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Inhibition of erythrocyte glutathione conjugate transport by polyethoxylated surfactants

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The surfactant Cremophor EL has recently been shown to reverse the multiple drug resistance phenotype (mdr1) created by over-expression of P glycoprotein, an ATP dependent membrane transport system. Oxidized glutathione and glutathione thioethers are transported out of cells by ATP dependent systems that are distinct from the P glycoprotein pathway. The present study shows that Cremophor EL and a number of other polyethoxylated surfactants inhibit the transport of 2,4-dinitrophenyl glutathione out of intact erythrocytes. The inhibition is not complete, suggesting that this inhibition may differentiate between different glutathione transporters. ATP concentrations are maintained within the cells incubated with Cremophor EL indicating that the inhibition is not mediated by metabolic deprivation.

Glutathione conjugate transport; Inhibition; Cremophor EL; Erythrocyte membrane; Human

1. INTRODUCTION

Glutathione plays a significant role in oxidation/reduction processes within cells and in the metabolism of a range of electrophilic compounds. ATP-dependent transport systems for oxidized glutathione (GSSG) and for glutathione conjugates have been described in membranes from a number of tissues [1–6] and are an important component of a cell's defence against oxidants and reactive electrophiles. The cysteinyl leukotrienes are important proinflammatory mediators and their transport across cell membranes by ATP dependent glutathione conjugate transport systems appears to be a significant factor in their regulation [6]. Little is known about the structure and function of the ATP-dependent glutathione transporters. There is good evidence that there are multiple forms with different affinities for GSSG or thioether conjugates [7,8] and it seems likely that there may be different forms expressed in various tissues [8].

P glycoprotein is another ATP-dependent transporter with broad specificity and is responsible for the efflux of a number of cytotoxic agents used in cancer therapy. The over-expression of P glycoprotein is responsible for the multiple drug resistance phenotype (MDR) [9,10]. Although glutathione transporters and P glycoprotein are considered to be independent, they have a number of features in common that suggest that they may be members of a large family of ATP-dependent transport-related proteins. Recently it was shown [11] that Cremophor EL was able to inhibit the action of P glycoprotein and reverse the MDR phenotype. Cremophor EL is commonly used as a vehicle for the i.v. administration of water-insoluble drugs and may therefore be a useful addition to chemotherapeutic protocols [12].

It was therefore of interest to determine if Cremophor EL inhibited other ATP-dependent membrane-transport processes. In the present investigation the effect of Cremophor EL and other related surfactants on glutathione conjugate transport across the erythrocyte membrane has been studied.

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2. MATERIALS AND METHODS

Blood was freshly obtained from male and female laboratory personnel and anticoagulated with heparin. The transport of 2,4-dinitrophenyl glutathione (GSDNP) from erythrocytes was determined as previously described [2,8]. Erythrocytes were exposed to 1.2 mmol/l of 1-chloro-2,4-dinitrobenzene for 15 min at 37°C, which results in the conversion of more than 70% of intracellular GSH to 2,4-dinitrophenyl conjugate. The erythrocytes were then washed in cold saline to remove excess substrate. The washed erythrocytes were resuspended with a packed cell volume of approximately 30% in phosphate buffered saline pH 7.2 containing 8 mM glucose. The efflux rate was determined by measuring the appearance of the GSH conjugate spectrophotometrically (340 nm) in the extracellular supernatant. As shown previously [2], efflux rates were linear for up to 90 min. Efflux during the first 60 min was used to calculate transport rates.

The surfactants were obtained from a number of sources and used in the dilutions indicated in the text. Cremophor EL and Solutol HS15 were originally obtained from BASF Fine Chemicals (Melbourne, Australia and Ludwigshafen, Germany, respectively) and were provided by Dr. D. Woodcock. CHAPS and Tween 80 were obtained from Sigma Chemical Company, Sydney, Australia.

ATP concentrations were determined in erythrocytes before and after 1 h exposure to a 10^{-3} dilution of Cremophor EL in PBS + 8 mM glucose at 37°C. Samples were precipitated in ice-cold 12% trichloroacetic acid and ATP concentrations in the supernatant were measured spectrophotometrically at 340 nm in a coupled reaction catalysed

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by phosphoglycerate kinase and glyceraldehyde 3 phosphate dehydrogenase. The reagents were obtained from Sigma and the procedure is described in detail in Sigma Technical Bulletin No. 366-UV.

3. RESULTS

Erythrocytes loaded with 2,4-dinitrophenyl glutathione (GSDNP) normally utilize an ATP-dependent system to remove the glutathione conjugate. Exposure of pre-loaded erythrocytes to the surfactant Cremophor EL resulted in substantial inhibition of GSDNP efflux. As shown in Fig. 1, this inhibition was partially concentration-dependent and reached a maximum of about 80%. The shape of the inhibition curve suggests that approximately 20% of the total transport activity is uninhibitable by Cremophor EL. The erythrocytes showed no evidence of hemolysis up to final concentrations of 10% v/v.

The effects of several other surfactants were evaluated (Table I). Tween 80 and Solutol HS15 were found to be even more potent inhibitors of GSDNP transport than Cremophor EL. In contrast, CHAPS had little effect at the same concentration. Other surfactants such as *n*-octylglucoside and *n*-dodecyl-*β*-D-maltoside were tested but caused substantial hemolysis at comparable concentrations.

The inhibition of GSDNP efflux was not readily reversible following the removal of Cremophor EL from the incubation medium. The GSDNP efflux rate was initially determined in erythrocytes treated with Cremophor EL and after 30 min the surfactant was removed by washing the cells in cold PBS plus glucose. As shown in Fig. 2, the rate of efflux from the washed erythrocytes remained inhibited when compared with control samples that had been washed and received identical treatment except for the initial exposure to Cremophor EL.

Previous studies [2] have shown that the transport of GSDNP out of erythrocytes is very sensitive to ATP depletion. In order to determine if the inhibition of GSDNP transport by Cremophor EL and related surfactants results from the depletion of intracellular ATP, erythrocytes were incubated in the presence and absence of a 1:1,000 dilution of Cremophor EL for an hour. As shown in Table II, there was no significant change in intracellular ATP concentrations.

4. DISCUSSION

The results show that some surfactants can significantly inhibit the efflux of GSDNP from crythrocytes. The effective compounds including Cremophor EL, Tween 80 and Solutol HS15 all share polyethoxyl hydrophilic side chains. In contrast, CHAPS which is relatively inactive as an inhibitor does not share this structural feature. Although other surfactants with differing structures were tested, they caused hemolysis of erythrocytes and the transport rates could not be evaluated.

The results obtained in this study are quantitatively similar to those obtained in the study of P glycoprotein and the efflux of daunorubicin [12]. The mechanism of this inhibition is not clear. Fluorescence polarization studies have shown that Cremophor EL causes a significant increase in cell membrane fluidity [12]. This disruption may alter the internal structure of the membrane and the arrangement of integral membrane proteins such as P glycoprotein and presumably the glutathione transporters. Alternatively P glycoprotein and the glutathione transporters may share some common structural feature that is specifically targeted by this polyethoxylated class of surfactants.

Washing red blood cells previously treated with Cremophor EL did not reverse the inhibition, suggesting that the surfactant binds relatively strongly to its targets in the membrane.

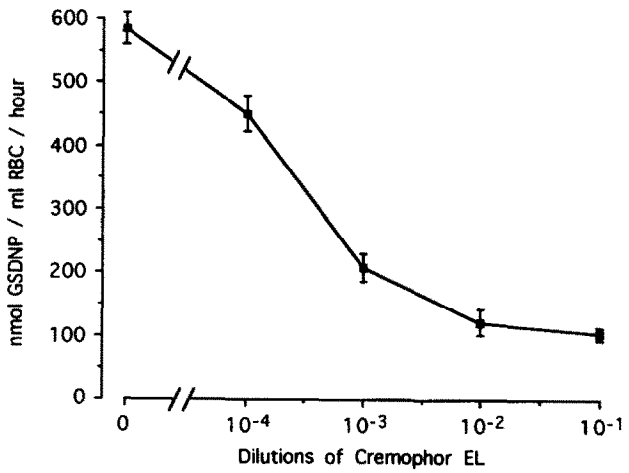

Fig. 1. The effect of increasing concentrations of Cremophor EL on the transport of GSDNP out of erythrocytes. All values are the mean of three experiments ± S.D.

Table I

Inhibition of erythrocyte GSDNP efflux by detergents

Detergent (diluted 1:1,000)	GSDNP (nmol/ml erythrocytes/h)
Control	563 ± 116
Cremophore EL (Polyethoxylated castor oil)	210 ± 22.6
Tween 80 (Polyoxyethylenesorbitan-monooleate)	63 ± 49
Solutol HS15 (Polyethoxylated 12-hydroxystearate 70%, polyethylene glycol 30%)	87 ± 58
CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulphonate)	510 ± 24

All values are the mean of three experiments ± S.D.

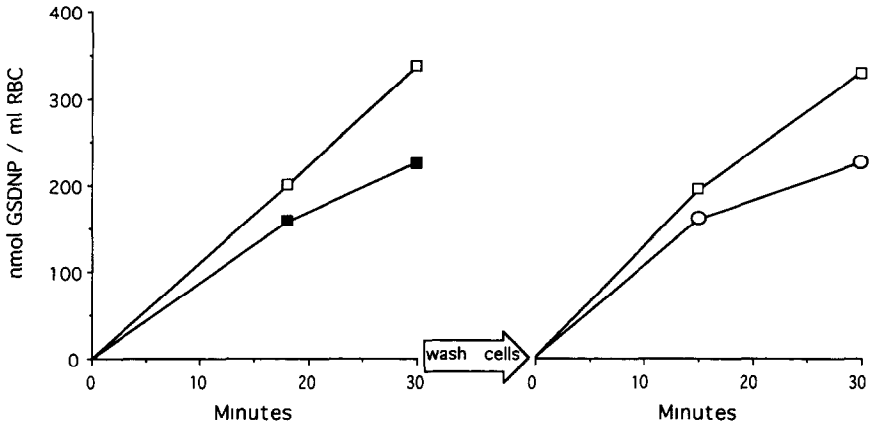

Fig. 2. The effect of removing Cremophor EL from the incubation medium on the reversibility of inhibition. Efflux of GSDNP from red blood cells (RBC) was determined for 30 min in the absence (□) or presence (■) of Cremophor EL at a dilution of 1:1,000. The cells were washed twice in 4 vols. of ice-cold PBS + glucose and the efflux rate redetermined in the untreated control cells (□) and the previously treated cells (○). The values presented are the mean of two experiments.

The transport of GSDNP out of erythrocytes is sensitive to metabolic depletion of ATP [2]. It seemed possible that the disorganization of the erythrocyte membrane by Cremophor EL and related compounds may have increased its permeability to sodium ions. This could lead to metabolic depletion of ATP by the increased activity of the sodium pump. Measurements of erythrocyte ATP concentrations after incubation with Cremophor EL failed to find any evidence of ATP depletion, indicating that this is not the mechanism by which Cremophor EL inhibits GSDNP transport.

There have been several previous suggestions that there may be multiple glutathione transporters in the erythrocyte membrane [7,8,13,14]. The observation that GSDNP transport is not completely inhibited by massive concentrations of Cremophor EL also supports those suggestions. It is possible that the residual activity (approximately 20%) is derived from a separate transporter that is not inhibited. This selective inhibition may allow the dissection of the different components of the glutathione transport systems.

Despite the possible general perturbation of the membrane, it is surprising that concentrations of Cremophor EL up to 10% (v/v) do not cause hemolysis. This observation supports the conclusion that Cremophor EL is a safe solubilizing agent for i.v. drug administration and may have clinical potential in reversing the MDR phenotype in some tumours.

It will be of interest to determine if Cremophor EL and related surfactants are capable of inhibiting other ATP dependent membrane transport processes. Furthermore it will be of importance to determine if possible therapeutic doses are sufficient to inhibit the transport and metabolism of the proinflammatory cysteinyl leukotrienes and thereby influence the course of inflammatory reactions.

Table II

ATP concentrations in erythrocytes incubated with Cremophor EL

Time (min)	No addition	Cremophor (1:1,000 dilution)
0	1.36 ± 0.099	1.32 ± 0.149
60	1.38 ± 0.080	1.47 ± 0.058

Mean of four experiments ± S.D. Concentrations expressed as μmol/ml erythrocytes.

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