Gender related differences in ATP-dependent transport of dinitrophenyl-glutathione conjugate across murine canalicular liver plasma membrane

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Abstract The present study reports gender related differences in ATP-dependent transport of dinitrophenyl-glutathione (GSH) conjugate (DNP-SG), a model GSH xenobiotic conjugate, across murine canalicular liver plasma membrane (cLPM). ATP-dependent transport of DNP-SG across female A/J mouse cLPM was mediated by two components, a high-affinity and a low-affinity component, with corresponding K_m of 18 μM (V_{max} 0.02 nmol/min·mg) and 500 μM (V_{max} 0.23 nmol/min·mg), respectively. On the other hand, only one component for the ATP-dependent transport of DNP-SG was observed in male mouse cLPM (K_m 130 μM; V_{max} 0.18 nmol/min·mg). Moreover, the rate of ATP-dependent transport of DNP-SG was markedly higher in the cLPM fraction of male mouse compared with that of the female. Presence of two transport components in female mouse cLPM, but only one system in the cLPM fraction of male mouse, was confirmed by measuring DNP-SG mediated stimulation of ATP hydrolysis (DNP-SG ATPase activity). To the best of our knowledge, the present study is the first report on gender related differences in ATP-dependent murine canalicular transport of GSH conjugates.

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Key words: Glutathione conjugate; Canalicular transport; Detoxification

1. Introduction

Glutathione (GSH) *S*-transferases (GSTs) are a superfamily of multifunctional proteins that can catalyze the conjugation of a wide variety of endogenous metabolites and xenobiotics with GSH generally leading to their detoxification [1,2]. In some cases, however, the products of the GST-catalyzed conjugation reactions (e.g. GSH conjugate of ethylene dibromide) are relatively more toxic than the parent compounds [3,4]. In liver, the resulting GSH conjugates are transported across canalicular membrane into bile [5,6]. The canalicular transport of GSH conjugates is believed to be an important step in overall scheme of xenobiotic detoxification [5,6]. Canalicular transport of GSH conjugates has been shown to be both ATP-dependent and -independent [7–10]. While relatively low molecular weight and hydrophilic conjugates are substrates only for the ATP-independent mechanism, the larger and hydrophobic conjugates are transported by both ATP-dependent and -independent systems [10].

In this communication, we report gender related differences in ATP-dependent transport of dinitrophenyl-glutathione conjugate (DNP-SG), GSH conjugate of the model electrophile 1-chloro-2,4-dinitrobenzene (CDNB), across murine canalicular liver plasma membrane (cLPM). The results of the present study reveal the presence of two components, a high-affinity and a low-affinity transport system, for the ATP-dependent transport of DNP-SG in female mouse cLPM. On the other hand, only one transport system is present for the ATP-dependent transport of DNP-SG in male mouse cLPM. Moreover, our results indicate that the rate of the ATP-dependent transport of DNP-SG is markedly higher in male mouse cLPM than in that of the female.

2. Materials and methods

2.1. Materials

Male and female A/J mice (8 weeks old) were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD, USA. Reagents including creatine phosphate, creatine kinase, CDNB, sucrose, isobutanol, benzene, ammonium molybdate, EGTA, ouabain and GSH were purchased from Sigma (St. Louis, MO, USA). [^{3}H]GSH (specific radioactivity 44.8 Ci/mmol) and [γ-^{32}P]ATP (specific radioactivity 3000 Ci/mmol) were purchased from DuPont NEN (Boston, MA, USA). The 96-well multi-screen filtration plates containing 0.45 μm nitrocellulose membrane were purchased from Millipore Corporation (Bedford, MA, USA).

2.2. Synthesis and purification of DNP-SG

Non-radiolabeled DNP-SG was synthesized according to the procedure described by Awasthi et al. [11]. The purity of the DNP-SG preparation was checked by reverse-phase HPLC using a Delta Pak C_{18} reverse-phase column (150×3.9 mm). The column was preequilibrated with 100% solvent I (5% acetonitrile/0.1% trifluoroacetic acid). The DNP-SG was eluted with 100% solvent I in 0–5 min, followed by a linear gradient of 0–100% solvent II (90% acetonitrile/0.1% trifluoroacetic acid) in 5–25 min at a flow rate of 1 ml/min. Under these conditions, DNP-SG eluted at a retention time of about 18.8 min [12]. Radioactive DNP-SG was synthesized by reacting 2 μmol CDNB with 0.25 μmol [^{3}H]GSH in 50 mM Tris/HCl, pH 7.4, in the presence of 0.1 mg of purified human placental GST π. The reaction mixture was incubated for 2 h at 37°C. The [^{3}H]DNP-SG was purified by preparative HPLC as described above. The purity of [^{3}H]DNP-SG preparation was checked by reverse-phase HPLC as described above.

2.3. Preparation of cLPM vesicles

The cLPM vesicles were prepared using sucrose density gradient according to the procedure of Meier and Boyer [13] with some modifications. Briefly, 50 g liver tissue from male or female mice was minced, suspended in 200 ml of ice-cold 1 mM sodium bicarbonate buffer, pH 7.4 (buffer A) and homogenized using a Type A glass homogenizer (seven strokes). The homogenate was diluted to 1 l with ice-cold buffer A, filtered twice through glass wool and centrifuged at 1500×*g* for 15 min. The pellet was collected, resuspended in 50% sucrose solution and agitated for 15 min to disrupt membrane aggregates. This preparation was layered at the bottom of a sucrose

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gradient containing 70 ml of 44%, 70 ml of 36.5%, and 40 ml of 8.5% sucrose solution, and centrifuged at 12 500 × *g* overnight. The turbid layer at the interface of 36.5% and 44% sucrose was collected, diluted 10-fold with ice-cold buffer A and centrifuged at 16 000 × *g* for 30 min. The pellet thus obtained was resuspended in buffer A containing 250 mM sucrose and homogenized using a Type B glass homogenizer. The homogenate (2 ml) was layered on top of a sucrose gradient containing 2.5 ml of 31%, 2.5 ml of 34%, and 3 ml of 38% sucrose solution. The samples were centrifuged at 200 000 × *g* for 3 h using a swinging bucket rotor, which resulted in three distinct bands and a pellet. The band at the top of 31% sucrose represented cLPM, which was aspirated using a Pasteur pipette. The cLPM fraction was diluted 10-fold with 10 mM Tris/HCl, pH 7.4, containing 250 mM sucrose (buffer B), and centrifuged again at 105 000 × *g* for 1 h. The pellet thus obtained was resuspended in 2 ml of buffer B, homogenized using a Type B glass homogenizer, and passed through a 27 gauge needle seven times to prepare cLPM vesicles. Cross-contamination of cLPM preparation with basolateral liver plasma membrane (bLPM) fraction was examined for both male and female mouse by determining Na^+,K^+-ATPase and Mg^{2+}-ATPase [14], and alkaline phosphatase [15] activities as described by Meier et al. [16]. The Na^+,K^+-ATPase activity, a marker enzyme for bLPM [16], could not be detected in either male or female mouse cLPM suggesting that these preparations were free from bLPM contamination. Moreover, the purity of the cLPM preparations from male and female mouse were comparable. Thus, the ratios of cLPM/homogenate Mg^{2+}-ATPase activity, an index of cLPM enrichment, were comparable for male and female mouse preparations.

2.4. Determination of [^{3}H]DNP-SG transport

Uptake of [^{3}H]DNP-SG by cLPM vesicles was measured using a rapid filtration technique. Briefly, the cLPM vesicles were passed through a 27 gauge needle five times, and kept on ice throughout the experiment. The incubation mixture in a final volume of 0.1 ml contained buffer B, 15 μg cLPM vesicle protein, 10 mM $MgCl_2$, 10 mM creatine phosphate, 12 units of creatine kinase, 1 mM EGTA, 1 mM ouabain and desired concentration of [^{3}H]DNP-SG (specific radioactivity 277 545 ± 82 985 cpm/nmol). The reaction mixture was preincubated for 5 min at 37°C and the reaction was initiated by the addition of 2 mM ATP (the pH of the ATP solution was adjusted to 7.4 with 0.1 N NaOH). The reaction mixture was incubated at 37°C for 5 min and aliquots (30 μl) were applied under suction to 0.45 μm nitrocellulose filters containing 0.3 ml of ice-cold stop buffer (10 mM Tris/HCl, pH 7.4, containing 250 mM sucrose and 100 mM NaCl). The filters were washed twice with 0.3 ml of stop buffer. Subsequently, the filters were blotted dry, punched out of the plates, and dissolved in scintillation fluid for radioactivity determination. In parallel controls, ATP was replaced with equiosmolar concentration of NaCl (3 mM), and ATP-dependent transport was calculated by subtracting the control transport values from those obtained in the presence of ATP. The kinetic parameters (K_m and V_{max}) were determined by measuring the transport as a function of varying DNP-SG concentration at a fixed concentration of ATP.

2.5. Determination of ATPase activity

The stimulation of ATP hydrolysis (ATPase activity) by DNP-SG in the presence of cLPM protein was measured by the method of Knowles and Leng [17] with some modifications described by us previously [18].

3. Results and discussion

Fig. 1 shows ATP-dependent transport of [^{3}H]DNP-SG as a function of varying cLPM protein concentration and incubation time. ATP-dependent uptake of [^{3}H]DNP-SG by cLPM vesicles increased with increasing protein concentration for both male (Fig. 1A) and female (Fig. 1B). Interestingly, the ATP-dependent transport of [^{3}H]DNP-SG was markedly higher in male mouse cLPM than in that of the female. For

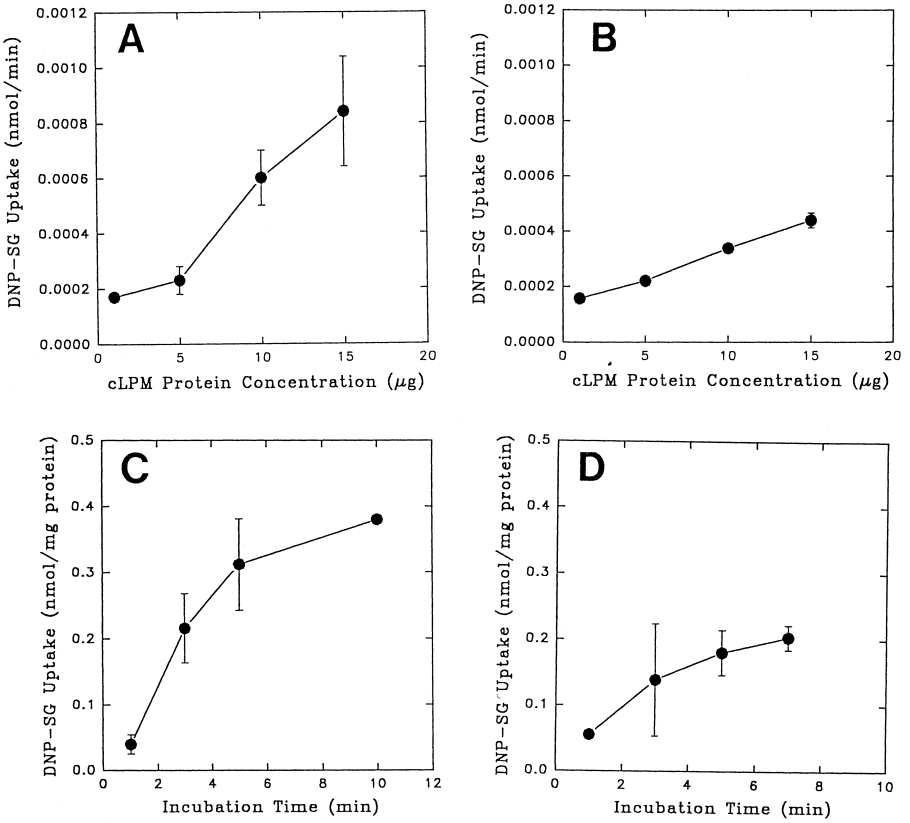

Fig. 1. Protein dependence for the ATP-dependent uptake of [^{3}H]DNP-SG by (A) male mouse cLPM vesicles, and (B) female mouse cLPM vesicles. Data are means ± S.E. of three determinations except for 1 μg cLPM concentration, where $n = 2$. Time course for the ATP-dependent transport of [^{3}H]DNP-SG across (C) male mouse cLPM, and (D) female mouse cLPM. Data are means ± S.E. of three determinations except for 1 min time point for male and 7 min time point for female, where $n = 2$.

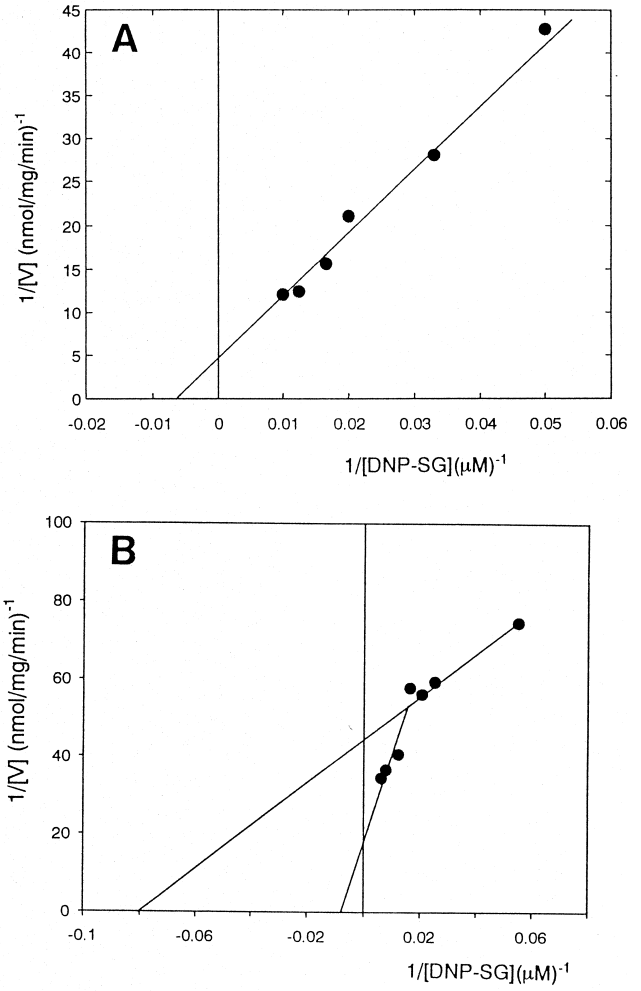

Fig. 2. Lineweaver-Burk plot of the ATP-dependent transport of [^{3}H]DNP-SG across (A) male mouse cLPM, and (B) female mouse cLPM. The [^{3}H]DNP-SG concentration was varied between 20 and 100 μM, whereas ATP concentration was kept constant at 2 mM. Data points are averages of triplicate determinations. Data for female mouse reproduced with permission from Srivastava et al. [12].

example, at 15 μg cLPM concentration, the ATP-dependent transport of [^{3}H]DNP-SG was about 2-fold higher in male mouse cLPM compared with the cLPM fraction of female mouse. As shown in Fig. 1C and D, the ATP-dependent transport of [^{3}H]DNP-SG was rapid and linear up to 5 min for both male and female mouse cLPM. The rate of the ATP-dependent transport of [^{3}H]DNP-SG was relatively higher in male mouse cLPM compared with the cLPM of female mouse.

As shown in Fig. 2A, the ATP-dependent uptake of [^{3}H]DNP-SG by male mouse cLPM vesicles followed Michaelis-Menten kinetics with K_m of 130 ± 40 μM and corresponding V_{max} of 0.18 ± 0.03 nmol/min·mg. On the other hand, the kinetic studies revealed presence of two components, a high-affinity and a low-affinity system, for the ATP-dependent transport of [^{3}H]DNP-SG across female mouse cLPM (Fig. 2B). The K_m values for the high-affinity and the low-affinity transport components of female mouse cLPM were 18 ± 5 μM (V_{max} 0.02 ± 0.012 nmol/min·mg) and 500 ± 200 μM (V_{max} 0.23 ± 0.15 nmol/min·mg), respectively. The ratio of V_{max}/K_m (after appropriate change of units), which is an index of transport efficiency, for the high-affinity and low-affinity components of [^{3}H]DNP-SG transport across female mouse cLPM were about 1 and 0.5, respectively, whereas the value was about 1.4 for male mouse cLPM. These results suggest that only high-affinity components of the ATP-dependent DNP-SG transporter may be expressed in male mouse cLPM.

Gender related differences in transport of DNP-SG across murine cLPM was confirmed by measuring DNP-SG mediated stimulation of ATP hydrolysis (DNP-SG ATPase activity). In agreement with the results of the transport studies, DNP-SG ATPase assays revealed a biphasic kinetics with female mouse cLPM (Fig. 3B), but not with the male mouse cLPM (Fig. 3A). The K_m values for the high-affinity and low-affinity components for DNP-SG ATPase in female mouse cLPM were 18 ± 9 and 484 ± 21 μM, respectively, and the corresponding V_{max} values were 20 ± 9 and 83 ± 23 nmol/min·mg. The K_m and V_{max} values for DNP-SG ATPase activity in male mouse cLPM were 40 ± 17 μM and 17 ± 5 nmol/min·mg, respectively.

The present study demonstrates that DNP-SG, a model

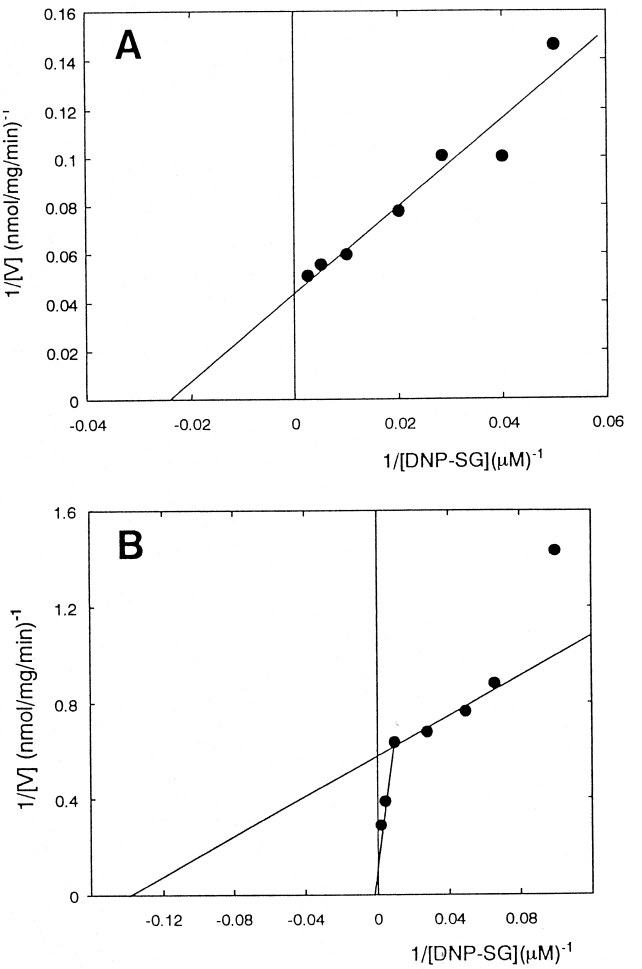

Fig. 3. Lineweaver-Burk plot of the stimulation of ATP hydrolysis (ATPase activity) by DNP-SG in the presence of (A) male mouse cLPM protein, and (B) female mouse cLPM protein. The concentration of the DNP-SG was varied between 15 and 400 μM. The non-specific ATP hydrolysis (basal control) was less than 5% of the experimental values. Data points represent averages of triplicate determinations. Data for female mouse reproduced with permission from Srivastava et al. [12].

compound of glutathione conjugates is transported differentially across male and female mouse cLPM. Previous studies on canalicular transport of DNP-SG have been carried out using male rat cLPM [7–10]. The kinetic constants and the initial transport rate for DNP-SG in male mouse cLPM, determined in the present study, are in agreement with those reported previously for DNP-SG transport across male rat cLPM [7–10]. The results of the present study indicate that only one transport system is present in male mouse cLPM for the ATP-dependent transport of DNP-SG. On the other hand, two components, a high-affinity and a low-affinity system, seem to be present for the ATP-dependent transport of DNP-SG in female mouse cLPM. Moreover, the rate of DNP-SG transport across male mouse cLPM is markedly higher than that of the female. Further evidence for the gender related differences in ATP-dependent transport of DNP-SG across murine cLPM derives from DNP-SG ATPase activity determinations. These assays reveal two components in female mouse cLPM but only one system in the cLPM fraction of male mouse. Even though gender related differences in the expression of GSTs have been documented in mouse and human tissues [19,20], the present study, to the best of our knowledge, is the first report on gender related differences in ATP-dependent canalicular transport of GSH conjugates.

In conclusion, the present study demonstrates that female mouse liver has systems which can eliminate both low and high concentrations of GSH conjugates. On the other hand, only one component seems to be present in male mouse liver for the ATP-dependent canalicular transport of GSH conjugates. However, the structural interrelationships between ATP-dependent GSH conjugate transporters of male and female mouse liver remain to be determined.

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