Quantitative Determination of Luminal and Abluminal Membrane Distributions of Transporters in Porcine Brain Capillaries by Plasma Membrane Fractionation and Quantitative Targeted Proteomics

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Received 2 December 2014; revised 30 January 2015; accepted 30 January 2015

Published online 20 February 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24398

ABSTRACT: Abluminal or luminal localization of transporter in plasma membranes at the blood–brain barrier (BBB) is critical for their physiological and pharmacological roles. Therefore, the purpose of this study was to develop a new method to investigate membrane localization of transporters, through quantitative measurement of protein expression levels in fractionated plasma membrane prepared from porcine brain capillaries. Luminal–abluminal distribution ratios were calculated from the results of quantitative targeted absolute proteomics of fractionated membranes, after correction for cross-contamination based on measurements of luminal and abluminal membrane markers. BCRP expression was greater at the luminal membrane than at the abluminal membrane, supporting the usefulness of the distribution ratio as a quantitative indicator of localization. The distribution ratios suggested luminal-dominant localizations of GLUT1 and OATP3A1, and abluminal-dominant localizations of ABCA1 and FATP1. For OATP3A1, ABCA1 and FATP1, these results require reconsideration of their functions at the BBB. Species differences were examined using expression levels normalized to Na^+/K^+–ATPase. BCRP expression is dominant over multidrug resistance 1 expression in porcine BBB, as in other primates including humans. This methodology for quantitative measurement of protein localization is expected to improve our understanding of the roles of transporters at the BBB, and should be applicable to other polarized cells. J Pharm Sci 104:3060–3068, 2015

Keywords: blood–brain barrier; transporter; membrane transport; localization; species differences; mass spectrometry; pharmacoproteomics (PPx); quantitative targeted absolute proteomics (QTAP); polarity

INTRODUCTION

The blood–brain barrier (BBB), which is formed by brain capillary endothelial cells linked via tight junctions, serves to separate neural tissue from blood. It possesses vectorial transport systems because of polarized expression of various transporter proteins, and these systems regulate the exchange of endogenous and exogenous compounds between brain and blood.[1,2] Multidrug resistance 1 (MDR1/P-gp/ABCB1) and breast cancer resistance protein (BCRP/ABCG2), which are representative ATP-binding cassette (ABC) transporters, are localized at the luminal membrane of the brain capillary endothelial cells in humans and rodents, in accordance with their function to restrict drug entry to the brain.[3–6] Amino acid transporter system A2 (ATA2/SLC38A2), a solute carrier (SLC) transporter, is involved in cellular uptake of small neutral amino acids, and is the major subtype of system A at the abluminal membrane in rodents.[7–10] Organic anion transporter 3 (OAT3/SLC22A8) is also localized at the abluminal membrane in rodents and mediates trans-BBB elimination of anions.[11]

Identification of the localization of transporters in brain capillary endothelial cells is essential to reach an understanding of their physiological and pharmacological roles at the BBB. So far, transporter localization has been analyzed by immunohistochemical methodologies, although this approach is limited by the availability of specific antibodies. In addition, there other issues, for example, there are conflicting reports about the localization of glucose transporter 1 (GLUT1/SLC2A1) in brain capillary endothelial cells: abluminal-dominant localization has been reported in rat,[12] and luminal-dominant localization in human.[13] Simpson *et al* explained this discrepancy in terms of restricted access of anti-GLUT1 antibody to the C-terminus of the transporter at the luminal membrane.[14] There are also conflicting reports on multidrug resistance-associated protein 5 (MRP5/ABCC5), with luminal-dominant, and abluminal-dominant or intracellular localizations suggested in humans and rats.[15,16]

The specificity and reactivity of antibodies are unavoidable issues in immunohistochemical analysis, and thus, alternative

Abbreviations used: ATA1, amino acid transporter system A1; ATA2, amino acid transporter system A2; ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; FATP1, fatty acid transport protein 1; GLUT1, glucose transporter 1; MCT1, monocarboxylate transporter 1; MDR1, multidrug resistance 1; MRP5, multidrug resistance-associated protein 5; OAT3, organic anion transporter 3; OATP3A1, organic anion transporting polypeptide 3A1; SLC, solute carrier.

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This article contains supplementary material available from the authors upon request or via the Internet at http://onlinelibrary.wiley.com/.

Journal of Pharmaceutical Sciences, Vol. 104, 3060–3068 (2015)

methodology is needed to validate the localization of proteins. One approach is membrane fractionation. Betz *et al* fractionated plasma membrane of bovine brain capillary endothelial cells, and compared the activity and cytochemical localization of enzymes, concluding that the luminal and abluminal membranes tended to be enriched in the lower- and higher-density layers, respectively.[17] They examined the membrane distributions of enzymatic activities, such as phosphatase and 5′-nucleotidase, to investigate functional differences between the luminal and abluminal sides of the BBB.[17] Fractionated membrane was also adopted in a functional study of amino acid transport at the abluminal membrane of the BBB.[9,10] However, membrane fractionation analysis can only reveal trends of distribution because of incomplete separation of luminal and abluminal plasma membranes.

We have established quantitative targeted absolute proteomics (QTAP) as a highly sensitive, simultaneous, antibody-free protein quantification method using liquid chromatography–mass spectrometry (LC–MS/MS).[18,19] We have employed QTAP to evaluate the absolute expression levels of ABC transporters and SLC transporters in brain capillaries of human, cynomolgus monkey, common marmoset, rat and mouse,[18–22] although these studies did not deal with localization. In the present study, we combined the two methodologies, fractionation and QTAP, to overcome the limitations of each method. Thus, QTAP analysis of fractionated plasma membrane of brain capillaries provides both protein expression levels and localization of transporters. The influence of cross-contamination of luminal and abluminal membranes can be corrected based on the absolute amounts of marker proteins that are specifically localized in luminal or abluminal membranes, and then the luminal–abluminal distribution ratios of each transporter can be calculated. This methodology makes it possible to achieve a comprehensive localization analysis of membrane proteins at the BBB and other polarized cells without the use of antibodies.

During method development, large amounts of brain tissues are required to prepare membrane fractions of brain capillary endothelial cells, which represent no more than 0.1% of brain volume.[23] We chose porcine brain, because the pig is a large domesticated animal with a low risk of transmissible spongiform encephalopathy[24]; it is widely used as an animal model for the *in vivo* and *in vitro* studies of the BBB,[25–29] and large amounts of porcine brain are readily available. Here, we describe the developed methodology and its application to obtain the localization ratios of transporters between luminal and abluminal plasma membranes of porcine brain capillaries.

MATERIALS AND METHODS

Isolation of Porcine Brain Capillaries

The contents of buffer solutions are given in Supplemental Information. Brain capillaries were prepared as described elsewhere.[21,30] Briefly, porcine brains were obtained at a wholesale market authorized by the Ministry of Health, Labour and Welfare of Japan, and packed in ice for transport to the laboratory. After removal of the surface blood vessels and meninges, brains were stored at −80°C. Brain blocks (~100 g), including gray matter and white matter, were minced in ice-cold solution A. The minced brain was homogenized with a 100-mL Teflon glass homogenizer in four volumes of solution A with 25 up-and-down hand strokes and no rotation, followed by centrifugation for 15 min at 1,000*g* and 4°C. The pellet was resuspended in solution A (similar volume to that used in the homogenization), and the suspension was mixed with an equal volume of 35% dextran-containing solution A, followed by centrifugation for 30 min at 4,500*g* and 4°C. The pellet was suspended in solution B and passed through 210- and 20-μm nylon mesh sheets. The porcine brain capillaries retained by the 20-μm nylon mesh sheet were suspended in solution B, and centrifuged for 10 min at 1,000*g* and 4°C. The pellet was rinsed with solution A, and stored as brain capillary-rich fraction at −80°C. Microscopic analysis was performed to confirm that the prepared samples contained predominantly brain capillaries (Supplemental Fig. 1), and the protein content was determined using a DC protein assay kit (Bio-Rad, Hercules, California).

Preparation of Fractionated Membrane of Porcine Brain Capillaries

The fractionated membrane was prepared as described elsewhere.[9,17] Briefly, brain capillary-rich fraction (~10 mg protein) was suspended in 1 mL hypotonic buffer, and homogenized with a loose-fitting Teflon-glass homogenizer (1,800 rpm, 10 strokes), and then a tight-fitting one (1,800 rpm, 40 strokes) on ice, followed by centrifugation for 10 min at 2,000*g* and 4°C. The resultant pellet was resuspended in 13 mL hypotonic buffer with a tight-fitting Teflon-glass homogenizer (1,800 rpm, 20 strokes), and disrupted by means of nitrogen cavitation (800 psi, 15 min, 4°C), followed by centrifugation for 10 min at 2,000*g* and 4°C. The supernatant was collected in a clean tube. Resuspension of the pellet, nitrogen cavitation, centrifugation, and collection of the supernatant were repeated three times. After addition of 1 M $MgSO_4$ (final concentration: 10 mM), the collected supernatant was centrifuged for 10 min at 3,000*g* and 4°C. The resultant supernatant was centrifuged for 60 min at 90,000*g* and 4°C, and a 25-gauge needle was used to suspend the resultant pellet in 3 mL Tris/sucrose/ethylenediaminetetraacetic acid (TSE) buffer. The suspension was layered on top of a discontinuous gradient (5, 10, 15 and 20% Ficoll in TSE buffer), and centrifuged for 2.5 h at 162,500*g* and 4°C. The fluffy materials at each of the interfaces between Ficoll layers were collected using a 200-μL micropipette whose top had been chopped off, without ruffling the interface, and rinsed in TSE buffer by centrifugation twice for 1 h at 90,000*g* and 4°C to remove Ficoll. The fractions from the interfaces between the 0% and 5%, 5% and 10%, 10% and 15%, and 15% and 20% Ficoll layers were assigned as fractions #1, #2, #3 and #4, respectively, and fractions #1 and #4 were used as the luminal- and abluminal-rich fractions, respectively, based on the previous report.[17] The pellets were each suspended in 150 μL TSE buffer using a 27-gauge needle, and stored as fractionated membranes at −80°C. The protein content was determined using a DC protein assay kit (Bio-Rad).

Quantification of Membrane Proteins by LC–MS/MS

Ten transporters and Na^+/K^+–ATPase were quantitated using the same probe peptides for human transporters as used in our previous studies on human, monkey, marmoset, mouse, and rat (Table 1).[19–22,31] We confirmed that identical sequences were present in the porcine orthologs listed on the Pig Genomic Informatics System (http://pig.genomics.org.cn) or *Sus scrofa* genome of