Radixin Influences the Changes in the Small Intestinal P-Glycoprotein by Etoposide Treatment

Takuro Kobori, Shinichi Harada, Kazuo Nakamoto, and Shogo Tokuyama*

Department of Clinical Pharmacy, School of Pharmaceutical Sciences, Kobe Gakuin University; 1–1–3 Minatojima, Chuo-ku, Kobe 650–8586, Japan.

Received June 25, 2013; accepted August 30, 2013

Previously, we reported that repeated oral administration of etoposide (ETP) increases P-glycoprotein (P-gp) expression in association with activation of ezrin/radixin/moesin (ERM) in the small intestine. Radixin has recently attracted attention for its critical role in the plasma membrane localization of certain drug transporters including P-gp by working as a scaffold protein. However, there have been no reports investigating that radixin really interacts with small intestinal P-gp and is involved in the mechanism by which the levels of P-gp are altered. Here, we examined whether radixin is involved in the increased P-gp expression in the small intestine after ETP treatment. Repeated oral treatment with ETP (10 mg/kg/day) for 7 d significantly increased ERM proteins bound to P-gp in the small intestine as determined by immunoprecipitation analysis. In particular, radixin but not ezrin or moesin bound to P-gp was dramatically increased in association with the up-regulation of P-gp in the small intestinal membrane, and radixin was highly colocalized with P-gp as measured by immunofluorescence analysis. In conclusion, radixin may contribute, at least in part, to an increase in the expression of the small intestinal P-gp upon induction with repeated oral treatment with ETP.

Key words P-glycoprotein; radixin; small intestine; ATP-binding cassette transporter

The expression levels and functional activity of plasma membrane proteins are not necessarily dependent upon their mRNA levels, as exemplified by P-glycoprotein (P-gp). While various transcriptional factors have been identified as key regulators of P-gp mRNA levels, little is known about the post-transcriptional regulation of this protein.

We have focused our previous studies on ezrin, radixin and moesin, all of which are involved to some degree in the post-transcriptional regulation of P-gp. Ezrin was originally identified as a component of microvilli from chicken intestinal brush borders. Radixin was isolated from rat liver as a component of the adherens junctions, while moesin was characterized as a heparin-binding protein from smooth muscle cells in the bovine uterus. These three proteins are closely related with ~75% amino-acid sequence homology, and together they comprise the ezrin, radixin and moesin (ERM) family. The high sequence homology of these proteins makes them difficult to distinguish from each other unless specific antibodies against each individual ERM member are used. Thus, ezrin, radixin and moesin have been considered as the ERM family as a whole in the majority of previous reports. The ERM play several critical roles in tissue morphology and organization as well as in cell motility by acting alone as a cross linker between the plasma membrane and the actin cytoskeleton. Recently, ERM have attracted much attention as scaffold proteins regulating the plasma membrane localization and functional activities of several drug transporters including P-gp. In fact, ERM have already been shown to be essential factors for the stable expression of P-gp in the plasma membrane and in the functional activity of P-gp in tissues such as brain, liver or cancer cell lines.

Previously, we reported that repeated oral administration of etoposide (ETP), an anticancer drug recognized as a substrate of P-gp, increases the protein expression of ERM by activating the Ras homolog gene family, member A (RhoA), and Rho-associated coiled-coil containing protein kinases (ROCK), leading to an increase in the P-gp expressions in membrane fractions of the small intestine. However, it remains unclear which ERM in the small intestine contributes the most to an increase in the P-gp expression under above experimental conditions. One of the reasons why this has been difficult to elucidate is that because of the high sequence homology and structural similarity among the ERM members, the development of commercially available specific antibodies against these proteins has been proven to be difficult until quite recently. Perhaps for this reason, there have been no reports describing the protein–protein interactions between P-gp and each ERM member in the small intestine except for ezrin. Although all ERM proteins are expressed in the small intestine, each ERM protein shows a different distribution in this tissue. Among the ERM proteins expressed in the small intestine, ezrin is believed to present most abundant. Ezrin is expressed in the epithelium and is the only ERM protein that has been proven to interact with P-gp by use of immunoprecipitation analysis. On the other hand, radixin is expressed in the liver and kidney, as well as in the epithelium of the small intestine, although not to the extent of ezrin. Furthermore, radixin has been just recently proposed to play a crucial role in regulating the membrane localization and functional activity of P-gp in the small intestinal by using mice lacking radixin. Since the small intestinal P-gp acts as a first barrier against the absorption of various substrate drugs administered via the oral route, it seems to be important to accumulate the evidence with regard to the regulatory factors for the plasma membrane localization of P-gp. However, there has been no direct evidence investigating the molecular interaction between P-gp and radixin in the small intestine and the role of radixin in the alteration mechanism of P-gp.

In this study, we examined whether radixin is involved in

© 2013 The Pharmaceutical Society of Japan
the increased expression of P-gp in the small intestine after repeated oral treatment with ETP, using both immunoprecipitation and immunofluorescence analysis.

MATERIALS AND METHODS

Ethical Approval of the Study Protocol All procedures were in accordance with the Guiding Principles for the Care and Use of Laboratory Animals as adopted by the Japanese Pharmacological Society. The study protocol was approved by the Animal Ethics Committee of Kobe Gakuin University, Kobe, Japan (approval number: A 090130-1).

Animals Male ddY mice (4–5 weeks old; Japan SLC, Inc., Shizuoka, Japan) were housed in an animal room maintained at 24°C and 55±5% humidity on a 12-h light–dark cycle (light phase, 08:00–20:00). Mice were provided with food and water ad libitum.

Drug Administration Etoposide phosphate (ETP; Sequoia Research Products, Pangbourne, U.K.) was administered as an aqueous solution. Mice were treated with ETP (10 mg/kg, p.o.) or vehicle alone (water) once a day for 7 d. Etoposide phosphate (Sequoia Research Products, Pangbourne, U.K.) was administered as an aqueous solution. Mice were treated with ETP (10 mg/kg, per os (p.o.)) or vehicle alone (water) once a day for 7d. The doses of ETP as well as the experimental schedule were chosen based on our previous publications.

Preparation of Membrane Fractions from Ileal Mucosa The preparation of membrane fractions from the ileal mucosa was conducted as described previously.

Preparation of Membrane Fractions from Ileal Mucosa

Western Blot Analysis The membrane fractions were diluted with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer containing 0.5 M Tris–HCl (pH 6.8), 10% SDS, 12% β-mercaptoethanol, 20% glycerol, and a drop of 1% bromophenol blue. Each sample was heated for 7 min at 97°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (7.5%). After SDS-PAGE, proteins were transferred via electrophoresis to a nitrocellulose membrane at 15 V for 50 min. Membranes were blocked with blocking buffer containing Tris-buffered saline (TBS, pH 7.6), 0.1% Tween 20, and 5% skimmed milk for actin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or, 5% bovine serum albumin (BSA for ezrin, radixin or moesin) (BSA; Sigma-Aldrich), for 60 min at room temperature. Membranes were incubated with primary antibodies for ezrin only (rabbit polyclonal antibody ab41672, 1:8000 dilution; Abcam; which can also detect phosphorylated form), radixin only (rabbit monoclonal antibody clone EP1862Y, 1:5000 dilution; Abcam), moesin only (rabbit monoclonal antibody clone EPR2428 (2), 1:8000 dilution; Abcam; which can also detect phosphorylated form) or actin (goat polyclonal antibody SC-1616, 1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) in each blocking buffer overnight at 4°C with gentle shaking. Membranes were then washed 10 times for 3 min each with TBS (pH 7.6) containing 0.1% Tween 20 (TBS-T). Membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-goat secondary antibody (1:2000 dilution for actin; Kirkegaard and Perry Laboratories, Guildford, U.K.) or anti-rabbit secondary antibody (1:1000 dilution for ezrin, radixin or moesin, Kirkegaard and Perry Laboratories) for 60 min at room temperature with gentle shaking and then washed 10 times for 3 min each with TBS-T. Visualization of the immunoreactive bands was undertaken using the Light-Capture system (ATTO, Tokyo, Japan) together with a Pierce ECL western blotting substrate (Thermo Scientific, Waltham, MA, U.S.A.). The signal intensity of each immunoreactive band was determined using CS Analyzer 3 software (ATTO). Each value for ezrin, radixin or moesin was normalized to the respective value for actin.

Immunoprecipitation Assays These experiments were performed as described previously with some modifications. Briefly, the mouse ileal membrane fractions were prepared in the same way as described above. The ileal membrane fractions were resuspended with 20 mM Tris–HCl buffer (pH 7.4). This lysate (total proteins: 2000 µg) was incubated with 20 µL Protein G Sepharose 4 Fast Flow (GE Healthcare) for 10 min at 4°C on a rotating wheel to remove non-specific binding proteins to Protein G Sepharose. The Protein G Sepharose was pelleted by centrifugation (19000×g for 20 min at 4°C), P-gp or multidrug-resistant protein (MRP) 2 was immunoprecipitated from the pre-cleared supernatants of the lysate using an anti-P-gp mouse monoclonal antibody (1:20 dilution; C219, Calbiochem, San Diego, CA, U.S.A) or anti-MRP2 rabbit polyclonal antibody (1:100 dilution; ab110374, Abcam, Cambridge, MA, U.S.A) with overnight incubation at 4°C on a rotating wheel. Next, 25 µL of protein G Sepharose was added into the lysate, which was then incubated for 3 h at room temperature on a rotating wheel. The beads were washed 5 times for 5 min each with 20 mM Tris–HCl buffer (pH 7.4). The immunoprecipitated beads were pelleted by centrifugation (2400×g for 1 min at 4°C) and the supernatants were carefully removed. After the resuspension of the immunoprecipitated beads in 30 µL SDS sample buffer, the beads were boiled at 97°C and pelleted by centrifugation (22000×g for 1 min at 4°C). The supernatant fractions were used for Western blot analysis to detect the tripartite molecular interactions between actin and P-gp as well as total ERM, ezrin, radixin or moesin or the same interactions between actin and MRP2 as well as radixin carried out as described previously with some modifications. Briefly, the supernatant including P-gp and proteins binding to P-gp or MRP2 and proteins bound to MRP2 obtained by using the above procedure, was heated for 7 min at 97°C and separated by SDS-PAGE (7.5%). Proteins were transferred via electrophoresis to a nitrocellulose membrane at 15 V for 50 min. Membranes were blocked with blocking buffer containing TBS-T and 5% BSA (for total ERM, ezrin, radixin or moesin) for 60 min, or with 5% skimmed milk (for P-gp, MRP2 and actin) for 2 h at room temperature. The membranes were incubated with primary antibodies for ezrin/radixin/moesin (rabbit polyclonal antibody, 1:1000 dilution; Cell Signaling Technology, Boston, MA, U.S.A), ezrin only (rabbit polyclonal antibody ab41672, 1:8000 dilution; Abcam), radixin only (rabbit monoclonal antibody clone EP1862Y, 1:5000 dilution; Abcam), moesin only (rabbit monoclonal antibody clone EPR2428 (2), 1:8000 dilution; Abcam), MRP2 (rabbit polyclonal antibody ab110740, 1:1000 dilution; Abcam), P-gp (mouse monoclonal antibody clone C219, 1:200 dilution; Calbiochem) and actin (goat polyclonal antibody clone SC-1616, 1:2000 dilution; Santa Cruz Biotechnology) in the relevant blocking buffer overnight at 4°C with gentle shaking. The membranes were then washed 10 times for 3-min each with TBS-T. Next, the membranes
were incubated with the following HRP-conjugated secondary antibodies: anti-mouse (1:1000 dilution for P-gp; Kirkegaard and Perry Laboratories), anti-goat (1:2000 dilution for actin; Kirkegaard and Perry Laboratories) or anti-rabbit (1:1000 dilution for total ERM, ezrin, radixin or moesin; Kirkegaard and Perry Laboratories) for 1 h (MRP2, total ERM, ezrin, radixin, moesin and actin) or 2 h (P-gp) at room temperature with gentle shaking, and then washed 10 times for 3 min each with TBS-T. Following analyses were carried out as described above.

**Immunofluorescence Analysis** Mice were deeply anesthetized with diethyl ether and perfused transcardially with phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Sections of ileal tissue were collected and fixed in 4% paraformaldehyde at 4°C overnight, then dehydrated in 10% sucrose at 4°C for 3 h followed by 20% sucrose at 4°C overnight. The following day, tissues were embedded and frozen in the Tissue-Tek optimal cutting temperature compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and held at −80°C until use. Frozen blocks were cut into 20 µm-thick sections with a cryostat (CM1850, Leica, Microsystems GmbH, Wetzlar, Germany), and mounted on a MAS-coated glass slide (S9115; Matsunami Glass Ind., Ltd., Osaka, Japan). The sections were then incubated in specific antibodies against P-gp (mouse monoclonal antibody C219, 1:200 dilution; Calbiochem) or radixin (rabbit monoclonal antibody clone EP1862Y, 1:200 dilution; Abcam), which were diluted in 1% BSA in PBS-T overnight at 4°C. The next day, sections were washed with PBS-T and incubated in secondary antibody conjugated with Alexa Fluor 488 (goat polyclonal anti-rabbit IgG, 1:200; Life Technologies, Inc., Carlsbad, CA, U.S.A.) for radixin, or Alexa Fluor 594 (goat polyclonal anti-mouse IgG, 1:200; Life Technologies, Inc.) for P-gp, both of which were diluted in 1% BSA and incubated at room temperature for 2 h. Finally, sections were washed with PBS-T, then cover-slipped with Fluoromount/Plus (Thermo Shandon Inc., Pittsburgh, PA, U.S.A.). Immunoreactive signals were detected with a Biorevo fluorescence microscope (Keyence, Osaka, Japan).

**Statistical Analyses** Data are the means ± S.E.M. Statistical significance was assessed using the unpaired Student’s t-test for single comparisons. A p value < 0.05 was considered significant.

**RESULTS**

Changes in the Level of P-gp and Total ERM Interaction in the Ileal Membrane Fraction after Repeated Oral Treatment with ETP In each vehicle-treated group, the protein expression of P-gp and total ERM immunoprecipitated with anti-P-gp antibody in the ileal membrane fractions were analyzed by immunoprecipitation followed by Western blot. Each panel represents typical Western blot images of P-gp (180 kDa), ERM (75–80 kDa) and actin (42 kDa) (A). Relative levels of P-gp and total ERM protein expressions were analyzed by the ratio of P-gp/actin (B) or ERM/actin (C). Each column represents the mean ± S.E.M. vehicle n = 6; ETP n = 6; * p < 0.05 vs. vehicle, unpaired Student’s t-test.
amount of P-gp immunoprecipitated with anti-P-gp antibody in mice that had undergone repeated oral treatment with ETP was significantly increased compared with that of vehicle-treated mice (Fig. 1B). Furthermore, the amount of total ERM in the membrane fractions of ileum co-immunoprecipitated with P-gp was significantly increased by repeated oral treatment with ETP, without affecting the levels of actin (Fig. 1C).

Changes in the Protein Expressions of Ezrin, Radixin or Moesin in the Ileal Membrane Fraction after Repeated Oral Treatment with ETP

In the ileal membrane fraction, the protein expressions of ezrin (Fig. 2A) or moesin (Fig. 2C) were similar between vehicle and ETP-treated groups. By contrast, the level of radixin in mice exposed to repeated oral treatment with ETP was significantly increased compared to that in vehicle-treated mice (Fig. 2B).

Changes in the Protein–Protein Interaction between P-gp and Ezrin, Radixin or Moesin in the Ileal Membrane Fraction after Repeated Oral Treatment with ETP

In each vehicle-treated group, the protein expression levels of ezrin, radixin, moesin, and P-gp as well as actin were detected in the immunoprecipitates from the ileal membrane fractions using anti-P-gp antibody (Figs. 3A, B, C). The amounts of P-gp immunoprecipitated with anti-P-gp antibody in mice exposed to the repeated oral treatment with ETP were significantly increased compared with that in vehicle-treated mice (Figs. 3A, B, C). Nevertheless, the amount of ezrin (Fig. 3D) or moesin (Fig. 3F) in the ileal membrane fractions co-immunoprecipitated with P-gp were not altered after repeated oral treatment with ETP. On the other hands, the amount of radixin in the same fractions co-immunoprecipitated with P-gp (Fig. 3E) was significantly increased by repeated oral treatment with ETP, without affecting the level of actin.

In vehicle-treated group, the protein expression levels of radixin and MRP2 as well as actin were detected in the immunoprecipitates from the ileal membrane fractions using anti-MRP2 antibody (Fig. 3G). The amount of MRP2 and radixin immunoprecipitated with anti-MRP2 antibody in mice exposed to the repeated oral treatment with ETP was similar with that in vehicle-treated mice (Figs. 3H, I).

**DISCUSSION**

In this study, we observed that after repeated oral treatment with ETP for 7 d, the protein expression levels of P-gp and total ERM, both of which are co-immunoprecipitated with anti-P-gp antibody, were significantly increased in the ileal membrane fraction. These results support our previous findings, which indicated that the protein expression of total ERM in this fraction was dramatically increased in mice treated with oral ETP for 7 d compared with those treated with the vehicle alone. However, this analysis alone is not sufficient to determine the degree to which each of the ERM proteins is altered by repeated oral treatment with ETP.

All three ERM proteins have been shown to interact with P-gp in different tissues. Furthermore, the protein expression of P-gp in the plasma membrane is known to be markedly reduced by genetic depletion of ERM in certain cell types. As is well documented using histological methods, ezrin or moesin are mainly localized on the epithelial cells of villi surfaces or the endothelial cells of the sub-epithelial...
capillaries, respectively. In contrast, radixin has been shown to be widely distributed in the epithelial and/or endothelial cells of the small intestine as indicated by immunofluorescence analyses. In recent years, some researchers have proposed that radixin functions as a scaffold protein for P-gp and other membrane transporters not only in the canalicular membrane of the hepatocytes, but also in the small intestine, similarly to ezrin. To give a specific example, the knockdown of radixin dramatically decreases the plasma membrane localization of P-gp in association with a decrease in its drug efflux activity in the hepatocytes, compared with the more moderate effect of ezrin or moesin knockdown.

Similarly, a deficiency or disturbed localization of radixin in the liver causes a marked decrease in the canalicular membrane localization of MRP2, suggesting that radixin works as a scaffold protein for MRP2. These alterations let to the impaired hepatic excretory function, which contributed to accumulations of total bilirubin in mice. Furthermore, some clinical studies have demonstrated that the canalicular membrane localization of radixin and MRP2 are both disturbed in patients with primary biliary cirrhosis and obstructive jaundice, showing similar behavior. In fact, using immunoprecipitation analysis, our study demonstrated that the protein expression of radixin but not ezrin or moesin, in addition to...
the levels radixin but not ezrin or moesin bound to P-gp in the ileal membrane fraction were both dramatically reduced in mice treated with oral ETP compared with those treated with the vehicle alone. These results are consistent with just recent finding that the plasma membrane localization and functional activity of P-gp in the small intestinal were dramatically reduced in mice lacking radixin[13] and that knockdown of radixin in Caco-2 cell lines, derived from human intestinal epithelium, markedly decreases the plasma membrane localization of P-gp.[14] indicating that radixin plays a critical role in modulating the protein expression of P-gp in the small intestine. In addition, our present results provide further evidence that among the three ERM proteins, radixin, has at least in part, impact on the protein expression level of P-gp in the small intestinal membrane. Contrary to our expectation, both the protein expression of MRP2 and radixin bound to MRP2 in the ileal membrane were not altered by ETP treatment, although radixin has been shown to play an important role in the plasma membrane localization of MRP2 under physiological condition.[14,17] Taken together, radixin may be a scaffold protein that responds to repeated oral treatment with ETP, particularly influencing the expression levels of P-gp but not for other transporters like MRP2 in the plasma membrane of the small intestine. To the best of our knowledge, this is the first report presenting insights into the protein–protein interaction between radixin and P-gp in the small intestine and the crucial role played by radixin in the increased expression of P-gp.

Using immunofluorescence analysis, we were able to confirm that P-gp and radixin were, at least in part, co-localized in the epithelial regions of the ileal villous surface. Surprisingly, P-gp was detected not only in moderate amounts in the epithelial region but also in marked amounts on the endothelial cells of the small intestine. Furthermore, at both regions, radixin was also highly co-localized with P-gp, possibly by working as a scaffold protein for P-gp. Together, these results provide further support for our hypothesis that radixin is involved in the increased expression of P-gp in the small intestine after repeated oral treatment with ETP.

In conclusion, our findings suggest that radixin can be interacted with P-gp in the plasma membrane of small intestine and may influence an increased expression of P-gp upon induction by repeated oral treatment with ETP.

Acknowledgments This study was supported, in part, by Grants-in-Aid and Special Coordination Funds from Kobe Gakuen University Joint Research (C).

REFERENCES


