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Inhibition of taurocholate efflux from rat hepatic canalicular membrane vesicles by glutathione disulfide

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In right-side out rat hepatic canalicular membrane vesicles glutathione disulfide (GSSG) inhibited the efflux of taurocholate approx. 70% in the presence or approx. 55% in the absence of a valinomycin-mediated K^+ diffusion potential; maximal inhibition occurred at 5 mM GSSG. The inhibition by GSSG was abolished by dithioerythritol. Neither dithioerythritol alone nor GSH inhibited taurocholate efflux. *S*-(2,4-Dinitrophenyl)glutathione and *N*-ethylmaleimide showed intermediate inhibitory effects.

Taurocholate transport; Glutathione disulfide; (Rat canalicular membrane vesicle)

1. INTRODUCTION

The hepatic clearance of bile acids depends upon transport across both basolateral and canalicular membranes. Efflux from the hepatocyte into the bile duct, i.e. the canalicular pole, is the rate-limiting step [1]. The in vivo excretion of bile acids can be mimicked by the efflux of taurocholate from isolated right-side out canalicular membrane vesicles [2,3]. Efflux is mediated by a Na^+-independent anion carrier and appears to be driven by the physiologic intracellular negative membrane potential of approx. -30 to -40 mV [2,3].

Previous work has shown that glutathione disulfide (GSSG) is specifically excreted into the bile [4]. In the perfused rat liver, thiol oxidants or hydroperoxides were able to inhibit the excretion of taurocholate leaving hepatic uptake of taurocholate unaffected [5]. Evidence was presented that the inhibition was not due to the thiol oxidants themselves but to the formation of intracellular GSSG [5]. In this study, we have investigated the influence of GSSG and related compounds on taurocholate transport using isolated right-side out canalicular membrane vesicles.

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2. MATERIALS AND METHODS

2.1. *Preparation of rat hepatic canalicular membrane vesicles*

Canalicular membrane vesicles were isolated from 8 to 10 male Wistar rat livers using a combination of rate zonal flotation and high speed centrifugation through a discontinuous sucrose gradient as described by Meier et al. [3,6]. At the conclusion of the various centrifugation steps, the canalicular membrane pellet was resuspended in a medium consisting of 100 mM sucrose, 0.2 mM Ca gluconate, 5 mM Mg gluconate, 100 mM K gluconate and 20 mM Hepes (pH 7.5). To generate a K^+-mediated diffusion potential, the K gluconate was replaced with tetramethylammonium gluconate (100 mM).

The enrichment relative to the liver homogenate (spec. act.) in the canalicular membrane preparation was measured for the following marker enzymes: Mg^{2+}-ATPase [7], 126-fold (1.8 ±

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0.2 U/mg protein); alkaline phosphatase [8], 48-fold (0.2 ± 0.01 U/mg protein); γ-glutamyltranspeptidase [9], 114-fold (25.1 ± 3.2 U/mg protein). The presence of contaminants was ascertained by measuring: NADPH-cytochrome *c* reductase [10], 0.08-fold (1.4 ± 0.4 U/mg protein); succinate cytochrome *c* reductase [11,12], 0.52-fold (3.3 ± 1.7 mU/mg protein); acid phosphatase [8], 1.86-fold (10.8 ± 1.3 mU/mg protein). Isolated canalicular membrane vesicles contained no measurable Na,K-ATPase [7] activity. Protein was measured by the method of Lowry et al. [13] using bovine serum albumin as a standard. Electron micrographs of the isolated canalicular membrane vesicles revealed a pure variable-sized population of membrane vesicles, virtually free of desmosomes, ribosomes and gap junctions, comparable to previous results reported by Meier et al. [6].

2.2. *Transport studies*

Transmembrane transport of [^{3}H]taurocholate was measured by a rapid filtration technique as in [6]. The vesicles were preloaded and revesiculated in the presence of 10 μM taurocholate and valinomycin (10 μg/mg protein). Efflux was started by the addition of 180 μl of incubation medium to 20 μl of preloaded membrane vesicles. Transport was terminated by the addition of 3 ml of ice-cold medium and subsequent rapid filtration was carried out through a 0.45 μm cellulose nitrate filter (Type SM11306, Sartorius GmbH, Göttingen, FRG), prefiltered with 1 ml of cold 1 mM taurocholate to diminish nonspecific filter binding. The filter was washed twice with 3.0 ml of the ice-cold medium and counted in a liquid scintillation counter. All incubations were performed in triplicate within one experiment.

2.3. *Materials*

GSSG, reduced glutathione (GSH), and ouabain were obtained from Boehringer (Mannheim, FRG); taurocholic acid, valinomycin, dithioerythritol, tetramethylammonium hydroxide and the gluconates from Sigma (St. Louis, MO); sucrose, gluconic acid lactone and *N*-ethylmaleimide from Merck (Darmstadt, FRG). All other chemicals and reagents (analytical grade) were purchased from either Sigma, Merck, Boehringer or Serva (Heidelberg, FRG). [^{3}H]Taurocholate (6.8 Ci/mmol) was obtained from NEN (Dreieich, FRG). A glutathione conjugate of 1-chloro-2,4-dinitrobenzene, *S*-(2,4-dinitrophenyl)glutathione was prepared according to the procedure of Awasthi et al. [14].

3. RESULTS

3.1. *Inhibition of taurocholate efflux from canalicular membrane vesicles by the addition of GSSG*

Taurocholate efflux from canalicular membrane vesicles can be stimulated by an artificially induced valinomycin-mediated K^+ diffusion potential (inside negative) indicating a potential-sensitive conductive pathway for the canalicular excretion of taurocholate ([6], see fig.1). Within the first 30 s,

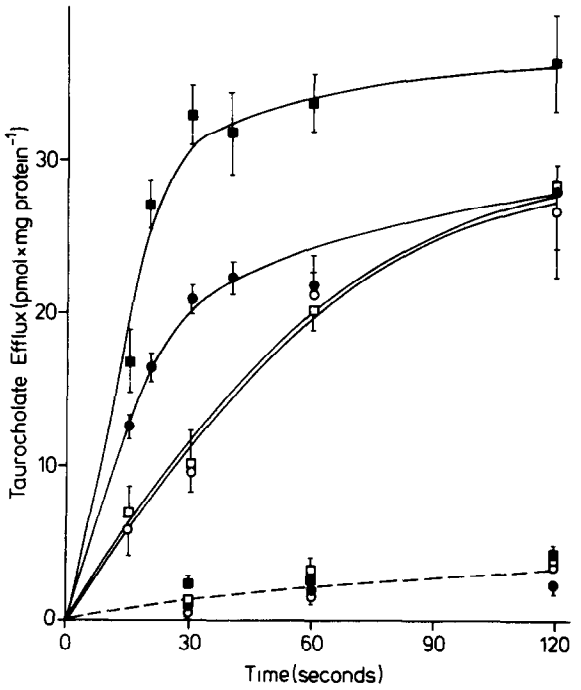

Fig.1. Taurocholate efflux from canalicular membrane vesicles. Transport studies were carried out at 25°C in the presence (■, □) and absence (●,○) of a K^+ diffusion potential (see section 2.3). Control (■,●) and 5 mM GSSG added (□,○). The transport studies were also performed at 0°C (– – –). Equilibrium (60 min) values (as pmol/mg protein) were statistically equivalent. At 25°C it was 60.6 ± 2.6 and at 2°C, 8.5 ± 0.9; means ± SE (n = 4–6, except the 30 s time point, n = 15–25).

there is a rapid and linear transport phase which then levels off. The addition of 5 mM GSSG, placed both intra- and extravesicularly, inhibits the efflux of taurocholate to the same level in both non-gradient and imposed valinomycin-mediated K^+ gradient conditions. In the case of the imposed gradient, the inhibition amounted to 70%. The activities are temperature-sensitive as transport is limited to very low levels at 2°C (fig.1).

3.2. *Concentration dependence of GSSG on taurocholate efflux*

Significant inhibition of taurocholate efflux by GSSG is seen at concentrations above 1 mM (cf. [15]) while the maximal inhibition of taurocholate efflux is reached at concentrations of 5 mM GSSG (fig.2; see fig.1, 30 s time point). The extent of inhibition is independent of the preincubation with the disulfide on ice. Starting the efflux 0, 5 and 30 min after preloading and vesiculation with taurocholate and GSSG had no influence on the transport activities and the concentration dependence of inhibition (fig.2).

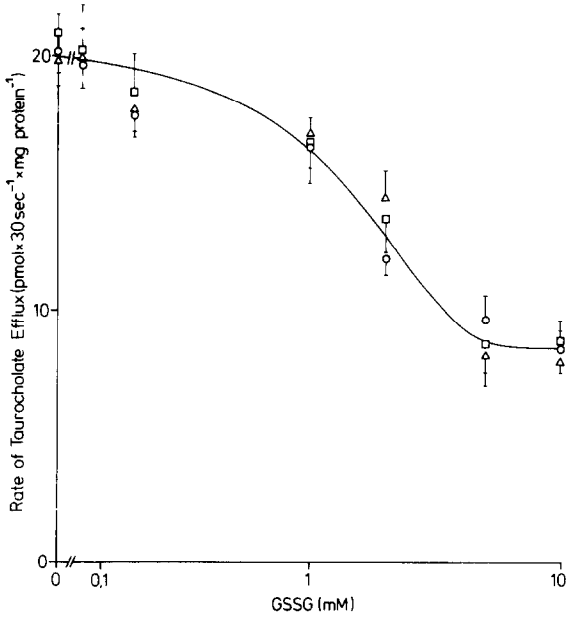

Fig.2. Concentration dependence of GSSG on taurocholate efflux from canalicular membrane vesicles. 30 s transports in the absence of a K^+ gradient were run at 25°C, following preincubation at 2°C for 0 min (○), 5 min (□) and 30 min (△) prior to the initiation of transport; means ± SE (n = 4–6).

Table 1

Influence of different forms of glutathione, dithioerythritol and *N*-ethylmaleimide on taurocholate efflux from canalicular membrane vesicles

Additions	Taurocholate efflux	
	pmol/mg protein per 30 s	%
None	23.3 ± 2.7	100
GSSG (5 mM)	8.3 ± 1.0	36
GSSG (5 mM) + dithioerythritol (7.5 mM)	24.7 ± 2.7	106
Dithioerythritol (7.5 mM)	22.1 ± 2.6	95
S-(2,4-Dinitrophenyl)glutathione (5 mM)	13.4 ± 3.2	58
GSH (5 mM)	24.7 ± 3.0	106
N-Ethylmaleimide (250 μM)	12.9 ± 3.0	55

Values represent transport rates during the first 30 s in the absence of a K^+ diffusion potential; means ± SE (n = 4)

3.3. *Specificity of GSSG inhibition on taurocholate efflux*

The inhibitory effects on taurocholate efflux of several compounds are presented in table 1. The addition of 7.5 mM dithioerythritol to the 5 mM GSSG was sufficient to abolish the inhibition by GSSG. Dithioerythritol alone did not influence the efflux of taurocholate. *S*-(2,4-Dinitrophenyl)-glutathione, at a concentration of 5 mM, showed intermediate inhibitory effects, whereas the addition of 5 mM reduced glutathione showed no inhibition compared to the control. The thiol-binding reagent, *N*-ethylmaleimide, at 250 μM, showed a partial inhibition indicating a possible involvement of protein -SH in the transport mechanism.

4. DISCUSSION

The use of photoaffinity probes substantiate distinct bile acid binding polypeptides in basolateral (54 and 48 kDa) and canalicular (100 kDa) membrane subfractions [16]. Inoue et al. [2] examined the transport of taurocholate from the luminal side into rat canalicular membrane vesicles and demonstrated a temperature-dependent, Na^+-independent transport which

followed saturation kinetics (approximate K_m for taurocholate = 43 μM and a V_{max} = 0.22 nmol/mg protein per 20 s at 37°C) and was inhibited by cholate and probenecid. Meier et al. [3] further investigated this carrier-mediated transport of taurocholate by measuring its efflux from right-side out rat canalicular membrane vesicles. In the isolated perfused rat liver, GSSG efflux occurs into the bile and was substantially increased with hydroperoxides or H_2O_2-generating compounds [4] and stimulation of GSSG formation inhibited the biliary excretion of taurocholate, leaving the net hepatic uptake from the sinusoidal compartment unaffected [5]. Using isolated canalicular membrane vesicles, Akerboom et al. [17] demonstrated that the transport of GSSG across the canalicular membrane though outside-to-inside, is also a carrier-mediated process. The findings presented in the present paper demonstrate that GSSG inhibits the efflux of taurocholate from canalicular membrane vesicles (fig.1). It is of interest that GSSG (5 mM) inhibited taurocholate efflux to similar rates in both non-K^+ gradient and imposed K^+ gradient conditions. During maximal inhibition by GSSG the transport rate was still substantially above the transport rate observed on ice.

Fig.1 also shows that the maximal separation between the control and the GSSG-inhibited transport occurred at 30 s, a time point utilized for the examination of the concentration dependence of GSSG on taurocholate efflux. The inhibition of taurocholate transport at 30 s was the maximum observed (fig.2). There was no effect of preincubation on ice on the concentration dependence of taurocholate efflux (fig.2).

The addition of dithioerythritol to the GSSG prior to its addition to the vesicles and transport media counteracted the inhibitory effect of GSSG (table 1). Dithioerythritol alone, as well as reduced glutathione, had no effect on the transport rate. Taken together, these observations allow the conclusion that the disulfide configuration is required for inhibition. The glutathione conjugate, *S*-(2,4-dinitrophenyl)-glutathione, showed intermediate inhibitory effects. It has been postulated that GSSG behaves like a 'glutathione *S*-conjugate of glutathione'; a homoconjugate handled in a similar way to the heteroconjugates [18]. Partial inhibition also was observed with the thiol-binding reagent, *N*-ethylmaleimide, indicating a possible involvement of protein -SH groups in the mechanism of taurocholate efflux. In conclusion, this work lends support to the hypothesis generated from the isolated perfused rat liver studies [5] that GSSG inhibits taurocholate transport across the canalicular membrane of the hepatocyte.

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