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Contribution of multidrug resistance-associated proteins (MRPs) to the release of prostanoids from A549 cells

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ABSTRACT

Previous studies indicated that several members of the multidrug resistance-associated protein (MRP) family mediate the transport of prostanoids. However, theimportance of MRPs in the release process of prostanoids has not been fully elucidated. In this study, we investigated the contribution of MRPs, including MRP1, MRP2, and MRP4, to the release process of the prostanoids from human lung adenocarcinoma epithelial A549 cells. The extracellular levels of PGE_2, $PGF_{2\alpha}$, and TXB_2 (a metabolite of TXA_2) were decreased by treatment with MRP inhibitors (dipyridamole, MK571, and probenecid). The studies using membrane vesicle suggest that the effects of the inhibitors were in part by inhibiting MRP4 function. The effects of knockdown of each MRP (MRP1, MRP2, and MRP4) were also investigated. The extracellular levels of PGE_2 and $PGF_{2\alpha}$ were significantly decreased after MRP4 knockdown. Our results suggest that MRPs including MRP4 contribute the release process of prostanoids in A549 cells.

1. Introduction

Prostanoids, including prostaglandin (PG) and thromboxane (TX), are important bioactive lipid mediators that contribute to various physiological and pathological processes including inflammation and cancer [1,2]. Overexpression of cyclooxygenase-2 (COX-2) and prostanoids is associated with a variety of cancers including lung, colorectal, prostate, and breast cancers, and non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to reduce the risk of these cancers [3]. Since there are multiple prostanoids and they have a variety of functions, the roles of prostanoids in physiological and pathological processes are complicated. Therefore, it is important to monitor the balance of prostanoids in the microenvironment. Furthermore, a better understanding of the regulation mechanism of prostanoids may contribute to the development of therapeutic targets.

Prostanoids are biosynthesized from arachidonic acid via the arachidonic acid cascade and then released from the intracellular space to the extracellular space, where they exert their biological actions in an autocrine or paracrine manner by binding to cell surface receptors [4]. Therefore, the release process might be important for prostanoid activity. Since prostanoids have a free carboxylic acid moiety, they are thought to possess a substantial negative charge at physiological pH. It has been reported that prostanoid permeability of the plasma membrane was low [5]. Transporters that act as efflux carriers are therefore possibly involved in the action of prostanoids. Although inhibition of the synthesis enzymes and receptors has been focused on, little attention has been given to the efflux mechanism of prostanoids in comparison with those processes [6].

To date, several studies using membrane vesicles have shown that several multidrug resistance-associated proteins (MRPs) including MRP4 have the ability to transport several prostanoids [7–9]. Reid et al. reported that MRP4 mediated the uptake of PGE_1 and PGE_2 in inside-out membrane vesicles derived from HEK293 cells or Sf9 cells, whereas MRP1, MRP2, MRP3 and MRP5 did not [7]. Rius et al. reported that MRP4 mediated the transport of $PGF_{2\alpha}$ and TXB_2 in addition to PGE_2 [8]. de Waart et al. showed that MRP1 and MRP2 were able to transport PGE_2 but that MRP3 was not [9]. Although these studies indicated that MRPs could transport several prostanoids, whether the transport by MRPs is actually involved in the release process from cells and whether this affects the mode of action of prostanoids has not been fully elucidated. Lin et al. reported the importance of murine Mrp4 in the release of PGE_2 from mouse embryonic fibroblast cells [10]. Lacroix-Pépin et al. reported

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the importance of bovine MRP4 in the transport of PGE_2 and $PGF_{2\alpha}$ under the regulation of oxytocin in the bovine endometrium [11]. Furthermore, Gelhaus et al. showed that MRP inhibitor probenecid treatment and MRP4 knockdown decreased extracellular PGE_2 in human bronchoalveolar H358 cells [12]. However, to our knowledge, there are no reports on the contribution of human MRPs to the simultaneous release of multiple prostanoids. Based on previous reports, we focused on MRPs, particularly MRP4, and investigated their involvement in prostanoid release.

MRP4 (ABCC4) is a member of the C subfamily of ATP-binding cassette transporters that transports a wide variety of drugs such as antiviral and antineoplastic drugs. MRP4 also transport endogenous substrates such as cyclic nucleotides, eicosanoids, urate, folic acid, and bile acids. It has been reported that MRP4 was found in various cell lines and several tissues including prostate, liver, testis, ovary, kidney, blood cells, and brain tissues [13]. A recent study showed that MRP4 protein was expressed in various human tissues, particularly in the kidney, testis, heart, and lung [14]. We previously reported that the human lung adenocarcinoma epithelial cell line A549 produced several prostanoids, including PGE_2, $PGF_{2\alpha}$, and TXB_2 (a metabolite of bioactive TXA_2), after stimulation with the calcium ionophore A23187 [15]. A549 cells are widely used in studies on alveolar epithelium function [16] and are also used as a model for non-small cell lung cancer (NSCLC) [17,18].

In this study, we investigated the importance of MRPs in the release of PGE_2, $PGF_{2\alpha}$, and TXA_2 from A549 cells. Our results suggest that MRP function is important for the release process of PGE_2, $PGF_{2\alpha}$, and TXA_2 and that MRP4 partly contributes to the release of these prostanoids from A549 cells.

2. Materials and methods

2.1. Chemicals

All prostanoids and deuterated prostanoids (PGE_2, $PGF_{2\alpha}$, TXB_2, PGE_2-d_4, $PGF_{2\alpha}$-d_4, and TXB_2-d_4) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Human recombinant COX-2 and arachidonic acid were purchased from Cayman Chemical. A23187, indomethacin, hematin, and (−)-epinephrine were obtained from Sigma–Aldrich (St. Louis, MO). Recombinant human interleukin-1β (IL-1β) was purchased from Peprotech (Rocky Hill, NJ). Other chemicals were purchased from Wako (Tokyo, Japan).

2.2. Cell culture

Human lung adenocarcinoma epithelial A549 cells and human airway epithelial Calu-3 cells were obtained from American Type Culture Collection (Rockville, MD). Human embryonic kidney HEK293/4.63 (MRP4-overexpressed) cells were kindly provided by Dr. Piet Borst (Netherlands Cancer Institute). A549 cells and HEK293/4.63 cells were kept in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal bovine serum. Calu-3 cells were kept in DMEM/F12 (Gibco/Invitrogen, Grand Island, NY) with 10% fetal bovine serum and 1% penicillin-streptomycin. All cells were grown at 37 °C under 5% CO_2. A549 cells (2×10^5 cells/well) and Calu-3 cells (1×10^6 cells/well) were seeded on 6-well plastic plates (Corning Costar Corp., Cambridge, MA). After the cells had grown to confluence, they were incubated overnight in a serum-free medium before each experiment. The cells were then treated with 1 mL of serum-free medium containing test reagents for a designated time. Duplicate sets of samples were harvested for determination of protein content to normalize the prostanoid levels. The cells were solubilized in 1% SDS/0.2 M NaOH and the protein content was determined using a Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) with bovine serum albumin as a standard.

2.3. Quantification of prostanoids by LC/MS/MS

For measurement of the released prostanoids, the medium was collected and stored at −80 °C until assay. Samples were prepared using Bond Elut® C18 solid-phase extraction cartridges (Agilent Technologies, Santa Clara, CA). Prostanoids were quantified by selected reaction monitoring (SRM) using an API 3200™ LC/MS/MS system (Applied Biosystems, Foster City, CA) with a Shimadzu Prominance 20A System (Shimadzu, Kyoto, Japan) as described previously [15].

2.4. RT-PCR analysis and quantitative real-time PCR

RT-PCR analysis and quantitative real-time PCR were performed as described previously [19]. Total RNA was prepared from cells using an ISOGEN (Nippon Gene, Japan) and an RNase-Free DNase Set (QIAGEN, Hilden, Germany). Single-strand cDNA was made from 1.0 μg of total RNA by reverse transcription using ReverTraAce (TOYOBO, Japan). PCR was performed using Hot Star Taq PCR (QIAGEN) with specific primers (sequences shown in Table 1) through 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. The PCR products were subjected to electrophoresis on a 2% agarose gel and then visualized by ethidium bromide staining. Quantitative real-time PCR was performed using an Mx3000P Real-time PCR System (Agilent Technologies) with GoTaq® qPCR Master Mix (Promega, Madison, WI) and specific primers (sequences shown in Table 1) through 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. The PCR products were normalized to amplified GAPDH, which was the internal reference.

2.5. Western blotting

Western blotting was performed as described previously [19]. The cells were washed with ice-cold PBS, scraped, and centrifuged at $1500 \times g$ for 5 min at 4 °C. The resulting pellet was suspended in ice-cold lysis buffer. The suspension was allowed to stand for 5 min on ice and was sonicated for 30 s at 4 °C. The suspension was then centrifuged at $14{,}000 \times g$ for 15 min at 4 °C. The supernatant was used for Western blotting. The protein concentration was determined using a Pierce® BCA Protein Assay Kit. Proteins were subjected to SDS-PAGE and transferred onto nitrocellulose membranes and then immunoblotted with antibodies. The primary antibodies used were as follows: mouse anti-MRP1 monoclonal antibody (Clone IU2H10, Abcam, Cambridge, UK), mouse anti-MRP2 monoclonal antibody (Clone M2III-5, Abcam), rat anti-MRP4 monoclonal antibody (Clone M4I-10, Abcam), mouse anti-COX-2 monoclonal antibody (Clone CX229, Cayman Chemical), and mouse anti-actin monoclonal antibody (Clone C4/MAB1501, Chemicon, Temecula, CA). The proteins bound to antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ). Scion image software was used to quantify the protein expression level.

2.6. MRP4 small interfering RNA (siRNA) and siRNA transfection

MRP1 siRNA (Oligo ID: HSS106712), MRP2 siRNA (HSS102057), MRP4 siRNA (HSS173510), and negative control (NC) (Stealth™ RNAi Negative Control Low GC Duplex) were purchased from Invitrogen. Transfection of siRNAs into A549 cells was performed using Lipofectamine™ RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions. Each siRNA duplex was diluted with 400 μL of Opti-MEM® I Reduced Serum medium. 4 μL of Lipofectamine™ RNAiMAX and diluted siRNA duplex as described above was mixed gently and incubated at room temperature for