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Fatty Acid–Binding Protein 5 Mediates the Uptake of Fatty Acids, but not Drugs, Into Human Brain Endothelial Cells

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ABSTRACT

The purpose of this study was to examine the involvement of fatty acid–binding protein 5 (FABP5), a lipid-binding protein expressed at the blood-brain barrier (BBB), in fatty acid and drug uptake into human brain endothelial cells. Following transfection with siRNA against hFABP5, human brain endothelial cell (hCMEC/D3) uptake of lipophilic ligands with varying affinity to FABP5 was assessed with intracellular concentrations quantified by liquid scintillation counting, HPLC, or LCMS/MS. The *in situ* BBB transport of [^{3}H]-diazepam was also assessed in wild type and FABP5-deficient mice. hFABP5 siRNA reduced FABP5 expression in hCMEC/D3 cells by 39.9 ± 3.8% (mRNA) and 38.8 ± 6.6% (protein; mean ± SEM), leading to a reduction in uptake of [^{14}C]-lauric acid, [^{3}H]-oleic acid, and [^{14}C]-stearic acid by 37.5 ± 8.8%, 41.7 ± 11.6%, and 50.7 ± 13.6%, respectively, over 1 min. No significant changes in [^{14}C]-diazepam, pioglitazone, and troglitazone uptake were detected following FABP5 knockdown in hCMEC/D3 cells. Similarly, no difference in BBB transport of [^{3}H]-diazepam was observed between wild type and FABP5-deficient mice. Therefore, although FABP5 facilitates brain endothelial cell uptake of fatty acids, it has limited effects on brain endothelial cell uptake and BBB transport of drugs with lower affinity for FABP5.
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Introduction

The transport of drugs from the bloodstream into the brain is a necessary step in the pharmacological treatment of neurological and neurodegenerative diseases.[1-3] However, the transfer of endogenous ligands and drugs from within the cerebral microvasculature into the central nervous system is often restricted due to the presence of the blood-brain barrier (BBB), a single layer of highly specialized endothelial cells which line brain capillaries.[4] The restrictive nature of the barrier results from 2 major factors: (1) interendothelial tight junctions between brain capillary endothelial cells (BCECs) preventing the paracellular diffusion of highly water soluble molecules into the brain parenchyma[5] and (2) efflux transporter proteins, such as P-glycoprotein and breast cancer resistance protein and metabolic enzymes within BCECs restricting the transcellular transport of both endogenous and exogenous molecules.[6] Drugs and endogenous molecules that permeate the BBB effectively, typically have physicochemical properties consistent with good passive permeability across BCECs (i.e., low molecular weight and moderate lipophilicity),[7] and contain structural features that allow them to undergo carrier-mediated transport into BCECs.[7,8]

A fundamental step in the translocation of lipophilic drugs across the BBB, regardless of whether they permeate the luminal membrane of BCECs via passive diffusion or by a carrier-mediated transport protein, is their subsequent passage through the aqueous cytoplasm of BCECs.[7,9] Once associated with the lipophilic luminal membrane of BCECs, the desorption of lipophilic drugs from this membrane into and across the aqueous cytoplasm to the abluminal membrane is likely to be thermodynamically

Abbreviations Used: BBB, blood-brain barrier; BCEC, brain capillary endothelial cells; DHA, docosahexaenoic acid; FABP, fatty acid–binding protein; hCMEC/D3, immortalized human brain endothelial cells; iLBP, intracellular lipid binding protein.

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unfavorable and may pose as a significant rate limiting step in the transport of these poorly water soluble drugs across the BBB.[10,11] The same situation is likely faced by endogenous lipophilic molecules permeating the BBB, including fatty acids. Fatty acids have been shown to permeate the lipophilic luminal membrane of brain endothelial cells by both passive diffusion and active transport mechanisms. In particular, fatty acid transport protein 1 has been implicated in uptake of various fatty acids[12] and for esterified docosahexaenoic acid (DHA), a significant involvement of a relatively new transporter, Mfsd2a has been implicated.[13] Regardless of the processes governing the luminal membrane transport of fatty acids, the subsequent movement of molecules from the luminal membrane to the abluminal membrane has been largely ignored. In many cell types, the transfer of endogenous lipophilic entities from plasma membranes into the cytosol, their cytosolic solubilization and their subsequent diffusion have been demonstrated to be facilitated by the aid of intracellular lipid–binding proteins (iLBPs).[14,15] Fatty acid–binding proteins (FABPs) represent one class of cytosolic proteins belonging to the iLBP family.[16,17] The human FABP family consists of 9 isoforms originally named based on the tissue in which they were first identified. This traditional nomenclature is however somewhat misleading as many tissues and cell types have been found to express multiple FABP isoforms. A numerical system is now used instead to refer to the 9 isoforms.[16,17] As suggested by their name, FABPs bind to fatty acids and thereby act as cellular chaperones for fatty acids in the cytoplasm.[17] FABPs are found most highly expressed in tissues with high demand for fatty acid consumption.[18-20] While FABP3, FABP5, and FABP7 have been found to be expressed in neurons and other brain parenchymal cells,[21] it is only recently, that the expression and function of FABPs at the BBB have been investigated.

FABP5 has been found to be expressed in primary human BCECs[22] and more recently, we have shown expression of FABP3, FABP4, and FABP5 in an immortalized human brain endothelial cell line (hCMEC/D3 cells)[23] and confirmed FABP5 expression at the mouse BBB.[24] Given the high expression of FABP5 at the human and murine BBB, we and others have assessed the functional role of FABP5 in trafficking various fatty acids across the aqueous cytoplasm of human BCECs.[24-26] Genetic downregulation of FABP5 in primary human BCECs resulted in a ~75%, ~46%, and ~50% reduction in the transport of palmitic, oleic, and linoleic acid, respectively.[25] Similarly, genetic knockdown of FABP5 in hCMEC/D3 cells has been demonstrated to result in a 14.1% reduction in [^{14}C]-DHA uptake.[24] The importance of FABP5 in BCEC uptake and BBB transport of DHA has been further investigated using mice lacking FABP5. In these studies, the uptake of [^{14}C]-DHA was reduced by 48.4% in BCECs from FABP5-deficient$^{-/-}$ mice relative to BCECs from wild type (WT) mice,[26] and furthermore, the *in situ* BBB transport of [^{14}C]-DHA was reduced 36.7% in FABP5$^{-/-}$ mice relative to WT mice. This reduction in BBB transport of [^{14}C]-DHA was associated with an attenuation in endogenous brain levels of DHA, and realizing the important role that DHA plays in the maintenance of cognitive function, cognitive dysfunction was observed in FABP5$^{-/-}$ mice.[26] It is therefore clear that FABP5 plays a significant role in the cytoplasmic trafficking of various endogenous fatty acids, however, whether this phenomenon extends to the trafficking of lipophilic drugs that bind FABP5 remains to be investigated. It has been shown that FABPs expressed at the small intestine have the capacity to traffic drugs across this membrane,[15,27,28] and therefore, FABPs at the BBB may play a similar role.

We have previously demonstrated that the 3 FABP isoforms present at the human BBB (FABP3, FABP4, and FABP5) are able to bind to various drugs, including benzodiazepines, fibrates, fenamates, thiazolidinediones, and propionic acid derived nonsteroidal inflammatory drugs in an isoform-specific manner and with varying affinities.[23] Given that FABP5 has been shown to be important in the cytoplasmic trafficking of fatty acids that bind FABP5 with high affinity, and that FABP5 binds to a wide panel of lipophilic drugs,[23] the aim of the present study was to investigate whether FABP5 exhibits a functional role in facilitating the uptake of drugs with varying binding affinity to FABP5. Using hCMEC/D3 cells, a genetic knockdown approach was taken to investigate the involvement of FABP5 in the uptake of 3 drugs with varying affinity to FABP5 (diazepam, pioglitazone, and troglitazone; Table 1). As positive controls and to ensure that genetic knockdown in FABP5 resulted in functional deficiency in FABP5, the hCMEC/D3 uptake of 3 fatty acids (lauric, oleic, and stearic acid) was assessed. For one drug (diazepam), *in vitro* studies were complemented with *in situ* studies, with the BBB transport of diazepam assessed in WT and FABP5$^{-/-}$ mice.

Materials and Methods

Materials

Cultureware was purchased from Corning Life Sciences (Tewksbury, MA). EBM-2 media and EGM-2 Single Quots Kit were purchased from Lonza (Walkersville, MD) and rat-tail collagen type I was purchased from BD Biosciences (Bedford, MA). Penicillin-streptomycin and fetal bovine serum were obtained from Invitrogen (Penrose, Auckland, New Zealand). Dulbecco's Phosphate–buffered saline (D-PBS) and Pierce BCA protein assay kit were purchased from Life Technologies (Mulgrave, Victoria, Australia). Bradford reagent and Precision Plus Protein Kaleidoscope® ladder were purchased from Bio-Rad (Hercules, CA). HiPerfect transfection reagent, hFABP5 FlexiTube siRNA (SI03145835), and Taqman primers and probes for hFABP3 (Hs00269758_m1), hFABP4 (Hs01086177_m1), hFABP5 (Hs02339439_g1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs02758991_g1) were purchased from Applied Biosystems (Foster City, CA). Rabbit anti-FABP5 polyclonal antibody and mouse anti-β-actin polyclonal antibody were obtained from Abcam (Cambridge, MA) and the secondary goat anti-mouse (800 nm) and donkey anti-rabbit (680 nm) antibodies were obtained from Licor (Lincoln, NE). [^{3}H]-diazepam, [^{14}C]-diazepam, [^{14}C]-lauric acid, [^{3}H]-oleic acid, [^{14}C]-sucrose, and [^{14}C]-stearic acid were purchased from American Radiolabelled Chemicals Inc. (St. Louis, MO). Ultima Gold liquid scintillation cocktail and scintillation vials were purchased from Perkin Elmer Life Sciences (Boston, MA). Pioglitazone and troglitazone were purchased from Adipogen (San Diego, CA). Thiazolyl blue tetrazolium bromide (MTT reagent) and Supelco Ascentis® Express C_{18} column were purchased from Sigma Aldrich (St. Louis, MO), whereas the Phenomenex Luna C18(2) column was purchased from Phenomenex (Torrance, CA). Dimethyl sulfoxide (DMSO) was

Table 1
Inhibition Constant K_i of the Lipophilic Ligands Against FABP5 (Mouse or Human) Alongside Their Calculated Log*p* Values

Compound	cLog*p*[a]	K_i
Lauric acid	5.1	mFABP5: 2.5 ± 0.53 μM[b]
Oleic acid	7.8	hFABP5: 1.6 ± 0.2 μM[c]
		mFABP5: 0.15 ± 0.04 μM[b]
Stearic acid	8.3	hFABP5: 0.29 ± 0.06 μM[c]
		mFABP5: 0.17 ± 0.04 μM[b]
Diazepam	2.9	hFABP5: 325 ± 12.0 μM[b]
Pioglitazone	3.6	hFABP5: 11.0 ± 0.06 μM[b]
Troglitazone	5.1	hFABP5: 1.00 ± 0.08 μM[b]

[a] cLog*p* was calculated using ChemBioDraw Ultra 13.0 (Cambridge Software, Cambridgeshire, UK).
[b] K_i was determined by an 8-anilinonaphthalene-1-sulfonic acid fluorescence displacement assay.[23,29]
[c] K_i was determined by a Lipidex method.[30]

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