Transport of Prostaglandin E₁ across Rat Erythrocyte Membrane

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Received December 1, 2007; accepted March 19, 2008; published online March 24, 2008

In this study erythrocyte transport of prostaglandin E₁ (PGE₁) was investigated by employing inside-out membrane vesicles prepared from rat erythrocytes. The uptake of [³H]PGE₁ in the presence of ATP was significantly higher than that of AMP, suggesting the involvement of an ATP-dependent efflux system in PGE₁ transport across the erythrocyte membrane. Coincubation of glutathione with ATP further stimulated the uptake of [³H]PGE₁. The uptake of [³H]PGE₁ in the presence of ATP and glutathione was temperature-sensitive, and various eicosanoids including PGE₂ and PGF_{2α} decreased the uptake. Multidrug resistance-associated protein (MRP) 4 substrates/inhibitors including methotrexate, indomethacin, taurocholic acid and indocyanine green significantly inhibited [³H]PGE₁ uptake. Western blot analysis revealed that Mrp4 is expressed in rat erythrocyte membrane. These results suggest that the release of PGE₁ from the erythrocyte into the blood circulation may be mediated by ATP-dependent efflux pump(s) such as Mrp4.

Key words prostaglandin E₁; multidrug resistance-associated protein; rat erythrocyte

Prostaglandins (PGs) are lipid compounds that are derived enzymatically from fatty acids and show various pharmacological actions in many tissues. In addition to platelet and endothelium, PGs have been shown to modulate various kinds of functions in the erythrocyte. Early study by Allen and Rasmussen¹⁾ showed that PGE₂ exhibited a biphasic response with a maximum decrease in erythrocyte deformability at a concentration of 10^{−10} M. Subsequently, the opposing effects of PGE₁ and PGE₂ on erythrocyte deformability were reported; PGE₁ and PGE₂ increased and decreased the deformability, respectively.^{2–4)} Furthermore, PGE₂ was shown to activate the non-selective cation channel (NSC) and the Ca²⁺-activated K⁺ channel (Gardos channel) in the erythrocyte.^{5–7)} It is suggested that PGs, which are produced by erythrocytes themselves, may induce the above-mentioned changes in erythrocyte functions, since mechanically stressed erythrocytes produce PGE₁ and PGE₂ following the induction of cyclooxygenase (COX)-2, which catalyzes the conversion of arachidonic acid to PGs.⁸⁾ Dutta-Roy and Sinha⁹⁾ demonstrated that highly specific PGE₁ binding receptors are located on the external surface of the erythrocyte membrane, and that cell surface sialic acid is directly involved in the binding of PGE₁. Therefore, in order to induce the physiological effects as signaling molecules, PGs must be released from the inside to the outside of the erythrocytes.

PGs are unsaturated fatty acids consisting of 20 carbon atoms having a cyclopentane ring in a molecule.¹⁰⁾ Since PGs are organic anions with the pK_a values of around 5, PGs are present as negatively charged molecules at physiological pH. Considering that the cell membrane lipid bilayer is generally impermeable to charged molecules, specific transporters are essential for the transport of PGs across the cell membrane. Our previous studies indicated that PGE₁ transport across the blood–brain barrier may be partly mediated by the members of the organic anion transporting polypeptide (Oatp) family.¹¹⁾ Furthermore, we showed that some specific transport systems mediate the transport of PGE₁ across the basolateral and brush-border membrane in renal proximal tubules.^{12,13)} Recently, it was reported that multidrug resistance-associated protein (MRP) 4, a member of the ATP-binding cassette

(ABC) transporter family, actively transports PGs, whereas MRP1, MRP2, MRP3 and MRP5 do not.¹⁴⁾ Since MRP4 is detected in human erythrocytes,¹⁵⁾ it is likely that MRP4 plays an important role in the release of PGs from the erythrocytes. However, to our knowledge, the exact mechanism of the efflux system of PGs in the erythrocytes is still unknown.

In this study, we examined the transport characteristics of PGE₁ in the erythrocytes, using inside-out membrane vesicles (IOVs) isolated from rat erythrocytes by which the efflux can be analyzed by uptake study under controlled conditions. The present observations suggest that PGE₁ is effluxed from rat erythrocytes in an ATP-dependent manner.

MATERIALS AND METHODS

Materials [5,6(n)-³H]PGE₁ (1.59 TBq/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). ATP disodium salt, AMP disodium salt and glutathione (GSH) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Creatine phosphokinase, phosphocreatine disodium salt and taurocholic acid sodium salt were from Nacalai Tesque (Kyoto, Japan). Methotrexate and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Indocyanine green was obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan). Unlabeled PGE₁ was the generous gift of Ono Pharmaceutical Company (Osaka, Japan). 15-keto PGE₁, PGE₂, PGF_{2α}, 6-keto PGF_{1α} and thromboxane B₂ (TXB₂) were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). M₄I-10, as the antibody for Mrp4, was obtained from MONOSAN (Uden, The Netherlands). All other chemicals used for the experiments were of the highest purity available.

Preparation of IOVs from Rat Erythrocytes Experiments with animals were performed in accordance with the Guide for Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University. IOVs were prepared from rat erythrocytes by a spontaneous, one-step vesiculation method, as de-

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scribed previously.¹⁶⁾ Briefly, fresh venous blood was drawn from Wistar rats (usually, 4 ml from one rat), and red blood cells were washed three times with 5 volumes of isotonic buffer (80 mM KCl, 70 mM NaCl, 10 mM HEPES, 0.1 mM EGTA, pH 7.5). The packed cells were lysed in 90 volumes of ice-cold hypotonic buffer (2 mM HEPES, 0.1 mM EGTA, pH 7.5) for 10 min. After centrifugation at $40000\times g$ for 20 min at 4 °C, the supernatant was removed. This step was repeated twice. The pelleted ghosts were suspended in half the packed cell volume of the hypotonic buffer, and incubated at 37 °C for 30 min. After the incubation, the suspension was dispersed by sucking 10 times through a fine needle (0.4 mm $\times$ 19 mm) with a plastic syringe. After addition of 10 ml of the suspension buffer (10 mM HEPES/Tris, 100 mM mannitol, pH 7.4), the suspension was centrifuged at $40000\times g$ for 20 min at 4 °C, and the supernatant was removed. This step was repeated twice. The final pellets containing IOVs were suspended in an appropriate volume of the suspension buffer by sucking 10 times through a fine needle (0.4 mm $\times$ 19 mm) with a plastic syringe, and stored at -80 °C until use. The fraction of IOVs in isolated membrane vesicles was estimated to be 51.2%, based on the activities of acetylcholinesterase, a membrane-bound ectoenzyme, in the presence or absence of Triton-X 100, which was consistent with the value (57.5%) reported previously by our laboratory.¹⁶⁾

Uptake Study The uptake of [³H]PGE₁ by IOVs was measured by a rapid filtration technique as described previously.¹⁷⁾ Briefly, IOVs (10 μ l) were preincubated for 10 min at 37 °C in the buffer consisting of 10 mM HEPES, 100 mM mannitol, 100 mM NaCl, pH 7.4, before the initiation of uptake. The reaction was initiated by the addition of the substrate mixture (40 μ l) to the membrane suspension at 37 °C or 4 °C. The substrate mixture was composed of 10 mM HEPES/Tris (pH 7.4), 80 mM KCl, 100 mM mannitol, 10 mM MgCl₂, 10 mM phosphocreatine, 100 μ g/ml creatine phosphokinase and [³H]PGE₁ in the presence of 1 mM ATP or AMP without or with 5 mM GSH. The final concentrations of ethanol and/or dimethyl sulfoxide (DMSO) used to dissolve eicosanoids was 1% or less. In a preliminary study, we confirmed that ethanol and DMSO within these concentrations had no effect on [³H]PGE₁ uptake by IOVs. At the stated time, incubation was stopped by diluting the reaction mixture with 1 ml of ice-cold stop solution comprising of 100 mM mannitol, 100 mM KCl and 10 mM HEPES/Tris (pH 7.4). The contents of the tube were immediately poured onto Millipore filters (HAWP, 0.45 μ m, 2.5 cm diameter), and then the filters were washed once with 5 ml of the ice-cold stop solution. Non-specific adsorption was estimated by adding 40 μ l of substrate mixture to 1 ml of ice-cold stop solution containing 10 μ l of IOVs, and was used for background correction.

Western Blot Analysis For immunoblot analysis, IOVs prepared from rat erythrocytes was mixed with a loading buffer. The samples were subjected to SDS-polyacrylamide gel electrophoresis with 8% polyacrylamide gels, and the proteins were transferred for 60 min to polyvinylidene difluoride (PVDF) membrane at 4 °C. The membrane was blocked in 5% (w/v) non-fat dry milk in phosphate-buffered saline (PBS-T; 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5) overnight at 4 °C. The membranes were washed twice for 5 min in PBS-T, once for 5 min

in PBS (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, pH 7.5), and were incubated with rat monoclonal anti-MRP4 antibody M₄I-10 (1:20 dilution). The membranes were washed twice in PBS-T, once in PBS, and were incubated with the horseradish peroxidase-labeled rabbit antibody to rat IgG (1:5000 dilution), washed twice in PBS-T, once in PBS, and visualized with enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Analytical Methods Protein concentration was measured by the method of Bradford¹⁸⁾ with bovine serum albumin as the standard. Statistical analysis was performed by Student's *t*-test or by the one-way analysis of variance with the Dunnett test for *post hoc* analysis. A difference of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

First, we examined the time-course of [³H]PGE₁ uptake by IOVs in the presence of 1 mM ATP or AMP. [³H]PGE₁ uptake in the presence of ATP was increased with incubation time and was significantly higher than that of AMP at all time points tested (Fig. 1). This result indicated that PGE₁ is effluxed by an ATP-dependent transport system in rat erythrocytes. Assuming that the intravesicular volume of IOVs from rat erythrocytes is 6.9 μ l/mg protein as described previously,¹⁶⁾ the intravesicular concentration of [³H]PGE₁ in the presence of ATP at 60 min was estimated to be 28.1 nM, which is higher than the initial extravesicular concentration of [³H]PGE₁ in the uptake solution (20 nM). Thus, it is likely that [³H]PGE₁ is transported across the erythrocyte membranes against a concentration gradient. In contrast, the intravesicular concentration of [³H]PGE₁ in the presence of AMP at 60 min (17.5 nM) did not exceed the initial extravesicular concentration of [³H]PGE₁.

Next, the effect of 5 mM GSH on [³H]PGE₁ uptake by IOVs was examined. The uptake of [³H]PGE₁ in the presence of both ATP and GSH was 2.6 times higher than that of ATP alone (Fig. 2A). In addition, the uptake of [³H]PGE₁ in the presence of ATP and GSH at 4 °C was markedly decreased, compared with that at 37 °C (Fig. 2B). These observations

Fig. 1. Time-Course of [³H]Prostaglandin E₁ (PGE₁) Uptake by Inside-Out Membrane Vesicles from Rat Erythrocytes

The uptake of [³H]PGE₁ (20 nM) was measured in the presence of 1 mM ATP (open circle) or AMP (closed circle). Each symbol represents the mean $\pm$ S.E. ($n=3$). ** $p < 0.01$; Significantly different from the value for the uptake in the presence of AMP.

Fig. 2. Effects of Glutathione (GSH) (A) and Temperature (B) on [³H]PGE₁ Uptake by Inside-Out Membrane Vesicles from Rat Erythrocytes

(A) The uptake of [³H]PGE₁ (20 nM) was measured in the presence of 1 mM ATP alone (open column) or in combination with 5 mM GSH (closed column). (B) The uptake of [³H]PGE₁ (20 nM) in the presence of 1 mM ATP and 5 mM GSH was measured at 37 °C or 4 °C. Each column represents the mean ± S.E. (n=3). **p<0.01; Significantly different from the value for the uptake in the presence of 1 mM ATP alone. ††p<0.01; Significantly different from the value for the uptake at 37 °C.

indicate that an ATP- and GSH-dependent efflux system is involved in PGE₁ transport across the erythrocyte membrane. So far, human erythrocytes have been reported to express several members of MRP family, mainly MRP1, MRP4 and MRP5.^{15,19} Recently, Reid *et al.*¹⁴ demonstrated that MRP4, but not MRP1, MRP2, MRP3 and MRP5, transports PGE₁ and PGE₂, by using inside-out membrane vesicles from Sf9 cells expressing these MRPs. In addition, they showed that PGE₁ and PGE₂ inhibited MRP4-mediated transport of estradiol 17β-D-glucuronide.¹⁴ Furthermore, we observed that PGE₁ significantly decreased ATP-dependent uptake of methotrexate, a substrate of MRP4, by rat IOVs.¹⁶ Regarding the GSH dependence, MRP4-mediated bile acid transport is a GSH-dependent transport process, which catalyzes cotransport of bile acid with GSH. In addition, it has been reported that depletion of intracellular GSH in HepG2 expressing MRP4 decreased the export of the MRP4 substrate cAMP from the cells.²⁰ These previous results concerning the expression and function of MRP4 may support that Mrp4, a rat homolog of human MRP4, is involved in the ATP- and GSH-dependent uptake of [³H]PGE₁ by rat IOVs. In contrast, Wielinga *et al.*²¹ indicated that intracellular GSH did not affect the efflux of cAMP and cGMP from MRP4-overexpressing cells. Klokouzas *et al.*¹⁵ also showed that GSH (0.5–4 mM) had no effect on the ATP-dependent uptake of cGMP by inside-out human erythrocyte membrane vesicles. Using IOVs from rat erythrocytes, we previously observed that GSH stimulated the uptake of 15 μM 2,4-dinitrophenyl-S-glutathione (DNP-SG), an MRPs substrate, whereas there was no significant effect of GSH on 300 μM DNP-SG uptake.¹⁶ Thus, the role of GSH in the function of MRP4/Mrp4 re-

Fig. 3. Effects of Various Eicosanoids on [³H]PGE₁ Uptake by Inside-Out Membrane Vesicles from Rat Erythrocytes

The uptake of [³H]PGE₁ (20 nM) was measured in the absence (control) or presence of various eicosanoids at a concentration of 100 μM. Each column represents the mean ± S.E. (n=3). *p<0.05, **p<0.01; Significantly different from control.

mains controversial. However, as described above, marked stimulation of ATP-dependent uptake of PGE₁ was observed in this study. In addition, GSH exists in the erythrocyte at a concentration of about 2 mM. Therefore, we think that GSH would be involved in the stimulation of PGs release from the erythrocyte, though further studies are needed to clarify the role of GSH in MRP4/Mrp4 function in the erythrocyte.

We next investigated the effects of various eicosanoids on [³H]PGE₁ uptake by IOVs. As shown in Fig. 3, [³H]PGE₁ uptake was significantly decreased by unlabeled PGE₁, 15-keto PGE₁, PGE₂ and PGF_{2α}, but not 6-keto PGF_{1α} and TXB₂. When the uptake values of [³H]PGE₁ by IOVs were expressed as percentage of control, those in the presence of PGE₁, PGE₂, PGF_{2α}, and TXB₂ were 45.6%, 50.0%, 64.2% and 90.1%, respectively. According to the report by Reid *et al.*,¹⁴ the rank order of inhibitory potency of these eicosanoids on MRP4-mediated transport is follows: PGE₁ ≅ PGE₂ > PGF_{2α} > TXB₂. Thus, the correlation between the present and previous results may further suggest the involvement of Mrp4 in the ATP-dependent uptake of [³H]PGE₁ by rat erythrocyte IOVs. In addition, PGE₂ release from the erythrocyte^{7,22} may be mediated by MRP4, since PGE₂ has been reported to be a substrate of MRP4 having almost the same affinity ($K_m=3.4 \mu\text{M}$) for MRP4 as that of PGE₁ ($K_m=2.1 \mu\text{M}$).¹⁴

Furthermore, we examined the effects of various MRP4 substrates/inhibitors on [³H]PGE₁ uptake in the presence of ATP and GSH (Fig. 4). Taurocholate (1 mM) and methotrexate (1.5 mM), which are reportedly transported by MRP4,^{23,24} significantly decreased [³H]PGE₁ uptake. Indomethacin (50 μM) and indocyanine green (100 μM), inhibitors of MRP4,^{14,21} significantly inhibited the uptake of [³H]PGE₁. The strong inhibitory effect by indocyanine green is in accordance with the previous finding that the inhibitor (100 μM) most potently decreased the MRP4-mediated transport among various compounds tested.²⁵ In addition, indomethacin may have greater influence on physiological responses, such as erythrocyte deformability, mediated by PGs than expected from the present result, since indomethacin, a non-steroid anti-inflammatory agent, may inhibit the biosyn-

Fig. 4. Effects of Various Inhibitors on [³H]PGE₁ Uptake by Inside-Out Membrane Vesicles from Rat Erythrocytes

The uptake of [³H]PGE₁ (20 nM) was measured without (control) or with 1.5 mM methotrexate (MTX), 50 μM indomethacin (IND), 1 mM taurocholic acid (TCA) and 100 μM indocyanine green (ICG). Each column represents the mean ± S.E. (n = 3–6). *p < 0.05, **p < 0.01; Significantly different from control.

Fig. 5. Immunoblot Analysis of Mrp4 in Rat Erythrocyte Membranes

Ten micrograms of inside-out membrane vesicles from rat erythrocytes were separated in SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane. Mrp4 was detected by Western blotting as described in Materials and Methods.

thesis of PGs by COX-2, which has been reported to be induced in the erythrocytes under mechanical stress.⁸⁾

As described above, MRP4 is reported to be expressed in human erythrocytes. However, as far as our knowledge, there has been no report showing the expression of Mrp4 in rat erythrocytes. In this study, immunoblotting was performed to investigate whether Mrp4 protein is expressed in rat erythrocyte membranes. As shown in Fig. 5, Mrp4 with the apparent molecular mass of 166 kDa was detected in the membrane from rat erythrocytes. The molecular size was well correlated with that of Mrp4 expressed in other rat tissues such as liver and kidney.^{26–28)}

In conclusion, we showed that [³H]PGE₁ was taken up by IOVs from rat erythrocytes in an ATP- and GSH-dependent manner. The uptake of [³H]PGE₁ in IOVs was inhibited by various MRP4 substrates/inhibitors. In addition, we detected the expression of Mrp4 in rat erythrocyte membranes. These observations suggest that the efflux of PGE₁ from rat erythrocytes may be, at least in part, mediated by Mrp4. However, further studies will be needed to clarify the efflux pump(s) involved in PGs release from the erythrocytes.

Acknowledgment This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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