Organic anion transport in choroid plexus from wild-type and organic anion transporter 3 (Slc22a8)-null mice

Sykes, Destiny, Douglas H. Sweet, Simon Lowes, Sanjay K. Nigam, John B. Pritchard, and David S. Miller. Organic anion transport in choroid plexus from wild-type and organic anion transporter 3 (Slc22a8)-null mice. Am J Physiol Renal Physiol 286: F972–F978, 2004. First published December 23, 2003; 10.1152/ajprenal.00356.2003.—The choroid plexus actively transports endogenous, xenobiotic, and therapeutic compounds from cerebrospinal fluid to blood, thereby limiting their exposure to the central nervous system (CNS). Establishing the mechanisms responsible for this transport is critical to our understanding of basic choroid plexus physiology and will likely impact drug targeting to the CNS. We recently generated an organic anion transporter 3—(Oat3)—null mouse, which exhibited loss of PAH, estrone sulfate, and taurocholate transport in kidney and of fluorescein (FL) transport in choroid plexus. Here, we measured the uptake of four Oat3 substrates by choroid plexus from wild-type and Oat3-null mice to establish one function of the choroid plexus is to remove potentially toxic neurotransmitter metabolites (7, 14, 23). Oat3 has been immunolocalized to the apical membrane of rat choroid plexus epithelial cells and to the basolateral membrane of rat and human renal proximal tubule cells, the correct locations to mediate the first step in transepithelial transport; concentrative uptake from CSF and blood, respectively (12, 16). Consistent with an important role for Oat3-mediated uptake in kidney and choroid plexus, recent experiments with tissue from Oat3 knockout mice show reduced uptake of PAH, estrone sulfate, and taurocholate in renal cortical slices and nearly complete inhibition of transport of the fluorescent organic anion fluorescein (FL) in intact choroid plexus (23).

In the present study, we examined further the mechanisms driving uptake of organic anions at the apical membrane of choroid plexus in tissue from wild-type and Oat3-null mice. We measured the transport of four organic anions, all shown to be substrates for transport by Oat3 expressed in heterologous systems (7, 14, 21, 23). As before (23), tissue accumulation and distribution of FL were followed using confocal microscopy, which provided information about both the apical and basolateral steps in transepithelial transport. In addition, uptake of radiolabeled PAH, estrone sulfate, and taurocholate was measured.

Four issues were addressed. First, we possess little information on transport of any organic anion in mouse choroid plexus. Such data will be of value as more transporter knockouts become available. Second, Oat3 is one of several organic anion transporters expressed at the apical membrane of choroid plexus (10), and compounds shown to be transported by Oat3 in expression systems could be substrates for multiple transporters in the native tissue. By comparing uptake in tissue from wild-type and Oat3-null mice as well as inhibition patterns in tissue from wild-type mice, we determined the contribution Oat3 makes to the apical uptake step for each of the four substrates studied. By this criterion, Oat3 accounted for essentially all mediated uptake of PAH and FL, about one-third of the uptake of estrone sulfate, but none of the uptake of taurocholate. Third, in the absence of Oat3 expression, transport of estrone sulfate and taurocholate was still mediated. In inhibition studies, we began to characterize the remaining components of organic anion uptake. Surprisingly, a substan-

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tial portion of this uptake is Na dependent, suggesting additional transporters that are directly or indirectly coupled to the Na gradient. Finally, although organic anion uptake by the kidney and choroid plexus is clearly concentric, initial experiments with rat Oat3 expressed in X. laevis oocytes failed to demonstrate energetic coupling to any ion gradient (14). Recent studies using X. laevis oocytes expressing rat Oat3 and rat renal cortical slices now show that Oat3, like Oat1 (24), can function as an organic anion/dicarboxylate exchanger that uses potential energy stored in the Na gradient to drive concentration uptake (indirect energetic coupling to the Na gradient; Ref. 21). In light of these findings, we also examined here the Na dependence of organic anion uptake in choroid plexus from wild-type and Oat3-null mice and found all Oat3-mediated transport to be Na dependent.

MATERIALS AND METHODS

Chemicals. FL was obtained from Molecular Probes. [3H]PAH (4.5 Ci/mmol), 3H-histidine sulfate (46 Ci/mmol), and 3H-taurine (2 Ci/mmol) were purchased from Dupont/New England Nuclear. All other chemicals were of reagent grade or better and were obtained from commercial suppliers.

Animals. Exon 3 of the murine Oat3 gene was replaced with an inverted neomycin cassette in C57BL/6J embryonic stem cells (3, 23). Recombinant embryonic stem cell clones were identified by Southern analysis, and chimeric mice were generated by blastocyst injection. Chimeric Oat3 mice were crossed with C57BL/6J mice, and F2 homozygous Oat3-null animals were backcrossed against the C57BL/6J strain. The Oat3-null mice appear healthy, are fertile, and do not exhibit any gross morphological abnormalities.

Mice were euthanized by CO2 inhalation. Working under a dissecting microscope, lateral choroid plexuses were isolated using Dumont 5 forceps inserted into each hemisphere of the brain. Tissue was transferred immediately to ice-cold, artificial cerebrospinal fluid (aCSF; 103 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, 10 mM glucose, 1 mM sodium pyruvate, pH 7.4), previously gassed with 95% oxygen-5% CO2. KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, 10 mM glucose, 1 mM sodium pyruvate, pH 7.4), previously gassed with 95% oxygen-5% CO2. In experiments designed to determine the Na dependence of transport, medium NaCl and NaHCO3 were replaced with choline chloride and choline bicarbonate, respectively (Na-free aCSF). All experiments were conducted according to protocols approved by the National Institute of Environmental Health Sciences Animal Use and Care Committee.

Confocal imaging. Each plexus was transferred to a covered, teflon microscopic imaging chamber, containing 1.0 ml of pregassed aCSF or Na-free aCSF with 1 μM FL and added effectors. Fluorescent compounds and inhibitors were added to the incubation medium as stock solutions in aCSF or DMSO. Preliminary experiments showed that the final concentrations of DMSO used (≤0.5%) had no significant effects on the uptake and distribution of FL. All transport experiments were conducted at room temperature (18–20°C) and all chambers with tissue were maintained in ziplock plastic bags containing 95% oxygen-5% CO2 under slight positive pressure until the tissue removed for confocal imaging; under these conditions, medium pH was constant for at least 1 h. While on the microscope, tissue was gassed with 95% oxygen-5% CO2 through a port in the chamber cover.

To acquire images, the chamber containing tissue was mounted on the stage of a Zeiss inverted confocal laser-scanning microscope (model 510 or 410) and viewed through a ×40 water immersion objective (numerical aperture = 1.2). A 488-nm laser line (Ar-ion laser) was used with a 505-nm dichroic mirror and a 515-nm long-pass emission filter were used. Low laser intensity was used to avoid photobleaching. With the photomultiplier gain set to give a subepithelial space fluorescence intensity of 150–200 (full scale 255) in control tissue loaded with 1 μM FL, autofluorescence of unloaded tissue was undetectable. Details of image acquisition and analysis are given by Breen et al. (4).

RESULTS

FL transport. In previous experiments using confocal imaging, we found that FL accumulation in cells and subepithelial and vascular spaces from Oat3-null mouse choroid plexus was 75% lower than in wild-type littermates (23). Thus Oat3 accounted for a substantial portion of FL transport in the mouse. We investigated further the mechanism of FL transport in wild-type and Oat3-null mice. Figure 1A shows a confocal micrograph of steady-state (45 min) FL accumulation in tissue from a wild-type mouse. The xy-section shown is from the middle of a stack of 45 optical sections taken 0.7 μm apart. As in the rat (4) and in agreement with previous initial findings for the mouse (23), fluorescence in the epithelial cells exceeded that in the medium and fluorescence in the vascular and subependymal spaces was greater than in the cells. Within the cells, fluorescence was both diffuse and punctate, with the punctate compartment being cytoplasmic; the large nucleus was devoid of punctate fluorescence. Present in the subepithelial space are small, intensely fluorescent blood vessels, which are easily identified by their nonfluorescent erythrocytes. Also shown in Fig. 1A are reconstructed xz- and yz-sections through the stack. They clearly show intense fluorescence in the tissue spaces below the epithelial cell layer, suggesting a continuous subependymal compartment in which FL is concentrated.

Measurements of average fluorescence intensity at steady state (45 min) showed that fluorescence in the subependymal and vascular spaces exceeded cellular fluorescence by a factor of about three (Fig. 2). The organic anions probenecid and estrone sulfate reduced accumulation of FL in cells and subependymal and vascular spaces by ∼70–80%. Removing medium Na reduced FL accumulation in both tissue compartments to about the same extent as probenecid and estrone sulfate, indicating that essentially all mediated uptake was Na dependent. As in the rat (4), increasing medium K reduced subependymal and vascular FL accumulation by 70% but doubled cellular accu-
mulation (Fig. 2), suggesting electrical potential (PD)-sensitive eflux at the basolateral membrane. Thus, as in the rat (4), FL transport across mouse choroid plexus involved two concentrative steps: Na-dependent uptake at the apical membrane followed by PD-driven eflux at the basolateral membrane.

In agreement with previous experiments, FL accumulation in both compartments of tissue from Oat3-null mice was reduced by ~80% compared with tissue from wild-type mice (Figs. 1, B and C, and 2). Moreover, in tissue from knockout mice, neither probenecid nor Na replacement significantly reduced uptake, indicating that no mediated or Na-dependent component of uptake remained when Oat3 expression was abolished (Fig. 2). Thus, in mouse choroid plexus, Oat3 appeared to be responsible for all mediated uptake of FL and FL transport on Oat3 was Na dependent.

PAH transport. Initial experiments with choroid plexus from wild-type mice indicated that accumulation of 10 μM [3H]PAH was approximately linear over the first 5 min of incubation and then reached a plateau after 30 min (not shown). Figure 3 shows that 5-min PAH uptake was reduced

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**Fig. 1.** Confocal sections of mouse choroid plexus after 45-min incubation in medium containing 1 μM fluorescein (FL). A: tissue from a wild-type mouse. The xy-section is a middle section from a stack of 45 taken 0.7 μm apart. Also shown are reconstructed xz- and yz-sections. Even at this relatively low magnification, epithelial cells are seen to contain punctate cytoplasmic sites of fluorescence; nuclei are clear of punctate sites. Some blood vessels contain trapped erythrocytes (red arrows), which are not fluorescent. Cell-free vessels are intensely fluorescent. B and C: tissue from an organic anion transporter 3 (Oat3)-null mouse. B: transmitted light. C: confocal image. Cellular and vascular fluorescence is substantially reduced in tissue from the Oat3-null mouse. Bars = 50 μm. B and C: same magnification.
from a control value of 0.188 ± 0.010 to 0.057 ± 0.005 and 0.046 ± 0.003 pmol/mg protein by 0.25 and 1 mM probenecid, respectively. Estrone sulfate and cimetidine, both Oat3 substrates (14), at 1 mM reduced uptake to 0.04 pmol/mg protein. Thus, by these criteria, nearly 80% of total uptake was mediated. PAH uptake was also Na⁺ dependent. Preincubation in Na-free medium or with the Na-K-ATPase inhibitor ouabain reduced uptake by 77% (Fig. 3).

In tissue from Oat3-null mice, PAH uptake averaged only 0.062 ± 0.004 pmol/mg protein, a value that is 68% lower than in tissue from wild-type mice (Fig. 3). Addition of 1 mM probenecid to the incubation medium did reduce PAH uptake further (P < 0.05), suggesting an minor component of uptake other than Oat3. We draw two conclusions from these uptake data for PAH: first, in tissue from wild-type mice, uptake is Na⁺ dependent, and this Na⁺-dependent component of uptake is equivalent to the probenecid-, estrone sulfate-, and cimetidine-sensitive component; second, knocking out Oat3 nearly abolished the mediated (and Na dependent) component of uptake. Thus, as with FL (above), it appears that in mouse choroid plexus specific PAH transport was both mediated by Oat3 and Na dependent.

Estrone sulfate transport. Estrone sulfate is a substrate for multiple organic anion transporters and several of these including Oat3 and Oatp3 are expressed in rat choroid plexus (2, 9, 16, 18); estrone sulfate is not an Oat1 substrate (23). Thus, in the choroid plexus, we would expect uptake of 0.2 μM estrone sulfate to be more complicated than FL or PAH. In tissue from wild-type mice, ~90% of the total 5-min uptake was mediated, i.e., inhibited by a 5,000-fold excess of unlabeled substrate or by 200 μM dehydroepiandrosterone sulfate (DHEAS; Fig. 4). Five millimolar PAH reduced uptake by 57%. Removing medium Na⁺ decreased uptake by 76%. Clearly, the major portion of mediated estrone sulfate uptake in tissue from wild-type mice was Na⁺ dependent, but a significant component was not.

Estrone sulfate uptake in tissue from Oat3-null mice was significantly reduced compared with tissue from wild-type mice (P < 0.05; Fig. 4); however, that reduction was only 33%. As with tissue from wild-type mice, a substantial fraction of total uptake in tissue from Oat3-null mice was inhibited by...
1 mM estrone sulfate and 200 µM DHEAS. Surprisingly, 5 mM PAH still inhibited uptake, indicating a PAH-sensitive component of uptake that was not on Oat3. As in tissue from wild-type mice, a substantial component of estrone sulfate uptake in tissue from Oat3-null mice was Na⁺ dependent (Fig. 4). Comparison of transport in tissue from wild-type and Oat3-null mice showed that the estrone sulfate-insensitive, DHEAS-insensitive, PAH-insensitive, and Na⁺-independent components were unchanged (Fig. 4), suggesting no compensatory upregulation of organic anion transporters. Thus, as with PAH, the portion of estrone sulfate transport lost when Oat3 was knocked out represented Na⁺-dependent uptake. Remaining uptake in tissue from Oat3-null mice was, in large part, mediated and partly Na⁺ dependent, suggesting at least three specific components of estrone sulfate transport in mouse choroid plexus.

**Taurocholate transport.** In tissue from wild-type mice, uptake of 1 µM taurocholate was reduced by ∼90% by 1 mM estrone sulfate, indicating that nearly all taurocholate uptake was mediated (Fig. 5). PAH at 5 mM did not significantly reduce taurocholate uptake, suggesting that Oat3 was not involved. Uptake was reduced by ∼69% when medium Na was replaced (Fig. 5). Consistent with the lack of effect of PAH on taurocholate uptake in wild-type mice, taurocholate uptake in tissue from Oat3-null mice was not significantly reduced compared with tissue from wild-type controls (Fig. 5). In tissue from Oat3-null mice, PAH at 5 mM was again without significant effect. Na replacement reduced uptake by 73%. Thus, although taurocholate uptake was Na-dependent and inhibited by estrone sulfate, it was insensitive to inhibition by PAH. Taurocholate transport on Oat3 was not detectable as judged by the lack of significant difference in uptake between wild-type and knockout tissue.

**DISCUSSION**

By controlling the efflux of xenobiotics and metabolic wastes from CSF, the choroid plexus plays an important role in protecting critical brain functions and regulating the extracellular environment of the central nervous system (CNS). Efflux transporters for organic anions are multispecific, and competition for transport is one mechanism that underlies drug-drug and drug-metabolite interactions. Thus understanding organic anion transport at the tissue, cellular, and molecular levels is essential to our ability to design more effective CNS-acting drugs and to intervene in disease progression.

A crucial obstacle to understanding the mechanisms that underlie xenobiotic export from brain by the choroid plexus is reconciling findings on transporter expression obtained from molecular/immunological studies with data on transport of individual compounds in the intact tissue or in vivo. In this regard, several organic anion transporters are known to be expressed in choroid plexus epithelial cells, including Oat1–3, Oatp1–3, and Mrp1 (2, 9, 10, 16, 18, 25). Of these, Oat1, Oat3, Oatp3, and possibly Oatp1 are localized to the apical membrane, and Mrp1 and Oatp2 are localized to the basolateral membrane. The cellular location of Oat2 has not been determined, and it is not yet clear whether Oat4, Oat5, and higher Oatp isoforms are expressed in the tissue. All these transporters exhibit broad and overlapping substrate specificities, making it difficult to unambiguously assign transporters to processes in intact tissues, even in those cases where transporters have been cloned and specificities and kinetics characterized thoroughly.

A complementary approach to mapping and characterizing transport pathways in complex tissues is through the use of knockout animals. We recently generated an Oat3-null mouse, which exhibited loss of organic anion transport in the kidney (PAH, estrone sulfate, and taurocholate) and choroid plexus (FL; Ref. 23). In agreement with immunolocalization studies, which place Oat3 at the basolateral plasma membrane in renal proximal tubule and the apical membrane in the choroid plexus (12, 16), loss of transport function was consistent with Oat3 mediating organic anion uptake from blood to kidney and from CSF to choroid plexus (23). In the present study, we examined in intact choroid plexus tissue from wild-type and Oat3-null mice the contribution of Oat3 to the uptake of four organic anions, all substrates for Oat3 (23), as well as the energetics of transport on Oat3.

Our data indicate that Oat3 was responsible for essentially all mediated uptake of FL and PAH. Knocking out Oat3 reduced total uptake of FL and PAH by ∼80%. Importantly, neither estrone sulfate nor probenecid at high concentrations reduced FL and PAH uptake below this level. FL and PAH are substrates for mouse Oat1 and Oat3, and mRNA for both paralogs has been detected in mouse and rat choroid plexus and kidney (23). Consistent with both paralogs mediating transport in the kidney, mediated PAH uptake in renal cortical slices from Oat3-null mice is partially reduced compared with slices from wild-type controls (23). In contrast, mediated uptake of PAH and FL is essentially abolished in choroid plexus tissue from Oat3 mice. Thus, the contribution, if any, of Oat1 to FL and PAH uptake in mouse choroid plexus would have to be below the limits of detection of our experiments (<10–15% of uptake).

With estrone sulfate, only about one-third of mediated transport was lost in tissue from Oat3-null mice. With taurocholate, no significant reduction in transport was seen in tissue from Oat3-null mice, a finding consistent with the lack of effect of PAH on taurocholate uptake. In contrast, previous experiments indicated that in mouse renal cortical slices, all uptake of estrone sulfate and taurocholate was mediated by Oat3, i.e., in slices from Oat3-null mice, probenecid and bromosulfophtha-
lein no longer significantly inhibited uptake (23). Both estrone sulfate and taurocholate are known to be substrates for a number of transporters, including members of the Oat and Oatp families (1, 6, 8, 17, 23). Clearly, tissue-specific patterns of organic anion transporter expression in mouse choroid plexus and kidney underlie the differences in the importance of Oat3 for estrone sulfate and taurocholate uptake in the two tissues. In the choroid plexus, in addition to the Oats, Oat3 has been localized to the apical membrane and thus is available to mediate uptake of large organic anions (13, 18); the possibility that Oat1 is expressed at the apical membrane of choroid plexus was recently cast in doubt (13, 18). In renal proximal tubule, no such alternative uptake pathway appears to be present (19).

Because Oat1 does not appear to play a major role in organic anion uptake in choroid plexus (above), one must consider other candidates for the Na-dependent component. Initial reports provided little information about the energetics of transport on Oat3 (14), but recent experiments with X. laevis oocytes injected with cloned rat Oat3 mRNA and rat renal cortical slices naturally expressing the transporter provide evidence for dependence on the Na gradient (23). Na dependence appears to be indirect, being the result of organic anion/dicarboxylate exchange on Oat3, and of Na-dicarboxylate cotransport. In the present study, we found for all substrates tested that the component of uptake lost by the knockout animals was clearly Na dependent. Although we have not previously tested for indirect coupling of Oat3 to Na in mouse choroid plexus, that mechanism of uptake has been confirmed for the cloned rat transporter in X. laevis oocytes (23), in rat and mouse kidney (Ref. 21; Chan LMS, Sweet DH, Walden R, Nigam SK, Beier DR, Miller DS, and Pritchard JB, unpublished observations), and in rat choroid plexus (Miller and Lowes, unpublished observations). Note that in the present experiments, we established Na dependence of organic anion transport by replacing medium NaCl and NaHCO\textsubscript{3} with choline Cl and choline HCO\textsubscript{3}, respectively. Previous experiments showed that choline uptake by rat choroid plexus is electrogenic (22, 26) and it is possible that the high choline concentrations used for Na replacement could have reduced organic anion transport through changes in membrane electrical potential rather than the Na gradient. We believe this is not the case because transport of FL and PAH in mouse choroid plexus (present study) and transport of estrone sulfate and taurocholate in rat choroid plexus (Miller DS and Lower, unpublished observations) were not reduced when medium potassium concentration was increased 10-fold, a maneuver that depolarizes by 55 mV (26).

The present experiments show that all the mediated transport of FL and PAH was both mediated by Oat3 and abolished when medium Na was replaced. This was not the case for the other two substrates studied. With estrone sulfate, tissue from Oat3-null mice exhibited a significant Na-dependent component of uptake. Thus, even after all Oat3-mediated transport was knocked out, Na-dependent transport was still evident. Moreover, transport of taurocholate, also an Oat3 substrate (23), was not reduced at all in choroid plexus from Oat3-null mice. Taurocholate transport was, however, both Na dependent and mediated (inhibited by estrone sulfate). Together, these findings suggest additional Na-dependent and -independent pathways are available for the uptake of organic anions. Consistent with this, we recently found evidence for an additional Na-dependent uptake pathway for large organic anions in rat choroid plexus. One possibility is that the Na-dependent component represents transport on Oat2. α-Ketoglutarate is an Oat2 substrate (20), suggesting that this transporter, like Oat1 and Oat3, could function as an organic anion/dicarboxylate exchanger and thus could be coupled energetically to the Na gradient in those tissues (like choroid plexus) that express both Oat2 and a Na-dicarboxylate cotransporter. Whether Oat2 is indeed an exchanger that could be energetically coupled to the Na gradient remains to be shown.

Finally, for estrone sulfate and taurocholate, there appears to be an additional Na-independent component of uptake. Estrone sulfate is transported by Oat1 and Oat3, both of which have been localized to the apical membrane of choroid plexus epithelial cells (2, 18). Uptake mediated by the Oat family of transporters is not Na dependent (11). At present, it is not clear how members of this family drive organic anion uptake into cells, although organic anion/glutathione exchange is one possibility (15).

In conclusion, in mouse choroid plexus, Oat3 was implicated in the uptake of PAH, FL, and estrone sulfate but not taurocholate. For the two substrates that are known to be handled by both Oat1 and Oat3 (PAH and FL), all mediated transport was on Oat3; an Oat1-mediated component could not be detected. Comparison of organic anion transport in choroid plexus from Oat3-null and wild-type mice demonstrated that all transport on this Oat paralog was Na dependent. However, for estrone sulfate and taurocholate, additional Na-dependent and -independent components of organic anion transport were found.

GRANTS

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