The International Journal of Biochemistry & Cell Biology 45 (2013) 326–334

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Long term myriocin treatment increases MRP1 transport activity

Peter Meszaros[a], Karin Klappe[a], Annie van Dam[b], Pavlina T. Ivanova[c], Stephen B. Milne[c], David S. Myers[c], H. Alex Brown[c], Hjalmar Permentier[b], Dick Hoekstra[a], Jan W. Kok[a,*]

[a] Department of Cell Biology, University Medical Center Groningen, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands
[b] Mass Spectometry Core Facility, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands
[c] Departments of Pharmacology and Chemistry, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

ARTICLE INFO

Article history:
Received 14 August 2012
Received in revised form 4 November 2012
Accepted 14 November 2012
Available online 23 November 2012

Keywords:
ABC transporter
Lipid raft
Myriocin
Sphingolipid

ABSTRACT

We investigated the effect of myriocin treatment, which extensively depletes sphingolipids from cells, on multidrug resistance-related protein 1 (MRP1) efflux activity in MRP1 expressing cells and isolated plasma membrane vesicles. Our data reveal that both short term (3 days) and long term (7 days) treatment effectively reduce the cellular sphingolipid content to the same level. Intriguingly, a two-fold increase in MRP1-mediated efflux activity was observed following long term treatment, while short term treatment had no impact. Very similar data were obtained with plasma membrane vesicles isolated from myriocin-treated cells. Exploiting the cell-free vesicle system, Michaelis–Menten analysis revealed that the intrinsic MRP1 activity remained unaltered; however, the fraction of active transporter molecules increased. We demonstrate that the latter effect is due to an enhanced recruitment of MRP1 into lipid raft fractions, thereby promoting MRP1 activity.

1. Introduction

ATP binding cassette (ABC) transporters were discovered because of their ability to render cancer cells multidrug resistant. Often cancer cells over express these proteins serving the purpose of defense against chemotherapeutic agents. Forty-nine ABC transporter coding genes have been found in the human genome, and this super family of membrane proteins has been subdivided into 9 subfamilies. These enzymes can transport (efflux) substrates, regulate ion channels, or regulate multiprotein channel-complexes (receptors). Because ABC transporters are membrane proteins and since they can alter pharmacological parameters of drugs, nutrients or other molecules, there is a great need for in vitro assays which can predict the interaction between (potential new) drugs and ABC transporters in the drug development process (Glavinas et al., 2004). Multidrug Resistance Protein 1 (MRP1 or ABCC1) is a member of the C subfamily of the ABC superfamily, and recognizes conjugated organic anions as substrates, such as cysteinyl leukotriene C_4 (LTC_4), estrone beta-glucoronide and estrone-sulphate. The energy required for active transport of these substrates is derived from ATP hydrolysis.

There are several factors which can influence the activity of ABC transporters. The precise localization of these proteins in connection to other proteins, such as actin as part of the cytoskeleton may play a role (Hummel et al., 2011). The lipid environment is important, especially when the transporter is localized to lipid rafts. These membrane domains are enriched in sphingolipids and cholesterol and vary in size from 20 to 200 nm. MRP1 is partially located in lipid rafts as shown previously (Klappe et al., 2009), and thus its function may be affected by major components of the lipid rafts, i.e. cholesterol and sphingolipids. In the case of cholesterol, there have been inconsistent reports published, ranging from a positive effect on MRP1-mediated efflux (Marbeuf-Gueye et al., 2007) to the absence of a correlation between cholesterol and MRP1 function (Meszaros et al., 2011). Other ABC transporters have been studied to various degrees with regard to their dependence on cholesterol and with varying outcomes, especially in the case of P-glycoprotein (Pgp or ABCB1) (Klappe et al., 2009).

Sphingolipids, the other important lipid component of lipid rafts, have not been extensively studied in relation to ABC transporter function. This may be due to the fact that cells have numerous species of sphingolipids, which are not always susceptible to experimental down-regulation. In a previous study we successfully depleted sphingolipids in baby hamster kidney

Abbreviations: ABC, ATP-binding cassette; BHK, baby hamster kidney; Cer, ceramide; 5-CF, 5-carboxyfluorescein; 5-CFDA, 5-carboxyfluorescein diacetate; CSA, cyclosporin A; FCS, fetal calf serum; GlcCer, glucosylceramide; HBSS, Hank's balanced salt solution; LacCer, lactosylceramide; LC–MS, liquid chromatography mass spectrometry; LPE, lysophosphatidylethanolamine; LTC_4, cysteinyl leukotriene C_4; MRP1, multidrug resistance-related protein 1; NIH, National Institutes of Health; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; Pgp, P-glycoprotein; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin-layer chromatography.

* Corresponding author. Tel.: +31 50 3632725; fax: +31 50 3632728.
E-mail address: j.w.kok@umcg.nl (J.W. Kok).

1357-2725/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biocel.2012.11.009

(BHK)-MRP1 cells, when treated with myriocin for 3 days (Klappe et al., 2010). There was no effect on MRP1 efflux pump activity by this treatment and the ability of MRP1 to associate with lipid rafts was unchanged. By contrast, the present study shows that prolonged (7 day) myriocin treatment did affect MRP1 function, increasing its efflux capacity. This was not related to myriocin-induced reduction of sphingolipid levels, which remained unaltered between 3 and 7 days of treatments. Our data demonstrate that the enhanced activity of MRP1 upon long term treatment with myriocin relates to an enhanced recruitment of MRP1 in lipid raft domains.

2. Materials and methods

2.1. Materials

All cell culture plasticware was obtained from Costar (Cambridge, MA). Cell culture media, Hank's balanced salt solution (HBSS), antibiotics, Hepes buffer, L-glutamine and trypsin were obtained from Gibco (Invitrogen, Paisley, UK). Fetal calf serum (FCS) was purchased from Bodinco (Alkmaar, The Netherlands), [^{3}H]LTC_4 and filter plates were purchased from PerkinElmer (Waltham, MA, USA). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Myriocin (ISP-1) was obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). The rat monoclonal anti-MRP1 (MRPr1) antibody was obtained from Alexis (Dedham, MD, USA). Prestained protein marker was procured from New England Biolabs (Ipswich, MA, USA). All other chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The BHK cell line stably expressing the human *MRP1/ABCC1* gene, named BHK-MRP1, was a gift from Dr. Riordan (Mayo Clinic Arizona, S.C. Johnson Medical Research Center, Scottsdale, AZ, USA (Chang et al., 1997)). Cells were grown as adherent monolayer cultures in Dulbecco's modified Eagle medium/NUT mix F-12 (1:1) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine, under standard incubator conditions (humidified atmosphere, 5% CO_2, 37 °C). The BHK-MRP1 cells were kept under selective pressure by growing them in the presence of 100 μM methotrexate.

2.3. Detection of MRP1-mediated efflux by flow cytometric analysis

MRP1 activity was determined using the compound 5-carboxyfluorescein diacetate (5-CFDA), which permeates the plasma membrane and upon cleavage of the ester bonds is transformed into the fluorescent anion 5-carboxyfluorescein (5-CF). The leukotriene D4 receptor antagonist and MRP inhibitor MK-571 was used to inhibit 5-CF efflux.

BHK-MRP1 cells were plated in 25 cm^2 flasks one day prior to the experiment and grown to confluence. Cells were harvested by trypsinization, washed with HBSS and incubated with 5-CFDA (0.5 μM in HBSS) at 10 °C for 60 min. Cells were transferred to ice and washed with ice-cold HBSS. To allow efflux, the cells were incubated at 37 °C for various time intervals. For the inhibitor control sample, the 37 °C incubation was performed in the presence of 20 μM MK571. All subsequent steps were performed on ice. The efflux of the fluorescent substrate was stopped by mixing the cell suspensions with ice-cold HBSS containing MK571 (final concentration 20 μM). The efflux by Pgp in National Institutes of Health (NIH) 3T3 MDR1 G185 cells was measured in a similar fashion, but using rhodamine 123 (10 μM in HBSS) as substrate and cyclosporin A (CSA; 10 μM) as a positive control for inhibition of Pgp-mediated efflux.

For both 5-CF and rhodamine123 the remaining cell-associated fluorescence was determined by flow cytometric analysis using an Elite^{TM} flow cytometer (Beckman Coulter, Miami, FL). For each sample, 5000 events were collected and analyzed using Win-list 6.0 software (Verity Software House Inc., Topsham, ME). With 5000 events, clear Gaussian fluorescence distributions were obtained with a calculated error of measurement of 1.4%. In all graphic representations of results, the values indicate the fluorescence remaining in the cells after a certain time window of efflux at 37 °C, as a percentage of the fluorescence present after loading the cells (=100%). In the latter case the cells were not allowed to efflux, as they were kept on ice and this was done for all conditions, so the values always indicate the fraction of the initial fluorescent substrate load that remains in the cells after a certain time period of efflux at 37 °C.

2.4. Isolation of detergent-free lipid raft

Detergent-free lipid rafts were isolated as described (Klappe et al., 2010).

2.5. Immunoblot analysis

Protein from equal volumes of the gradient fractions was processed as described (Klappe et al., 2010).

2.6. Sphingolipid depletion

In order to deplete the sphingolipid content, cells were grown in the presence of 0.5 μM myriocin for 7 days (long term). In order to be able to isolate membrane vesicles, after 7 days the cell culture was scaled up by growing cells in roller bottles for an additional 3 days (in the presence of myriocin). Hence, in the case of membrane vesicle studies the myriocin incubation was 7 + 3 days. A 4 + 3 day incubation protocol (i.e. scaling up the culture after 4 days of myriocin treatment) was not sufficient to obtain the same reduction in sphingolipids compared to the 7 day experiments. The sphingolipid content was determined by liquid chromatography mass spectrometry (LC–MS) as described (Klappe et al., 2010). Control cells were incubated and washed similar to the myriocin-treated cells.

2.7. Isolation of membrane vesicles from BHK-MRP1 cells

BHK-MRP1 cells were washed with HBSS, trypsinized, harvested and centrifuged (3000 rpm, 3 min), and then frozen in liquid nitrogen and stored in −80 °C. Pellets of $\sim 8 \times 10^8$ frozen cells were used to isolate membrane vesicles as described (Meszaros et al., 2011).

2.8. Measurement of inside-out vesicle ratio

Determination of the inside-out vesicle ratio was based on 5'nucleotidase activity and performed as described (Meszaros et al., 2011).

2.9. Measurement of MRP1-mediated ATPase and transport activities

Vanadate sensitive ATPase activity was measured as described (Meszaros et al., 2011). Values were normalized for graphical representation to maximal activity in control conditions (=100%). For transport of LTC_4 into membrane vesicles, vesicles were incubated as described (Meszaros et al., 2011). Values were normalized for graphical representation to maximal activity in control conditions (=100%).

2.10. Preparation of LPE

1-Docosahexaenoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (22:6 LPE) and 1-arachidonoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (20:4 LPE) were generated by treating 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (22:6/22:6 PE) and 1,2-diarachidonoyl-*sn*-glycero-3-phosphoethanolamine (20:4/20:4 PE), respectively, with phospholipase A2 (Avadhani et al., 2006). Six milligrams of 22:6/22:6 PE or 20:4/20:4 PE was incubated with 0.2 mg phospholipase A2 from *Naja mossambica mossambica* in the presence of 200 mM Tris pH 8, 10 mM $CaCl_2$, and 3 mg/ml sodium deoxycholate at 37 °C for 2 h. Lipids were extracted with chloroform:methanol:acetic acid (50:50:1) and purified by thin-layer chromatography (TLC) using chloroform:methanol:water (65:25:4) as the mobile phase. Chloroform:methanol (1:1) was used to extract LPE from the silica. The concentration of the extracted LPE was determined by phosphate analysis.

2.11. Loading cells or membrane vesicles with PS, PC, or LPE

Cells were loaded with LPE as follows: an appropriate amount of 20:4 or 22:6 LPE in chloroform was dried under nitrogen and then dissolved in 5 μl ethanol. This LPE solution was added to 1.5×10^6 cells in 750 μl of HBSS. The final concentration of LPE was 5, 10, or 20 μM, which is equivalent to 12.5×, 25×, or 50×, respectively, of the concentration of endogenous LPE. Cells were incubated for 1 h at 4 °C with LPE.

To load membrane vesicles with LPE, 100 or 200 nM 20:4 LPE or 22:6 LPE (which is equivalent to 40× or 80×, respectively, of the concentration of endogenous LPE) in chloroform/methanol was dried under nitrogen, dissolved in 10 μl ethanol and added to 500 μg membrane vesicles in 610 μl (final volume) of isotonic buffer. Membrane vesicles were incubated for 1 h at 37 °C, centrifuged (30,000 rpm, 45 min, 4 °C) and subsequently resuspended in isotonic buffer.

To obtain membrane vesicles with increased PS or PC levels, intact cells were loaded with the lipid of interest followed by preparation of membrane vesicles from these cells. Cells were incubated with methyl-β-cyclodextrin (M-β-CD) loaded with 18:1/18/1 PS or 18:1/18:1 PC as described earlier (Meszaros et al., 2011) for 30 or 60 min in serum-free medium at 37 °C (10 mM concentration), and subsequently membrane vesicles were prepared as described above.

2.12. Quantification of lipids

Quantification of glycerophospholipids was achieved by the use of a LC–MS technique employing synthetic (non-naturally occurring) diacyl and lysophospholipid