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Modulation of multidrug resistance-associated proteins function in erythrocytes in glycerol-induced acute renal failure rats

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Keywords
acute renal failure; erythrocytes; inside-out erythrocyte membrane vesicles; multidrug resistance-associated proteins; uraemic toxin

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Abstract

Objectives Evaluation of the function of multidrug resistance-associated proteins (MRPs) expressed in erythrocytes and screening of endogenous MRPs modulator(s) in glycerol-induced acute renal failure (ARF) rats.

Methods Concentrations of 2,4-dinitrophenyl-S-glutathione (DNP-SG), a substrate for MRPs, in erythrocytes after administration of 1-chloro-2,4-dintrobenzene (CDNB), a precursor of DNP-SG, were determined in control and ARF rats. The screening of endogenous MRPs modulator(s) was performed using washed erythrocytes and inside-out erythrocyte membrane vesicles (IOVs) *in vitro*.

Key findings Accumulation of DNP-SG in erythrocytes was observed in ARF rats. Uraemic plasma components exhibited a greater inhibitory effect on DNP-SG uptake by IOVs than control plasma components and increased the DNP-SG accumulation significantly in washed erythrocytes. Several protein-bound uraemic toxins at clinically observed concentrations and bilirubin significantly inhibited DNP-SG uptake by IOVs. In washed erythrocytes, bilirubin (10 μM) and L-kynurenine (100 μM), a precursor of kynurenic acid being MRPs inhibitor, increased DNP-SG accumulation significantly.

Conclusions Glycerol-induced ARF rats contain various MRPs inhibitors in plasma, and membrane-permeable MRP substrates/inhibitors including their precursors inhibit the MRPs function in erythrocytes cooperatively.

Introduction

The elimination of renal disposition drugs is suppressed under renal failure due to the suppression of glomerular filtration rate or transporter-mediated renal secretion. The elimination of non-renal disposition drugs is also suppressed due to the alteration of transport and metabolism under renal failure.[1,2] Under renal failure, various endogenous compounds including uraemic toxins are accumulated in central circulation, and the alteration of transport and metabolism is thought to be caused by uraemic toxins.[3–5] Uraemic toxins are classified into three groups, and middle molecules and protein-bound solutes of uraemic toxins are thought to exert various effects on the function and expression of transporters.[6–8] For example, deproteinized uraemic serum from end-stage renal failure patients increased pravastatin accumulation and decreased the expression of mRNA of multidrug resistance-associated protein (MRP) 2 significantly in Caco-2 cells.[5]

Multidrug resistance-associated proteins family, one of ATP-binding cassette (ABC) efflux transporter superfamily, contains 13 members from MRP1 to MRP13 (*ABCC1-13*), and widely expressed in normal tissues such as intestine, brain, lung, liver, kidney and skeletal muscle.[9] ABC efflux transporters including P-glycoprotein (*ABCB1*), MRPs family and breast cancer resistance protein (BCRP, *ABCG2*) have a role to prevent the influx of endogenous and exogenous xenobiotics into cells and facilitate the efflux from cells to prevent the intracellular accumulation of such xenobiotics as a host defence-detoxification mechanism together with various metabolic enzymes.[10,11] Among them, MRPs transport relatively hydrophilic compounds such as methotrexate, pravastatin and etoposide and various conjugated compounds including glucuronide,

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glutathione and sulphate conjugate of various endogenous and exogenous compounds.[5,9,11] Previously, the MRP function in various tissues was evaluated in rats treated with bilirubin, an endogenous MRPs substrate, intravenously.[12] Evaluation was made by measuring the tissue concentrations of 2,4-dinitrophenyl-S-glutathione (DNP-SG), a substrate of MRPs, after intravenous administration of 1-chloro-2,4-dinitrobenzene (CDNB), a precursor of DNP-SG. CDNB can penetrate biomembranes easily by simple diffusion due to its high lipophilicity and is rapidly metabolized to DNP-SG by glutathione S-transferase (GST) in tissues. In that study, significantly higher DNP-SG concentrations were observed in the brain, liver, jejunum and skeletal muscle in bilirubin-treated rats, suggesting that bilirubin at a higher concentration suppresses MRP-mediated efflux transport systemically.

In this study, the effect of acute renal failure (ARF) on MRPs function in erythrocytes was examined in rats, as erythrocytes express MRP1, MRP4 and MRP5[13,14] and the effect of ARF on MRP function in erythrocytes is not yet evaluated. ARF was induced by intramuscular injection of 50% glycerol after 24-h water deprivation,[15] where the pathogenic mechanisms are due to the ischaemic injury and tubular nephrotoxicity including haemolysis.[16] In haemolytic uraemic syndrome, the high concentration of indirect bilirubin has also been observed in the concentration range from 0.98 to 8 mg/dl (16.8–136.8 μM) in patients.[17] Haemolytic uraemic syndrome is involved in all cases of renal dysfunction.[18] The screening of endogenous modulating compounds against MRPs function was performed using inside-out erythrocyte membrane vesicles (IOVs) and washed erythrocytes *in vitro*.

Materials and Methods

Materials

1-Chloro-2,4-dinitrobenzene, reduced glutathione (GSH), uric acid, uridine, urea, D(−)-mannitol, quinolic acid and Blood Urea Nitrogen B-Test Wako were purchased from Wako Pure Chemicals (Osaka, Japan). 1-Fluoro-2,4-dintrobenzene (FDNB) and hippuric acid sodium salt were obtained from Tokyo Kasei (Tokyo, Japan). Bilirubin, indoxyl sulphate potassium salt, indole-3-acetic acid, kynurenic acid, L-kynurenine, p-cresole, xantine, cytidine, creatine, creatinine and inosine were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). QuantiChrom™ Bilirubin Assay Kit was purchased from BioAssay Systems (Hayward, CA, USA). 3-Carboxy-4-methyl-5-propyl-2-furanopropanoic acid (CMPF) was purchased from Cayman Chemical (Ann Arbor, MI, USA). All other chemicals used were of the highest purity available.

Synthesis of 2,4-dinitrophenyl-S-glutathione

2,4-Dinitrophenyl-S-glutathione, a GSH-conjugated metabolite of CDNB, was synthesized using FDNB according to Hinchman *et al.*[19] Briefly, 2.5 mmol FDNB dissolved in 2.5 ml methanol was slowly mixed with 3.75 mmol GSH dissolved in 1 M $KHCO_3$ (12.5 ml) under stirring. After 15-min incubation at room temperature, the solution was filtered and acidified to approximately pH 2 with diluted HCl. The precipitate was collected by vacuum filtration and was washed with a sufficient amount of distilled water to remove any extra GSH. DNP-SG synthesized was chromatographically pure, as well as those in our previous study.[20,21]

Animal study

Male Sprague–Dawley (SD) rats aged 7–9 weeks old weighing 270–350 g were used. The protocol of the experiments was reviewed and approved in advance, and experiments with animals were performed in accordance with the *Guide for Animal Experimentation* from the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima International University, which is in accordance with the Guidelines for Proper Conduct of Animal Experiments from the Science Council of Japan. The licence number of this animal study was AE15-005. ARF was induced by an injection of 50% glycerol (10 ml/kg) into the leg muscles after a 24-h period of water deprivation in SD rats.[15] Control rats received the same volume of saline. The induction of ARF states was confirmed by determining the concentrations of blood urea nitrogen (BUN), indoxyl sulphate, direct (conjugated) and indirect (unconjugated) bilirubin in plasma 24 h after glycerol treatment.

Evaluation of multidrug resistance-associated protein function in erythrocytes in rats *in vivo*

Multidrug resistance-associated protein function in erythrocytes was evaluated by determining the DNP-SG concentration in erythrocytes after administration of CDNB in rats. Control and ARF rats were anaesthetized by an intraperitoneal injection of pentobarbital (40 mg/kg) and received CDNB (30 μmol/kg) intravenously from the jugular vein, or by a constant rate infusion (6 μmol/2 ml/h) via the cannulae (polyethylene tubing PE50; Natsume Seisakusho, Tokyo, Japan) inserted at the femoral vein. Blood (0.4 ml each) was collected periodically from jugular vein. A part of blood sample was centrifuged at 8000*g* for 15 min to obtain plasma sample. A part of blood sample was filled into haematocrit tubes (75 mm; Drummond Scientific Company, Broomall, PA, USA) to measure haematocrit value. Haematocrit tubes containing blood sample were

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centrifuged at 15 000 g using haematocrit rotor (Kubota 3220; Kubota, Tokyo, Japan). The concentration of DNP-SG in whole blood was measured after haemolysis of blood sample (50 μl) by adding 250 μl of distilled water. The concentration of DNP-SG in erythrocytes was calculated using the following equation: $C_e = [C_w - C_p \times (1 - H_t)]/H_t$, where C_e, C_w, C_p and H_t represent the erythrocytes concentration, whole blood concentration, plasma concentration of DNP-SG and haematocrit, respectively.

Preparation of inside-out erythrocyte membrane vesicles

Inside-out erythrocyte membrane vesicles were prepared from rat erythrocytes by a spontaneous, one-step vesiculation method.[22] Briefly, rat erythrocytes were obtained by washing fresh whole blood with fivefold volume of isotonic buffer (70 mM NaCl, 80 mM KCl, 10 mM HEPES, 0.1 mM EGTA, pH 7.5) three times. The packed erythrocyte cells were lysed by 60-fold volume of ice-cold hypotonic buffer (2 mM HEPES, 0.1 mM EGTA, pH 7.5), and pellet ghosts were obtained by centrifugation (40 000*g*, 20 min, 4 °C). The pellet ghosts were suspended with a half volume of hypotonic buffer and incubated at 37 °C for 30 min, and were dispersed in 10 ml of the suspension buffer (100 mM mannitol, 10 mM HEPES/Tris, pH 7.4), The suspension was centrifuged (40 000*g*, 20 min, 4 °C) to remove the supernatant. The pellet including IOVs was re-suspended in suspension buffer and was stored at −80 °C until use. The fraction of IOVs in all vesicles was estimated to be 37.5% by measuring the acetylcholine esterase activity in the absence and presence of 0.2% Triton-X.[22]

Collection of plasma components

Plasma obtained from control and ARF rats was deproteinized by mixing with the same volume of acetonitrile. After centrifugation at 1500*g* for 10 min, the supernatant containing acetonitrile (50%) was collected and evaporated to dryness with evaporator (EYELA TVE-1000; Tokyo Rikakikai, Tokyo, Japan). The residue was stored in freezer kept at −20 °C until use.

Screening of multidrug resistance-associated proteins modulator using inside-out erythrocyte membrane vesicles *in vitro*

Effects of various endogenous compounds on MRP-mediated DNP-SG uptake were examined using IOVs in the same manner as reported.[22] Briefly, IOVs (5 mg protein/ml) were incubated with DNP-SG (20 μM) in the presence of 1 mM ATP or 1 mM AMP at pH 7.4 and 37 °C, and various compounds such as plasma components and uraemic toxins were added to the incubation medium. Concentrations of uraemic toxins used were the concentrations reported in various renal failure patients.[23] The compounds examined were dissolved in incubation medium containing a small amount of dimethyl sulfoxide (DMSO), and the final concentration of DMSO in incubation medium was adjusted at 1%. Incubation was made for 30 min, and the ATP-dependent uptake of DNP-SG in IOVs was calculated by subtracting the uptake in the presence of AMP from that in the presence of ATP.

Screening of multidrug resistance-associated proteins modulator using washed erythrocytes *in vitro*

Blood was obtained from untreated control rats and washed three times with fivefold volume of ice-cold isotonic medium (140 mM NaCl, 5 mM KCl, 20 mM Tris/HCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 5 mM glucose, pH 7.4) by repeating centrifugation and dilution with isotonic medium. Haematocrit of washed erythrocytes was adjusted to 0.45 by adding ice-cold isotonic medium to packed erythrocytes and centrifuging it using haematocrit rotor (Kubota 3220; Kubota) to confirm the haematocrit value. The erythrocytes suspension was incubated for 1 h on ice. After incubation, 100 μl of isotonic medium containing endogenous compounds (uraemic toxin or plasma components) was added to the washed erythrocytes suspension (100 μl), and the suspension was incubated at 37 °C for 15 min. The accumulation study of DNP-SG in erythrocytes was started by adding 100 μl of CDNB (30 μM) dissolved in isotonic medium containing a small amount of DMSO. The final concentrations of CDNB and DMSO in the washed erythrocytes suspension were 10 μM and 1%, respectively. After 15 min, the incubation was stopped by adding threefold volume of ice-cold isotonic medium. The suspension was centrifuged at 1500*g* and 4 °C for 10 min, and the procedure was repeated twice. After haemolysis of erythrocytes pellet by adding fivefold distilled water, protein and DNP-SG concentrations were determined.

Sample analysis

Concentration of blood urea nitrogen (BUN) was determined using Blood Urea Nitrogen B-Test Wako. Concentrations of total and direct bilirubin were determined using QuantiChrom™ Bilirubin Assay Kit. Concentration of protein was measured by Bradford method using bovine serum albumin as the standard.[24] After deproteinization of DNP-SG samples by adding perchloric acid (final concentration was more than 4%) and indoxyl sulphate samples by adding twofold volume of methanol, the concentrations of DNP-SG and indoxyl sulphate were determined by HPLC using a column of J-Pak Wrapsil RP-18 (Jasco Engineering, Tokyo,

Japan). Mobile phases were a mixture of 1% acetic acid and acetonitrile (85 : 15 (v/v)) for DNP-SG and a mixture of pH 4.5, 0.2 M acetate buffer, and acetonitrile (93 : 7 (v/v)) for indoxyl sulphate, respectively. DNP-SG was detected at UV 365 nm, and indoxyl sulphate was detected by fluorometer at Ex. 280 nm and Em. 375 nm.

Table 1 Biochemical data of control and acute renal failure (ARF) rats

	Control	ARF
BUN (mg/dl)	13.7 ± 0.70	124 ± 6.94**
Indoxyl sulphate (μM)	3.63 ± 0.61	103 ± 10.6**
Total bilirubin (μM)	1.76 ± 0.09	29.1 ± 7.96*
Direct bilirubin (μM)	1.01 ± 0.12	3.01 ± 0.62*

BUN, blood urea nitrogen. ARF rats were induced by intramuscular injection of 50% glycerol (10 ml/kg, v/v) after 24-h water deprivation in rats. Data were obtained 24 h after the treatment with saline (Control) or 50% glycerol (ARF). Each value represents the mean ± SE of results from three to four rats. $^{**}P < 0.01$ and $^{*}P < 0.05$: significantly different from the value for control.

Statistical analysis

Data were presented as the mean ± SE. Statistical analysis was performed by Student's *t*-test, or by one-way analysis of variance followed by the Tukey test for multiple comparisons. A difference of $^{*}P < 0.05$ was regarded as significantly different from the corresponding value.

Results

Biochemical data of control and acute renal failure rats

To confirm the induction of ARF states, plasma concentrations of BUN, indoxyl sulphate and bilirubin were determined in control and ARF rats (Table 1). In ARF rats, concentrations of BUN and indoxyl sulphate were approximately ninefold and 28-fold higher than those in control rats, respectively. The plasma concentrations of total and direct bilirubin, endogenous MRPs substrate/inhibitor, in ARF rats were also significantly higher than those in control rats.

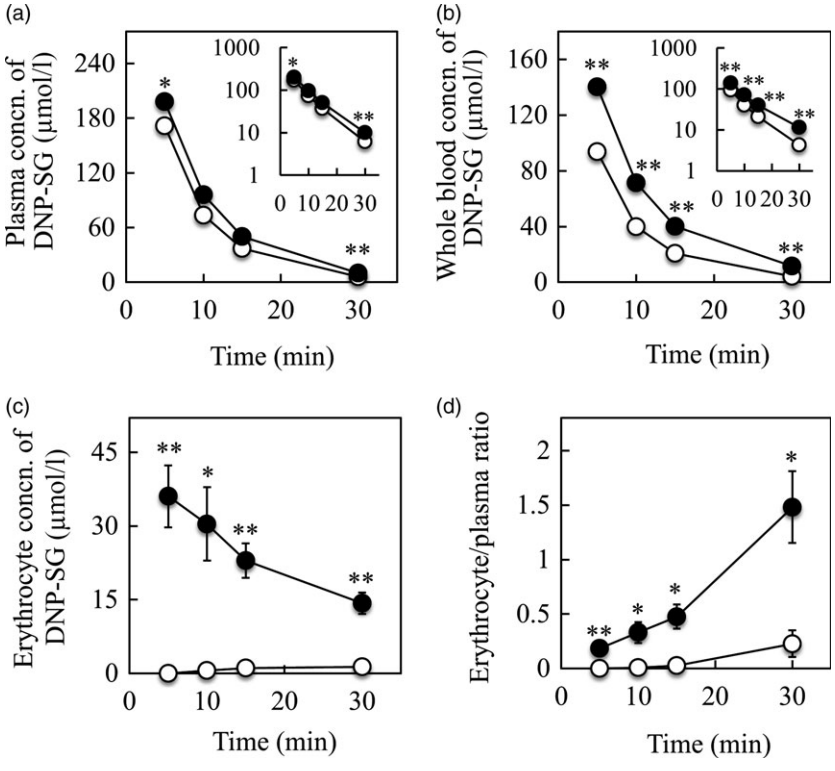

Figure 1 Concentrations of 2,4-dinitrophenyl-S-glutathione in plasma (a), whole blood (b) and erythrocytes (c), and erythrocyte/plasma concentration ratio (d) after intravenous administration of 1-chloro-2,4-dintrobenzene (30 μmol/kg) in control and acute renal failure rats. Rats received saline (control) or 50% glycerol (acute renal failure) at a volume of 10 ml/kg and were used 24 h after treatment. Open and closed circles represent the results in control and acute renal failure rats, respectively. Each value represents the mean ± SE of results from three rats. $^{*}P < 0.05$, $^{**}P < 0.01$: significantly different from the value for control.

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Accumulation of 2,4-dinitrophenyl-S-glutathione in erythrocytes in acute renal failure rats

1-Chloro-2,4-dinitrobenzene was administered intravenously or by a constant rate infusion, and concentrations of DNP-SG in erythrocytes were determined in control and ARF rats (Figures 1a and 2a). The difference in DNP-SG plasma concentrations between control and ARF rats was small. In contrast, ARF rats exhibited significantly higher blood concentrations of DNP-SG compared to control rats, indicating that DNP-SG is accumulated in erythrocytes in ARF rats (Figures 1b, 1c, 2b). Although there was no relationship in plasma concentrations between DNP-SG and indoxyl sulphate, an uraemic toxin, the higher indoxyl sulphate concentrations resulted in the higher DNP-SG accumulation in erythrocytes in ARF rats (Figure 2d).

Effect of plasma components on 2,4-dinitrophenyl-S-glutathione accumulation in washed erythrocytes

2,4-Dinitrophenyl-S-glutathione concentrations in washed erythrocytes after application of CDNB were determined (Figure 3). Plasma components (corresponding to 10% deproteinized plasma) obtained from control rats exerted no significant effect on DNP-SG accumulation in washed erythrocytes. In contrast, plasma components (corresponding to 10% deproteinized plasma) obtained from ARF rats significantly increased DNP-SG accumulation in erythrocytes by approximately 3.5-fold of control, indicating that plasma of ARF rats contains some MRP modulator(s).

Screening of multidrug resistance-associated proteins modulator using inside-out erythrocyte membrane vesicles *in vitro*

MK-571 and probenecid, both are typical MRP inhibitors, inhibited the DNP-SG uptake by IOVs significantly (Figure 4). Bilirubin, an endogenous MRPs substrate but not uraemic toxin, also inhibited DNP-SG uptake by IOVs significantly. Plasma components obtained from control and ARF rats significantly inhibited the DNP-SG uptake by IOVs in a concentration-dependent manner, and greater inhibitory effects were observed with ARF plasma components (Figure 4). All small water-soluble solutes of uraemic toxins examined in this study exerted no significant effect on DNP-SG uptake by IOVs (Figure 5a). In contrast,

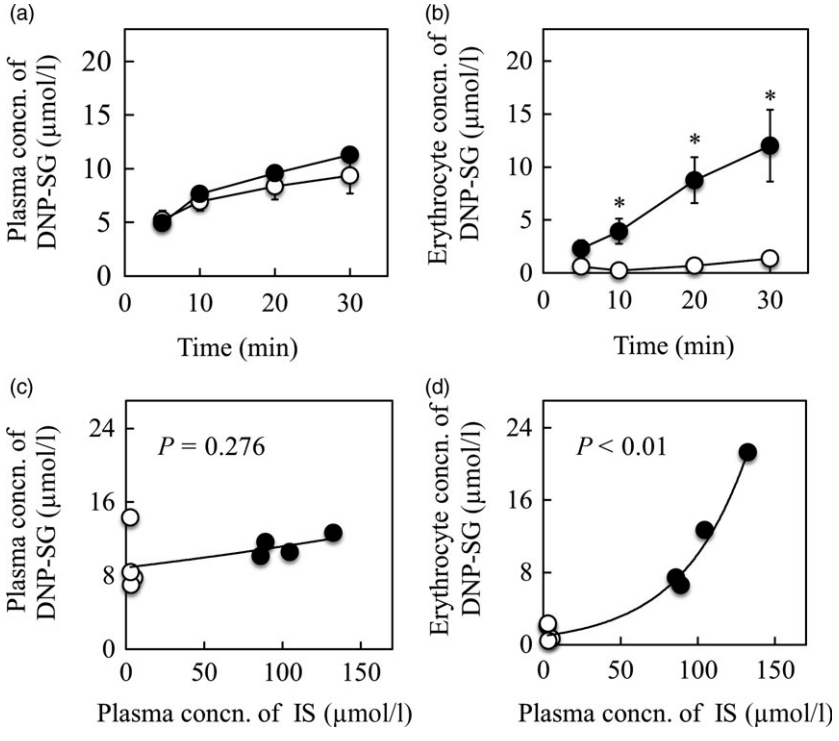

Figure 2 Concentrations of 2,4-dinitrophenyl-S-glutathione in plasma (a) and erythrocytes (b) during the constant rate infusion of 1-chloro-2,4-dinitrobenzene (6 μmol/2 ml/h), and the relationship of 2,4-dinitrophenyl-S-glutathione concentrations in plasma (c) and erythrocytes (d) with plasma concentrations of indoxyl sulphate at 30 min in control and acute renal failure rats. Rats received saline (control) or 50% glycerol (acute renal failure) at a volume of 10 ml/kg and were used 24 h after treatment. Open and closed circles represent the results in control and acute renal failure rats, respectively. Each value represents the mean ± SE of results from four rats. *$P < 0.05$: significantly different from the value for control.

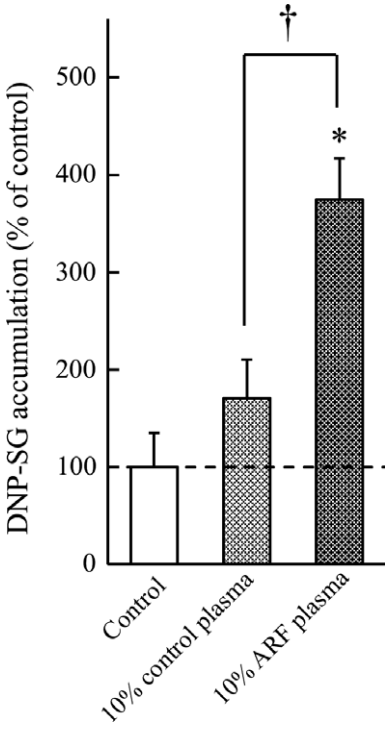

Figure 3 Effect of plasma components obtained from control and acute renal failure rats on 2,4-dinitrophenyl-S-glutathione accumulation after application of 1-chloro-2,4-dintrobenzene in washed erythrocytes. Control value represents the concentration of 2,4-dinitrophenyl-S-glutathione in washed erythrocytes in the absence of plasma component in medium (37 °C). Plasma components were obtained from deproteinized plasma of control and acute renal failure rats (24 h after glycerol treatment). Concentrations of 2,4-dinitrophenyl-S-glutathione in erythrocytes were determined 15 min after application of 1-chloro-2,4-dintrobenzene (10 μM). Each value represents the mean ± SE of results from four trials. $*P < 0.05$: significantly different from the value for control. $^{\dagger}P < 0.05$: significantly different from the value for control plasma.

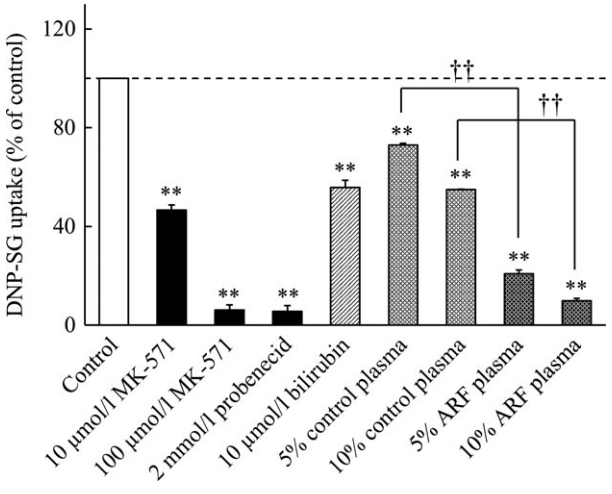

Figure 4 Effects of MK-571, bilirubin, probenecid and plasma components obtained from control and acute renal failure rats on 2,4-dinitrophenyl-S-glutathione uptake by inside-out erythrocyte membrane vesicles. Control represents the results in the absence of any multidrug resistance-associated protein substrate/inhibitor in the medium (37 °C). Plasma components were obtained from deproteinized plasma of control and acute renal failure rats (24 h after glycerol treatment). Inside-out erythrocyte membrane vesicles were incubated with 2,4-dinitrophenyl-S-glutathione (20 μM) for 30 min at 37 °C. Each value represents the mean ± SE of results from three trials. $**P < 0.01$: significantly different from the value for control. $^{\dagger\dagger}P < 0.01$: significantly different from the value for control plasma.

protein-bound solutes of uraemic toxins such as p-cresol, hippuric acid, indoxyl sulphate, indole-3-acetic acid, kynurenic acid and CMPF significantly inhibited DNP-SG uptake by IOVs (Figure 5b).

Screening of multidrug resistance-associated proteins modulator by washed erythrocytes *in vitro*

MK-571 and bilirubin significantly increased DNP-SG accumulation in washed erythrocytes after application of CDNB (Figure 6a). Protein-bound solutes of uraemic toxins exerted no significant effect on DNP-SG accumulation in washed erythrocytes (Figure 6b), different from the case of IOVs (Figure 5b). Among them, however, L-kynurenine, a precursor of kynurenic acid, and CMPF showed an increasing tendency of DNP-SG accumulation in washed erythrocytes.

Different effects of kynurenic acid and L-kynurenine on MRP function between IOVs and washed erythrocytes

Kynurenic acid inhibited DNP-SG uptake by IOVs in a concentration-dependent manner, whereas L-kynurenine, a precursor of kynurenic acid, did not show any inhibitory effect on DNP-SG uptake even at a concentration of 100 μM (Figure 7a). In contrast, kynurenic acid did not show any significant effect on DNP-SG accumulation in washed erythrocytes, but L-kynurenine clearly increased the DNP-SG concentration in erythrocytes in a concentration-dependent manner (Figure 7b).

Discussion

The effect of glycerol-induced ARF state on MRP function in erythrocytes was evaluated in rats. In control rats *in vivo*, DNP-SG was not accumulated in erythrocytes after administration of CDNB (Figures 1c and 2b), possibly due to the rapid metabolism of CDNB to DNP-SG by GST in erythrocytes followed by rapid

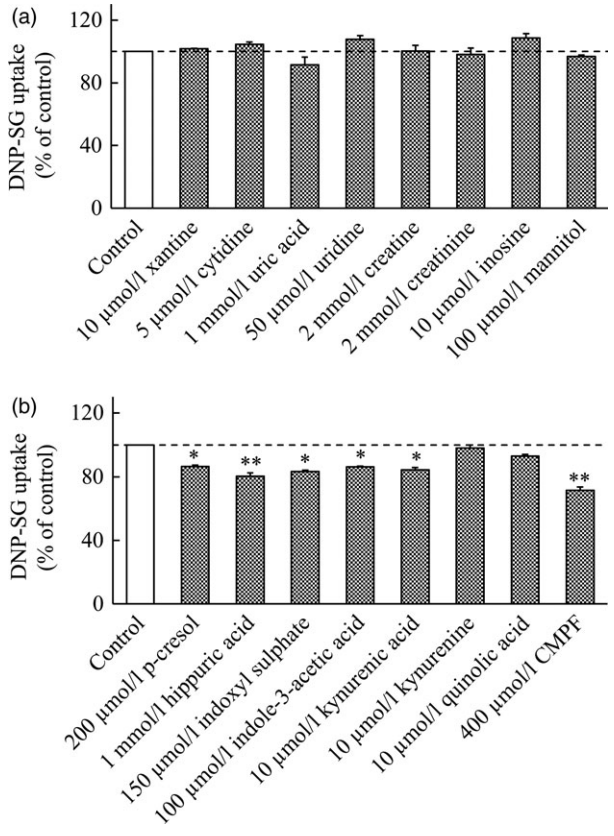

Figure 5 Effects of small water-soluble solutes (a) and protein-bound solutes (b) of uraemic toxins on 2,4-dinitrophenyl-S-glutathione uptake by inside-out erythrocyte membrane vesicles (IOVs). Inside-out erythrocyte membrane vesicles were incubated with 2,4-dinitrophenyl-S-glutathione (20 μM) for 30 min at 37 °C. Each value represents the mean ± SE of results from three trials. $*P < 0.05$, $**P < 0.01$: significantly different from the value for control.

MRPs-mediated efflux of DNP-SG from erythrocytes. In contrast, in glycerol-induced ARF rats, DNP-SG was greatly accumulated in erythrocytes (Figures 1c and 2b), indicating that the MRPs-mediated efflux transport of DNP-SG in erythrocytes was suppressed in glycerol-induced ARF rats. The screening of modulator(s) for MRPs function was performed using IOVs and washed erythrocytes (without plasma protein) *in vitro*. IOVs of erythrocytes would be used as a simple and rapid screening tool of MRPs modulators against erythrocytes.[22,25] However, IOVs technique does not involve the membrane permeability process of testing modulating compounds. To evaluate both of the membrane permeability and MRP inhibitory effect of test compounds, washed erythrocyte technique is preferable, in which the maximal membrane permeability and therefore the maximal MRP modulating effect can be obtained, because of the absence of plasma protein binding, different from the case of *in vivo* condition. In addition, the metabolism of CDNB to DNP-SG in plasma is eliminated, different from the case in *in vivo* condition. In IOVs, plasma components obtained from control and ARF rats significantly increased DNP-SG accumulations, and ARF plasma components showed greater DNP-SG accumulation, indicating that MRP inhibitors are contained in plasma of control and ARF rats, and the amount is greater in ARF rats than untreated control rats (Figure 4). In the uptake study of DNP-SG by IOVs, most of protein-bound uraemic toxins examined inhibited DNP-SG uptake significantly, in addition to endogenous MRP substrate bilirubin (Figure 5). In washed erythrocytes, 10% plasma components obtained from ARF rats exhibited great accumulation of DNP-SG than that obtained from control rats (Figure 3). These results would further suggest that membrane-permeable MRP inhibitors are present in ARF plasma at a greater amount than control rats. Among various test compounds, bilirubin alone increased DNP-SG accumulation, and uraemic toxins of protein-bound solutes examined did not increase DNP-SG accumulation significantly (Figure 6). The discrepancy of inhibitory effects of protein-bound solutes between IOVs and washed erythrocytes was considered to be derived from the membrane permeation process of protein-bound solutes. In inhibiting DNP-SG uptake by IOVs, the membrane permeation of uraemic toxin is not necessary, because MRP transporter is located outside of erythrocyte vesicles. In contrast, to inhibit MRP function in erythrocytes, inhibitors must enter inside of erythrocytes across erythrocyte membranes. In washed erythrocytes, L-kynurenine and CMPF showed an increasing tendency of DNP-SG accumulation, although the effect did not reach significant difference (Figure 6). To examine the possible membrane permeation of L-kynurenine, the inhibitory effects of kynurenic acid and L-kynurenine were compared in IOVs and washed erythrocytes by increasing their concentrations. Kynurenic acid significantly inhibited the DNP-SG uptake by IOVs in a concentration-dependent manner, but exerted no significant effect on DNP-SG accumulation in washed erythrocytes. In contrast, L-kynurenine exerted no significant effect on DNP-SG uptake by IOVs but increased DNP-SG accumulation significantly in washed erythrocytes in a concentration-dependent manner. Based on these results, L-kynurenine is considered to be taken up by erythrocytes and metabolized to kynurenic acid in erythrocytes. In contrast, kynurenic acid being MRPs inhibitor cannot penetrate erythrocyte membrane. L-Kynurenine is known as a substrate for L-amino acid transporter, and L-amino acid transporter is expressed in erythrocyte membranes.[26] Also, kynurenine aminotransferase, a metabolic enzyme converting L-kynurenine to kynurenic acid, exists in erythrocytes.[27,28] In addition, CMPF also tended to increase DNP-SG

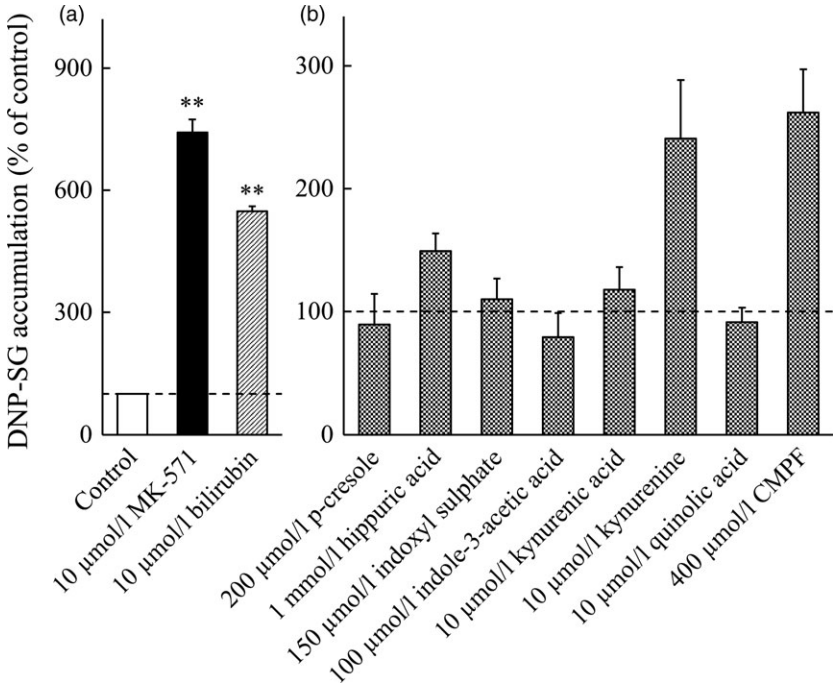

Figure 6 Effects of MK-571 and bilirubin (a) and various protein-bound solutes of uraemic toxins (b) on 2,4-dinitrophenyl-S-glutathione accumulation after application of 1-chloro-2,4-dintrobenzene in washed erythrocytes. Control value represents the results in the absence of multidrug resistance-associated protein substrate/inhibitor in the medium (37 °C). Concentrations of 2,4-dinitrophenyl-S-glutathione in erythrocytes were determined 15 min after application of 1-chloro-2,4-dintrobenzene (10 μM) at 37 °C. Each value represents the mean ± SE of results from three trials. $^{**}P < 0.01$: significantly different from the value for control.

accumulation in erythrocytes (Figure 6). The partition coefficient (log P) of CMPF between hydrochloric acid and octanol is 1.2, and the distribution coefficient (log D) between octanol and phosphate buffer at pH 7.4 is −0.59, suggesting the possibility of membrane permeation by simple perfusion due to its slight hydrophobic character.[29,30]

Among various endogenous substances examined in the present study, bilirubin significantly suppressed MRP-mediated transport in IOVs and washed erythrocytes. The plasma concentrations of total bilirubin and direct bilirubin were significantly increased in glycerol-induced ARF rats, compared to control rats (Table 1), possibly due to the haemolysis caused by glycerol. Indirect bilirubin (unconjugated bilirubin) is a compound with high permeability through lipid bilayer membrane.[31,32] Recently, the uphill transport of indirect bilirubin from plasma to the liver was also reported.[32] Indirect bilirubin is metabolized to glutathione-conjugated bilirubin, a MRP substrate, by glutathione S-transferases (GST) in hepatic cytosol.[33,34] GST also exists in rat erythrocytes.[35] Thus, it may be considered that a part of bilirubin distributed into erythrocytes by passive diffusion is conjugated by glutathione, and both indirect and direct bilirubin may inhibit MRP function in erythrocytes. In normal condition, however, bilirubin binds to albumin extensively at the level of more than 99.9%,[32] indicating that the unbound bilirubin concentration in plasma is very low. However, it is also known that the protein binding of bilirubin is decreased in renal patients, and CMPF was suggested as one of the substances which contribute to the decreased binding capacity of bilirubin in uraemic serum.[36] In general, bilirubin is excreted into the intestinal lumen by biliary excretion, not into urine under normal healthy condition, and the concentration of bilirubin in plasma increases only under hepatic failure state. However, as described already in the introduction, the high concentration of indirect bilirubin has also been observed in haemolytic uraemic syndrome patients,[17] and the haemolytic uraemic syndrome is involved in all cases of renal dysfunction.[18] In the present study, the plasma concentration of bilirubin, in addition to uraemic toxins such as BUN and IS, was significantly increased in glycerol-induced ARF rats, possibly by haemolytic action of glycerol. It has been reported that the P-glycoprotein function in the liver was significantly suppressed in glycerol-induced rats, in which the biliary excretion of rhodamine 123, a typical substrate for P-glycoprotein, was significantly decreased in ARF rats.[15,37] Thus, it

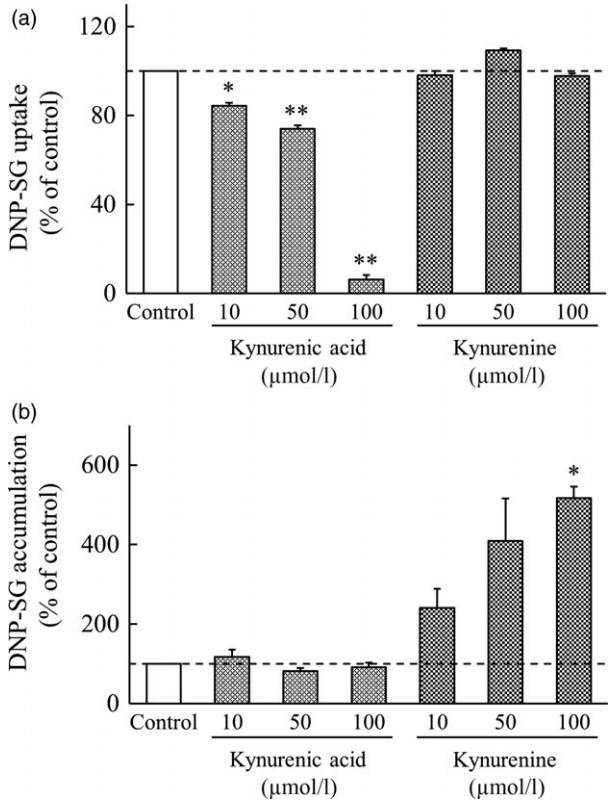

Figure 7 Effects of kynurenic acid and L-kynurenine on 2,4-dinitrophenyl-S-glutathione uptake by inside-out erythrocyte membrane vesicles (a) and on 2,4-dinitrophenyl-S-glutathione accumulation after application of 1-chloro-2,4-dintrobenzene in washed erythrocytes (b). Inside-out erythrocyte membrane vesicles were incubated with 2,4-dinitrophenyl-S-glutathione (20 μM) for 30 min at 37 °C. Concentrations of 2,4-dinitrophenyl-S-glutathione in erythrocytes were determined 15 min after application of 1-chloro-2,4-dintrobenzene (10 μM) at 37 °C. Each value represents the mean ± SE of results from three trials. $*P < 0.05$, $**P < 0.01$: significantly different from the value for control.

may be necessary to examine the effect of glycerol-induced ARF state on the function of MRP2 expressed on the bile canalicular membrane, to clarify the mechanism of the increased plasma level of bilirubin in glycerol-induced ARF rats. In addition, it will be important to examine the effect of the bilirubin accumulation in plasma on renal function such as glomerular filtration rate (GFR) and tubular secretion.

The suppression of MRP function in erythrocytes under ARF state may not be explained only by several endogenous compounds including bilirubin, L-kynurenine and CMPF, by considering their unbound concentrations in plasma. However, various endogenous compounds are accumulated in plasma under ARF states, and they would increase their unbound fractions by displacing the protein binding each other. Further study is necessary to clarify the contribution of protein binding under ARF state.

Conclusion

In the present study, DNP-SG, a typical MRP substrate, was found to be greatly accumulated in erythrocytes after application of CDNB, a precursor of DNP-SG, in glycerol-induced ARF rats, possibly due to the suppression of MRPs-mediated efflux transport in erythrocytes. Some membrane-permeable endogenous substances such as bilirubin, L-kynurenine and CMPF were thought to have suppressive effects on MRP function in erythrocytes cooperatively. These results would suggest that renal failure state can cause accumulation of MRPs substrates in erythrocytes.

Declaration

Conflict of interest

The Author(s) declare(s) that they have no conflict of interest to disclose.

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