Fluorescein-Methotrexate (FL-MTX) Transport in Dogfish Shark, *Squalus acanthias*, Choroid Plexus

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The choroid plexus (CP) comprises the blood-cerebrospinal fluid (CSF) barrier, separating brain extracellular fluid from blood. Being the main site of CSF production, it regulates brain homeostasis, provides essential nutrients and removes metabolic wastes and xenobiotics. Previous imaging and flux chamber experiments with dogfish shark CP demonstrated active blood-to-CSF transport of two small organic anions, fluorescein (FL) and 2,4-dichlorophenoxyacetic acid (2,4-D)[7]. Confocal imaging showed that transepithelial transport of FL involved two concentrative steps: apical uptake into the cells and basolateral efflux into blood vessels. The same two-step mechanism has been proposed for FL in rat and mouse CP[2,5,6]. Here we examine mechanisms driving transport of a larger, fluorescent organic anion, FL-MTX, in shark CP as well as one type of signaling event that regulates it.

Lateral CP were excised into gassed (99% O_2/1% CO_2), elasmobranch Ringer solution (ER, in mM: 280 NaCl, 6 KCl, 4 $CaCl_2$, 3 $MgCl_2$, 1 NaH_2PO_4, 0.5 Na_2SO_4, 350 urea, 72 trimethylamine oxide, 2.5 glucose, 8 $NaHCO_3$ and pH 7.8). For incubation and measurements, tissue was transferred to Teflon chambers containing ER with 2 μM FL-MTX without (controls) or with effectors. Tissue distribution of substrate was followed using confocal microscopy (Zeiss Pascal confocal microscope, 488 nm laser excitation, 40x, 1.2 NA water immersion objective) and measured using NIH Scion Image Software, as previously described[7].

FL-MTX accumulated rapidly in CP, with steady state distributions achieved after 1 h. At steady state, cellular fluorescence approximated medium fluorescence, but blood vessels and lateral intercellular spaces were intensely fluorescent, i.e. at least 5 times medium levels. Vessel fluorescence was reduced by more than 50% when medium NaCl was replaced with N-methylglucamine, when Na,K-ATPase was inhibited by 500 μM ouabain or when metabolism was blocked with NaCN; increasing medium K from 6 to 30 mM had no effect. Thus, transepithelial transport of FL-MTX was concentrative, Na-dependent and driven by metabolism, but it appeared to be insensitive to changes in electrical potential difference (PD). Transport into blood vessels was also reduced by other organic anions, including, MTX, MK-571, leukotriene C_4, taurocholate, p-aminohippurate (PAH), probenecid and estrone sulfate. These compounds minimally affected cellular MTX accumulation. Surprisingly, 10-50 μM digoxin reduced vessel accumulation of FL-MTX, but increased cellular accumulation by more than 2-fold. The digoxin-induced increase in cellular fluorescence was blocked when tissue was incubated in Na-free medium or with ouabain, MTX, probenecid, PAH or taurocholate. One explanation for these inhibition data is that transepithelial transport is a two-step process, involving mediated uptake followed by efficient efflux into blood vessels. With most inhibitors, uptake was affected first, causing minimal changes in normally low cellular fluorescence, but large decreases in vessel fluorescence. Digoxin, preferentially reduced efflux, substantially increasing cellular accumulation. This showed that the first step in FL-MTX transport was mediated and Na-dependent.

Further evidence for two-step FL-MTX transport was provided by experiments concerned with the effects of signaling by protein kinase C (PKC). PKC was previously implicated in the regulation of organic anion transport in teleost renal tubules[3,4]. Incubating shark CP with nanomolar concentrations of phorbol ester (phorbol-12-myristate-13-acetate, PMA) reduced cellular and blood vessel accumulation of FL-MTX; these effects were concentration dependent. Bis-indolylmaleimide (BIM), a PKC-selective inhibitor that by itself did not affect transport, blocked PMA effects . Thus one or more PKC isoforms regulate FL-MTX uptake. It is not clear from these experiments whether the reduction in FL-MTX accumulation in blood vessels was a consequence of inhibition of the apical uptake step alone or whether the basolateral efflux step was also affected.

Previous confocal imaging experiments with CP from rat, mouse and shark have shown that transepithelial FL transport involves two concentrative steps in series, the first being Na-dependent and most likely mediated by organic anion transporter isoform 3 (Oat3) and its shark ortholog and the second being electrical potential dependent[3,6-8]. For the larger organic anion, FL-MTX, a different pattern of distribution within CP was observed in tissue from mouse[5], rat[1] and shark (present study). Apical uptake was not concentrative, but basolateral efflux clearly was. In all three species, transepithelial transport was Na-dependent and inhibited by other organic anions, but it was not PD-sensitive. These findings might lead one to assume that apical uptake of FL-MTX was not mediated. However, in rat and shark, experiments with digoxin revealed mediated, Na-dependent FL-MTX uptake at the apical membrane. Since this drug is a potent inhibitor of rat organic anion transporting polypeptide isoform 2 (Oatp2) and since that transporter is known to be expressed on the basolateral membrane of rat CP, it is likely that digoxin increased cellular FL-MTX by blocking efflux mediated by Oatp2 in rat and by its ortholog in shark.

Identifying the Na-dependent, apical uptake step is problematical. In mammalian CP, the only transporter that could be responsible for Na-dependent organic anion uptake at the basolateral membrane is Oat3[1,6], but CP from Oat3-null mice showed no reduction in FL-MTX transport[5]. In shark, an Oat3-like transporter is suggested by studies with FL and 2,4-D[7,8], but it is not known whether this transporter also handles FL-MTX. Clearly, further studies are needed to determine the molecular basis of organic anion transport in shark CP and to identify the hormones that activate PKC.
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1. Breen CM, Sykes DB, Baehr C, Fricker G, and Miller DS. Fluorescein-methotrexate transport in rat choroid plexus analyzed using confocal microscopy. *Am. J. Physiol.*, submitted.
2. Breen CM, Sykes DB, Fricker G, and_Miller DS. Confocal imaging of organic anion transport in intact rat choroid plexus. *Am. J. Physiol.* 282:F877-85, 2002.
3. Masereeuw R, Terlouw SA, Van Aubel RAMH, Russel FGM, and Miller DS. Endothelin B receptor mediated regulation of ATP-driven drug secretion in renal proximal tubule. *Mol. Pharm.* 57:59-67, 2000.
4. Miller DS. Protein kinase C regulation of organic anion transport in renal proximal tubule. *Am J Physiol.* 274:F156-64, 1998.
5. Sweet DH, Miller DS, Pritchard JB, Fujiwara Y, Beier DR, and_Nigam SK. Impaired organic anion transport in kidney and choroid plexus of organic anion transporter 3 (Oat3 (Slc22a8)) knockout mice. *J. Biol. Chem.* 277:26934-43, 2002.
6. Sykes D, Sweet DH, Lowes S, Nigam SK, Pritchard JB, and_Miller DS. Organic Anion Transport in Choroid Plexus From Wild-Type and Organic Anion Transporter 3 (Slc22a8)-Null Mice. *Am. J. Physiol.* in press, 2004.
7. Villalobos AR, Miller DS, and_Renfro JL. Transepithelial organic anion transport by shark choroid plexus. *Am. J. Physiol.* 282:R1308-16, 2002.
8. Villalobos AR, Miller DS, and_Renfro JL. ion dependence of organic anion transport across dogfish shark (Squalus acanthias) choroid plexus. *Bull MDIBL.* 42:125-6, 2003.