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Long term myriocin treatment increases MRP1 transport activity

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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.

Keywords

ABC transporter; Lipid raft; Myriocin; Sphingolipid

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 sub-divided 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 C₄ (LTC₄), estrone beta-glucuronide 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 (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), [³H]LTC₄ 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% CO₂, 37 °C). The BHK-MRP1

cells were kept under selective pressure by growing them in the presence of 100 μ M 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 cm² 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 μ M 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 μ M 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 μ M). 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 μ M in HBSS) as substrate and cyclosporin A (CSA; 10 μ M) 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™ 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 μ M 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 LTC₄ 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 CaCl₂, 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- α -cyclodextrin (M- α -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 standards as previously described (Klappe et al., 2010; Myers et al., 2011). Quantification of sphingolipids was achieved by using a LC–MS technique employing C₁₂ fatty acid homologues of sphingolipids as described (Klappe et al., 2010).

2.13. Statistical analysis

Statistical analysis was performed using Student's *t*-test, with values of $P < 0.05$ indicating statistical significance unless otherwise noted.

3. Results

3.1. Depletion of sphingolipids with myriocin

In intact BHK-MRP1 cells, both short term (3 days) and long term (7 days) treatments with myriocin yielded the same extent of sphingolipid depletion, i.e. $22.1 \pm 1.6\%$ and $23.0 \pm 1.9\%$ compared to controls. The headgroup composition of the residual sphingolipids was comparable between short and long term myriocin treatments (Fig. 1). Long term myriocin treatment resulted in levels of Cer that were twice that of short term myriocin treatment ($P = 0.03$). However, it should be noted that in absolute amounts Cer is a minor sphingolipid, while LacCer and SM together make up $91.7\% (\pm 0.78\%; n = 3)$ of the sphingolipid pool. C16:0, C24:0, and C24:1 fatty acids were by far the most abundant in all sphingolipids (Klappe et al., 2010). Upon myriocin treatment, changes in the relative amounts of these three species occurred for all sphingolipids, and C16:0 species were relatively more abundant compared to C24:0 and C24:1 species. Furthermore, there were no differences in the acyl chain distribution between short and long term myriocin treatment (Table 1). Finally, membrane vesicles derived from long term myriocin-treated BHK-MRP1 cells have a similar extent of sphingolipid depletion, i.e. $22.7 \pm 6.0\%$ versus the control condition.

3.2. MRP1 shows increased activity in long term myriocin-treated BHK-MRP1 cells

In order to determine whether myriocin treatment affected the function of MRP1, we tested MRP1-mediated 5-CF efflux activity. BHK-MRP1 cells were loaded with 5-CFDA, and efflux activity was determined on the basis of fluorescence retention in the cells, monitored over a time span of 10 min at 37 °C. Treatment of the 5-CFDA-loaded cells with the MRP1 inhibitor MK571, used as a positive control for inhibition of MRP1-mediated efflux activity, showed a 5-CF retention of more than 90% over a 10 min time interval (Fig. 2A and B). Interestingly, compared to the untreated cells (Fig. 2A, continuous line), MRP1 appeared substantially more active (Fig. 2A, dotted line) in long term myriocin-treated cells. Thus, compared to control cells, after 5 min of efflux time, an approximately 50% enhanced net efflux of 5-CF was seen in the treated cells. Remarkably, short term treatment of the cells with myriocin did not affect efflux, its kinetics being identical to that of the control condition (Fig. 2B; see also Klappe et al., 2010). Our data therefore indicate that long term myriocin incubation did enhance the MRP1 activity in contrast to short term treatment, despite the lack of further depletion of the sphingolipid content. To investigate whether this increase in MRP1 activity is specific, the same experiment was performed in NIH 3T3 MDR1 G185 cells over expressing Pgp. The data demonstrate that contrary to MRP1, Pgp-mediated efflux activity was not sensitive to myriocin treatment, but was readily inhibited by the Pgp inhibitor CSA that was used as a positive control (Fig. 2C).

3.3. MRP1 shows increased activity in membrane vesicles isolated from long term myriocin-treated cells

The effect of myriocin on MRP1 function could have been due to confounding factors in the complex background of the intact cell. To overcome the complexity of the cell system, plasma membrane vesicles were isolated from myriocin-treated BHK-MRP1 cells. To make sure that myriocin treatment did not affect the inside-out ratio of MRP1 in the isolated membrane vesicles, we measured this ratio. Differences in the inside-out ratio would result in differences in both maximal substrate transport across the vesicular membrane and ATPase activity, simply because of differences in MRP1 accessibility in the assay. However, the inside-out vesicle ratios, determined as described in Section 2, were found to be very similar in myriocin-treated and control conditions (Fig. 3A). The control inside-out vesicle content was $22.8 \pm 3.5\%$. Moreover, as verified by immunoblot analysis (Fig. 3B), no significant degradation of MRP1 could be detected, implying that MRP1 integrity was not changed by myriocin treatment.

In this simplified system for MRP1 function analysis, we subsequently measured transport of LTC₄ (Fig. 2D) into the membrane vesicles by MRP1 as well as its ATPase activity (Fig. 2E). Both parameters of MRP1 activity showed substantially higher values in membrane vesicles isolated from myriocin-treated cells than in vesicles derived from control cells (Fig. 2D and E and Table 2). Accordingly, these data are entirely consistent with those presented for the intact cells. Michaelis–Menten kinetic analysis of LTC₄ transport in the vesicular model system revealed that while the K_m values were similar, the V_{max} value of myriocin-treated samples was nearly twofold higher than that for the control condition (Table 2).

The enhanced activity of MRP1 after long term myriocin treatment could be due to up-regulation of MRP1 levels during the treatment. However, the levels of MRP1 in treated versus control intact cells as well as in the cell-derived membrane vesicles were found to be similar: the ratio of the level of MRP1 normalized to actin in treated versus control intact cells was 1.20 ± 0.38 ($n = 22$; $P = 0.64$) and for membrane vesicles this ratio was 1.03 ± 0.11 ($n = 18$; $P = 0.65$).

3.4. MRP1 shows increased association with lipid rafts in long term myriocin-treated cells

We have previously shown that MRP1 is enriched in lipid rafts (Hummel et al., 2011; Klappe et al., 2010). Here we tested the effect of long term myriocin treatment on the association of MRP1 with these domains, using a detergent-free method for isolation of lipid rafts. Gradient fractions 1–2 as well as fractions 3–4 obtained with this method contain floating membranes, which are highly enriched in cholesterol and sphingolipids and have very low protein content, thus fitting the criteria for lipid rafts (Klappe et al., 2010). Intriguingly, MRP1 showed a significantly higher extent of association with lipid raft fractions 3–4 only after long term myriocin treatment (Fig. 4A, black bars) compared to control cells (Fig. 4A, white bars) and not after short term myriocin treatment (Fig. 4, striped bars). This increase represents a shift from the non-raft fractions 7–9, indicating recruitment of MRP1 from non-raft membrane to lipid raft domains. This is in accordance with the effect of myriocin on MRP1 efflux function, which only occurred after long term myriocin treatment (Fig. 2A and B). Src (Fig. 4B) and caveolin (Fig. 4C) displayed increased association to fractions 3–4 already after short term myriocin treatment, and this was maintained for caveolin after long term treatment.

3.5. Myriocin-treated cells show slight differences in glycerophospholipid profile

It is conceivable that in response to the loss of sphingolipids after long term myriocin treatment, compensatory mechanisms might be triggered that result in a change in the glycerophospholipid profile. Therefore LC–MS was used to analyze the

glycerophospholipids in terms of headgroup and fatty acid composition as well as the total amount of this lipid pool. Short term treatment showed no significant changes in the molecular fraction of major glycerophospholipid classes and relatively few species with significant changes in absolute amount (Klappe et al., 2010). Further, we found no significant ($P < 0.05$) changes in the total glycerophospholipid content (data not shown) or in the molecular fractions of phosphatidic acid (PA), phosphatidylinositol (PI), PS, or PC after long term myriocin treatment (see Supplementary Table S1). The molar fraction of phosphatidylglycerol (PG) was somewhat higher and that of PE somewhat lower after long term myriocin treatment (see Supplementary Table S1). No significant changes were observed in the total content of the lyso-forms of PE, PI PS, or PC (see Supplementary Table S1). Several relatively long-chain LPE species appeared to be more abundant in long term myriocin-treated cells, although the high fold values did not reach statistical significance (Table 3 and Supplementary Table S1). A similar trend of (not significantly) increased long-chain LPE species was observed in membrane vesicles isolated from long term myriocin-treated cells (see Supplementary Table S1). In this context it should be mentioned that the LPE contributes only 2–5% of the total lipid mass (data not shown). PS and PC together contribute about 60% of the total lipid mass. Although the total content of PS or PC species did not differ from control, in both these lipid classes as well as for PG there were several species which were more abundant in long term myriocin-treated cells (Table 3 and Supplementary Table S1). Finally, neither the absolute nor the relative overall lipid composition of raft fractions isolated from long term myriocin-treated cells showed statistical differences compared to control (data not shown).

3.6. Loading cell membranes with LPE or loading membrane vesicles with LPE, PS, or PC does not increase MRP1 activity

We reasoned that if the enhanced activity of MRP1 after long term myriocin treatment is a direct effect of increases in one or more of the lipids as reported in the previous paragraph, a similar effect might be achieved by their exogenous addition. Accordingly, we examined the effects of exogenous addition of LPE, PS, and PC on MRP1 activity in control and myriocin-treated cells and membrane vesicles derived from cells. First, we loaded control cells and isolated membrane vesicles derived from control cells with LPE. However, the efflux activity of MRP1 measured in intact cells actually decreased with stepwise increases of the LPE concentration used for loading of the cells (Fig. 5, white bars). Membrane vesicles derived from control BHK-MRP1 cells loaded with LPE showed a similar effect; both vesicular transport and vanadate sensitive ATPase activity were stepwise reduced with increasing concentrations of LPE (Fig. 6A and B).

To properly evaluate the potential effect of the phospholipids following myriocin treatment, short term treated cells (3 days) were used as well, because they have very similar sphingolipid levels as 7 day treated cells. In these short term treated cells, potential changes in MRP1-mediated efflux upon the exogenous addition of phospholipids might best simulate changes triggered inherently after long term treatment. Similarly as in control cells we observed an inhibition of MRP1 efflux activity (Fig. 5, black bars).

Next we focused on a potential effect of PS. PS-enriched membrane vesicles from control cells were prepared by treatment with 18:1/18:1 PS loaded methyl- β -cyclodextrin. Table 4 shows the efficiency of this procedure. The lipid loaded vesicles were indeed enriched in PS 36:2; however, they were also enriched in PS 36:1 and PS 36:0. These PS enriched membrane vesicles did not show enhanced MRP1 transport or vanadate-sensitive ATPase activities (Fig. 6C and D). Rather, a decreased activity was measured, as was the case when LPE was added. Similar results, i.e. decreased activity of MRP1, were obtained when membrane vesicles were loaded with 18:1/18:1 PC (data not shown).

4. Discussion

In this study we show that MRP1 activity as an efflux pump is increased after long term (7 days) myriocin treatment. This was demonstrated in intact BHK-MRP1 cells with a 5-CF efflux assay, and corroborated in membrane vesicles isolated from long term myriocin-treated cells. The latter results are important, as they show that the myriocin effect is not a result of confounding factors associated with myriocin treatment in the complex system of the intact cell. Rather, an increase of MRP1 activity is also apparent in relatively simple membranes containing the transporter. We show that short term (3 days) myriocin treatment does not affect MRP1 function in spite of the fact that the residual cellular levels of sphingolipids after either 3 or 7 day treatment are very similar, both in terms of headgroup and fatty acid composition. An apparent difference occurred only in the low abundance sphingolipid class Cer, the residual level of which was twice as high after long term versus short term myriocin treatment. It is difficult to conceive that a smaller reduction of Cer level after long term treatment affects MRP1 activity, while a larger reduction after short term treatment does not. Moreover, LacCer and SM were by far the most abundant sphingolipid classes and together accounted for more than 90% of the sphingolipid pool. The shift in relative abundance of C24:0 and C24:1 fatty acid containing sphingolipid species towards the dominance of C16:0-containing species observed after short term myriocin treatment also occurred after long term treatment. In conclusion, it is not likely that the long term myriocin effect on MRP1 activity is a result of a further loss or change of sphingolipids compared to short term treatment.

We therefore investigated other potential mechanisms underlying the long term myriocin effect. An increased expression (up-regulation) of MRP1, differences in the protein's stability (occurrence of degradation), or a different inside-out ratio of the transporter in the membrane vesicle system would be obvious reasons for differences in MRP1 activity in treated versus non-treated cells or membrane vesicles derived from these cells. However, these options were all experimentally excluded. We have previously observed that the extent of lipid raft association of MRP1 is an important factor for its activity (Hummel et al., 2011), reflecting the relevance of a particular environment for optimal transporter activity. Therefore we studied the lipid raft association of MRP1 at conditions following long term myriocin treatment. Interestingly, the data revealed a significant increase in the association of MRP1 to lipid raft fractions 3–4 (Fig. 4). This only occurred after long term myriocin treatment and not after short term treatment, which is entirely consistent with the effect of myriocin on MRP1-mediated efflux. More specifically, the results indicate a shift in the association of MRP1 from fractions 7–9 to fractions 3–4 under these conditions. These observations thus suggest that the total amount of MRP1 in a raft environment is increased and this may (partially) explain the higher efflux activity measured. We speculate that MRP1 needs a certain physical environment, i.e. a more tightly packed lipid surrounding, for its optimal conformation.

A particular advantage of the membrane vesicle system that we exploited in this study is that it allowed us to perform Michaelis–Menten kinetic analysis on the LTC₄ transport data. The analysis showed that the K_m value in the case of long term myriocin treatment was similar to control, which indicates that the intrinsic transport activity of individual MRP1 molecules was not changed. In contrast, the V_{max} value nearly doubled and this may indicate that there is a larger fraction of active transporter molecules under conditions of long term myriocin treatment. This interpretation would be entirely consistent with the observation that there is a higher extent of lipid raft association of MRP1, a condition that promotes MRP1 activity. It cannot be excluded, however, that MRP1 has properties that correspond to V-type allosterism, meaning that allosteric regulators affect V_{max} rather than K_m values (Fenton, 2008).

In previous work we have excluded a particular role for sphingolipids (Klappe et al., 2010) or cholesterol (Meszaros et al., 2011) in regulation of MRP1 activity. In this study we therefore focused on a potential effect of phospholipids, taking also into account that specific phospholipids could act as allosteric regulators of MRP1-function (Pál et al., 2007). Indeed, using LC–MS we established that several changes in certain species of glycerophospholipids had occurred among LPE, PS, and PC species. If these lipids were affecting MRP1 function, it would be reasonable to assume that their exogenous addition to control cells could mimic such an effect on MRP1. Surprisingly, instead of an enhancement of MRP1 activity, a substantial decrease in its activity was observed upon exogenous addition of selected LPE, PS, or PC species to both myriocin-treated and untreated cells. This effect could not have resulted from a complex interplay between exogenously added glycerophospholipids and endogenous sphingolipids, because a similar decrease of MRP1 function was observed when glycerophospholipids were added to myriocin-treated, and thus sphingolipid depleted, cells. Rather than being due to allosteric effects of phospholipids on MRP1 structure, we interpret the negative effect of the exogenously added phospholipids on MRP1 function to result from a disturbance of the integrity of the MRP1-containing membrane domains, including rafts, since ABC transporters are known to be affected in their function by lateral membrane perturbations (Arima et al., 2004). Indeed, it has been well-established that exogenous inclusion of lysolipids and or (saturated) phospholipids in the lateral plane of a bilayer may trigger local curvature changes, alterations in membrane hydration, or modulate catalytically important motions of proteins, thereby negatively affecting protein function (Hunter et al., 1999; Pecheur et al., 2000).

In conclusion, we have shown that long term myriocin treatment increases MRP1 activity as an efflux pump. This effect is not related to depletion of sphingolipids, but rather to an increased association of MRP1 with lipid rafts. The impact of contributions via allosteric interactions with specific glycerophospholipid species, potentially promoting MRP1 activity, can be excluded.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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
LTC₄	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

Fig. 1.

Long term (7 day) myriocin treatment does not affect the headgroup composition of sphingolipids differently from short term (3 day) treatment. The effect of myriocin treatment (striped bars: short term; black bars: long term) on sphingolipid levels in BHK-MRP1 cells was established by LC-MS. Results for each lipid as well as the sum of all sphingolipids are expressed as a percentage of those for untreated cells (100%) and are indicated as means $\pm$ SD ($n = 3$). GlcCer: glucosylceramide; Cer: ceramide; LacCer: lactosylceramide; SM: sphingomyelin. It should be noted that in absolute amounts Cer is a minor sphingolipid, while LacCer and SM together account for 91.7% ($\pm 0.78\%$; $n = 3$) of the sphingolipid pool.

Fig. 2.

Long term myriocin treatment increases the activity of MRP1. (A and B) Efflux assays were performed in long term (7 day) (A) versus short term (3 day) (B) myriocin-treated samples and their respective controls using 5-CF as a substrate for MRP1 in BHK-MRP1 cells. (C) Efflux assay in long term (7 day) myriocin-treated and control NIH 3T3 MDR1 G185 cells using rhodamine 123 as substrate for Pgp. The Y-axis represents the remaining fluorescent substrate relative to the starting value at $t = 0$ min (=100%). Data represent the mean $\pm$ SD ($n = 3$). At 10 min the inhibitor controls are also presented, using MK571 for MRP1 and CSA for Pgp. These values range between 85 and 100%. (D and E) Vesicular transport of LTC₄ (D) and ATPase activity of MRP1 (E) in isolated membrane vesicles generated from long term (7 + 3 days) BHK-MRP1 cells (see Section 2). Data represent the mean percentage relative to maximal activity of the control $\pm$ SD ($n = 9$). In all panels: solid line: control, untreated cells; dotted line: myriocin-treated cells or membrane vesicles derived from these cells.

Fig. 3.

Increased MRP1 activity is not an artifact of variable inside-out vesicle ratio or protein degradation. (A) Myriocin treatment was performed independently in triplicate with corresponding controls, and for all samples membrane vesicles were isolated. The inside-out vesicle content of membrane vesicles derived from control (white columns) and myriocin-treated cells (black columns) is shown. Values are expressed relative to control (=100%) and represent the mean $\pm$ SD of three independent experiments. (B) Immunoblot analysis of isolated membrane vesicles shows that MRP1 is intact. Myriocin treatment was performed independently in triplicate with corresponding controls, and for all samples membrane vesicles were isolated. For all membrane vesicle samples 10 μ g of protein was loaded on the gel. The sample named "Degradation" is a positive control for degradation of MRP1, which was generated by cholesterol-oxidase treatment of membrane vesicles for 30 min at 37 °C and shows fragments of MRP1. The marker bands are from top to the bottom 175, 80, 58, 46, 30, 25, and 17 kDa.

Fig. 4.

Effects of myriocin treatment on detergent-free lipid raft localization of MRP1, caveolin, and Src in BHK-MRP1 cells. (A–C) BHK-MRP1 cells were treated with myriocin for either 3 or 7 days and lipid raft association of MRP1 was determined. Values indicate the percentage of MRP1 (A), Src (B), and caveolin (C) found in the gradient fractions, relative to the total amount in the entire gradient (9 fractions). White bars, control cells; striped bars, short term (3 day) myriocin-treated cells; black bars, long term (7 day) myriocin-treated cells. Data represent the mean $\pm$ SD of three independent experiments. Asterisks indicate values that are significantly different from the control condition ($P < 0.05$, as determined by Student's *t*-test).

Fig. 5. LPE loading of BHK-MRP1 cells decreases the efflux activity of MRP1. Control cells (white bars) and short term myriocin-treated cells (black bars) were loaded with 20:4 LPE or 22:6 LPE at 5, 10, or 20 μ M concentrations for 1 h at 4 °C. The same experiment was done at 37 °C and gave similar results (data not shown). The cells were allowed to efflux for 5 min. Data represent the remaining fluorescence of 5-CF relative to the 0 min value and are normalized to control (=1) or myriocin-treated cells (=1), respectively. Data represent the mean $\pm$ SD of three independent experiments.

Fig. 6.

Loading the membrane with LPE or PS does not increase the activity of MRP1 in membrane vesicles. (A and B) Membrane vesicles derived from control BHK-MRP1 cells were loaded with 20:4 LPE. Results using 22:6 LPE were similar (data not shown). (C and D) Intact control BHK-MRP1 cells were loaded with 18:1/18:1 PS, followed by membrane vesicle isolation. Vesicular transport (A and C) or vanadate-sensitive ATPase activity (B and D) of MRP1 in these vesicles was measured. In all panels: solid line: control, dotted line: 100 mM 20:4 LPE or 30 min 18:1/18:1 PS loading, dashed line: 200 mM 20:4 LPE or 60 min 18:1/18:1 PS loading. Values are expressed as the mean percentage relative to maximal activity of the control $\pm$ SD ($n = 3$).

Table 1

Fatty acid composition of major sphingolipids from BHK-MRP1 cells after short term (3 day) or long term (7 day) treatment with myriocin.

	<u>Short term myriocin treatment</u>		<u>Long term myriocin treatment</u>	
	Fold	<i>P</i> value	Fold	<i>P</i> value
GlcCer C16:0	1.75	1.4×10^{-5}	1.30	8.6×10^{-5}
GlcCer C24:1	0.38	8.2×10^{-5}	0.58	2.4×10^{-3}
GlcCer C24:0	0.37	3.6×10^{-4}	0.66	5.8×10^{-4}
Cer C16:0	1.43	5.0×10^{-3}	1.17	1.8×10^{-2}
Cer C24:1	0.58	1.6×10^{-3}	0.32	1.4×10^{-4}
Cer C24:0	0.54	1.3×10^{-2}	0.27	9.7×10^{-4}
LacCer C16:0	1.20	8.2×10^{-2}	1.70	3.6×10^{-5}
LacCer C24:1	0.64	4.7×10^{-3}	0.43	1.5×10^{-5}
LacCer C24:0	0.86	2.3×10^{-1}	0.57	2.4×10^{-4}
SM C16:0	1.08	2.7×10^{-4}	1.13	1.2×10^{-3}
SM C24:1	0.43	4.2×10^{-5}	0.42	6.8×10^{-6}
SM C24:0	0.67	4.1×10^{-4}	0.61	1.0×10^{-3}

LC-MS was used to analyze the major sphingolipid species of BHK-MRP1 cells after long versus short term myriocin treatment. Fold indicates the ratio of the amount of a given sphingolipid species in myriocin-treated cells divided by the amount in control cells, with a value of 1 indicating no change under myriocin treatment. Data represent the means of 3 independent experiments. Values in bold indicate where levels in myriocin-treated and control conditions are significantly ($P < 0.05$) different as determined by Student's *t* test.

GlcCer: glucosylceramide; Cer: ceramide; LacCer: lactosylceramide; SM: sphingomyelin.

Table 2

MRP1 activity in isolated membrane vesicles.

	Control	Myriocin treatment	<i>P</i> value
ATPase activity (nmol Pi/mg/min)	27.4 ± 3.8	40.2 ± 7.8	0.004
Vesicular transport (pmol/mg/min)	25.9 ± 6.3	44.8 ± 11.0	0.002
K_m (nM)	628.6 ± 236.7	706.1 ± 214.7	0.602
V_{max} (pmol/mg/min)	225.4 ± 43.2	457.0 ± 101.8	0.001

Membrane vesicles were isolated from long term (7 + 3 days) myriocin-treated BHK-MRP1 cells (see Section 2). Assays were then performed for ATPase activity and vesicular transport, and Michaelis–Menten kinetic analysis was applied to the vesicular transport data. Data represent the mean ± SD of 3 independent membrane preparation experiments, each performed in triplicate. Values in bold indicate statistical significance between myriocin-treated and control conditions ($P < 0.05$) as determined by Student's *t* test.

Table 3

Major changes in lipid species after long term (7 day) myriocin treatment.

Lipid	Fold	P value
LPE 18:1	1.92	0.320
LPE 20:4	4.92	0.126
LPE 20:1	2.26	0.241
LPE 22:5	4.12	0.144
Total LPE	2.26	0.242
PS 40:2	1.25	0.001
PS 40:4	1.63	0.001
PS 40:5	1.43	<0.001
Total PS	1.03	0.586
PC 32:0	1.34	<0.001
PC 34:0	1.11	0.027
PC 36:1	1.09	0.020
Total PC	1.02	0.447

LC-MS was used to analyze the glycerophospholipid composition of BHK-MRP1 cells in terms of headgroup and fatty acid composition. Glycerophospholipids are indicated with the sum of C-atoms of the two fatty acids and the total number of double bonds (notation X:Y – total number of carbons X in the two fatty acid chains, and total number of double bonds Y). Fold indicates the ratio of the molecular fraction of a given glycerophospholipid in myriocin-treated samples divided by the molecular fraction in control samples. Several species of PS and PC showed differences from the control conditions after long term (7 days) myriocin treatment. The results for the sum of all lipid species in selected lipid classes are also indicated. Data represent the mean of 3 independent experiments. Values in bold indicate species with significant ($P < 0.05$) differences between myriocin-treated and control conditions as determined by Student's *t* test.

Table 4

PS content of 18:1/18:1 PS loaded membrane vesicles.

	PS 30 min		PS 60 min	
	Fold	P value	Fold	P value
PS 36:2	1.85	<0.001	1.38	<0.001
PS 36:1	1.44	0.002	1.32	0.003
PS 36:0	2.87	0.009	2.33	0.034

BHK-MRP1 cells were loaded with 18:1/18:1 PS by incubating them with lipid loaded methyl- β -cyclodextrin for 30 or 60 min at 37 °C. Subsequently, membrane vesicles were prepared and analyzed for glycerophospholipids by LC-MS. PS species with 36 carbons are shown. Fold indicates the ratio of the amount of a given glycerophospholipid in myriocin-treated samples divided by the amount in control samples. Data represent the mean of 3 independent experiments. Values in bold indicate lipids with significantly ($P < 0.05$) increased levels in PS loaded membrane vesicles compared to control conditions as determined by Student's *t* test.