-SG uptake (not shown). These results support previous findings, suggesting that GSSG and glutathione conjugates share the same transport system [10,17]. Furthermore, it is also apparent from fig.4 that glutathione conjugates were transported more efficiently than GSSG. Consistent with this interpretation is the observation that infusion into the perfused liver of 1-chloro-2,4-dinitrobenzene, which is conjugated in the liver to form DNP-SG, reduced the biliary excretion of GSSG, presumably due to competition for the canalicular secretory process [17].

Table 1

Kinetics of ATP-stimulated *S*-dinitrophenyl glutathione transport in liver plasma membrane vesicles

K_m(DNP-SG)	9 μM
V_{max}	21 pmol·mg protein^{-1}·h^{-1}
K_m(ATP)	320 μM
Mg^{2+} requirement	millimolar
pH optimum	7.4

Plasma membrane fraction (≈90 μg protein/ml) was incubated with labeled DNP-SG in 50 mM Tris-HCl buffer, pH 7.4, supplemented with 1 mM ouabain and 1 mM EGTA, as described in section 2

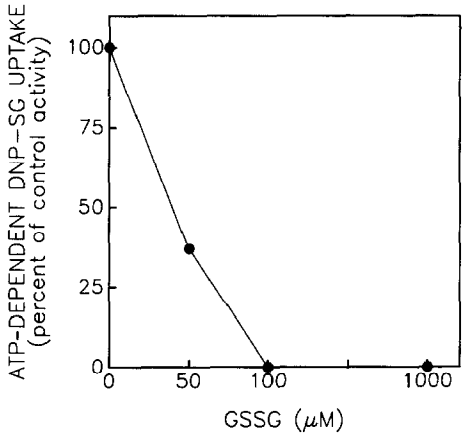

Fig.4. Effect of glutathione disulfide on ATP-stimulated *S*-dinitrophenylglutathione uptake by liver plasma membrane vesicles. Incubation was performed as described in the legend to fig.1 with the exception that increasing GSSG concentrations were added to the incubation medium. Rate of DNP-SG uptake in the absence of GSSG was 20 pmol·mg protein^{-1}·h^{-1}. One experiment typical of four.

The molecular mechanisms underlying the ATP-dependent extrusion of glutathione conjugates and GSSG in hepatocytes need further characterization. However, the similarities between the kinetic parameters observed for DNP-SG-stimulated ATP hydrolysis (K_m = 6 μM) [10] and DNP-SG transport (K_m = 9 μM; present study) in hepatic plasma membrane vesicles support the assumption that the GS-stimulated ATPase activity is involved in the extrusion of glutathione conjugates and GSSG from liver cells. Recently, a similar ATPase activity has been described by Kondo et al. in erythrocytes [18], and studies in our laboratories have identified a GSSG-ATPase activity in human polymorphonuclear leukocytes (Nicotera, P., unpublished). Thus, it appears that the extrusion of GSSG and glutathione conjugates may be mediated by this, or similar ATPase(s) in several different cell types.

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