TRANSPORT OF A FLUORESCENT BILE SALT DERIVATIVE IN HEPATOCYTES FROM LITTLE SKATE (RAJA ERINACEA)

David S. Miller[1], Gert Fricker[2], Thomas W. Simmons[3], John H. Henson[4], David Hager[5], Ursula Schramm[2], Gerhart Kurz[6], Jonathan Filmore[5] and James L. Boyer[7]

[1]NIH-NIEHS, Research Triangle Park, NC 27709
[2]Sandoz Pharma AG, Ch-4002 Basle, Switzerland
[3]Department of Biology, Indiana University of PA, Indiana, PA 15705
[4]Department of Biology, Dickinson College, Carlisle, PA 17013
[5]Cornell University, Ithaca, NY 14853
[6]Institut für Organische Chemie und Biochemie, Universität Freiburg, D-7800 Freiburg, Germany
[7]Department of Medicine and Liver Center, Yale University School of Medicine, New Haven, CT 06510

In most vertebrates, the liver actively transports bile acids, such as cholyltaurine (TC), from blood to bile and much of the work on bile acid transport has focused on a Na-dependent mechanism (Boyer et al., Ann. Rev. Physiol. 54:415, 1992). In the liver of the little skate (Raja erinacea), TC secretion is Na-independent (Smith et al., Amer. J. Physiol. 252:G479, 1987), thus, providing an important model in which Na-independent mechanisms driving bile acid transport can be elucidated. In the present study, we used a fluorescent bile salt derivative, (N-{7-(4-nitrobenzo-2-oxa-1,3-diazol)}-7ß-amino-3a,12a-dihydroxy-5ß-cholan-24-oyl)-2-aminoethanesulfonate (NBD-NCT), epi-fluorescence microscopy and video imaging techniques to measure bile acid uptake and distribution in skate hepatocyte clusters with patent canalicular spaces.

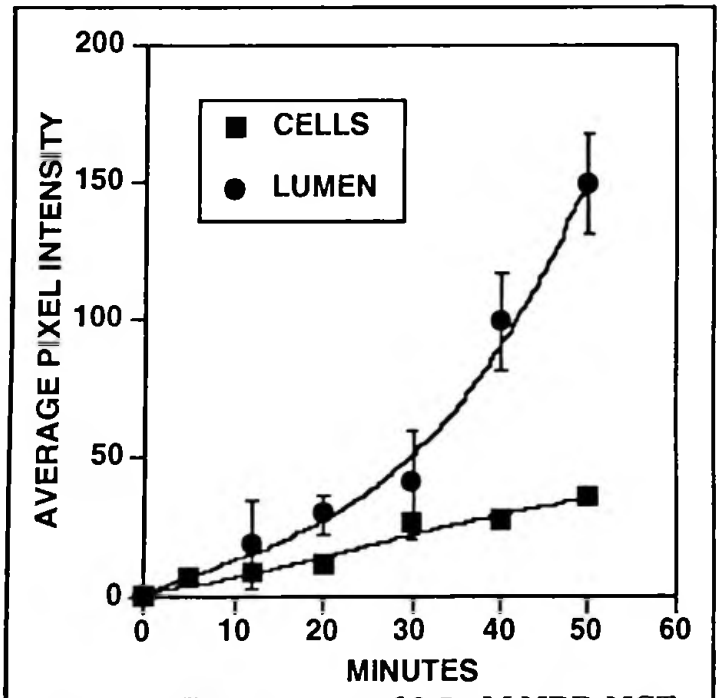
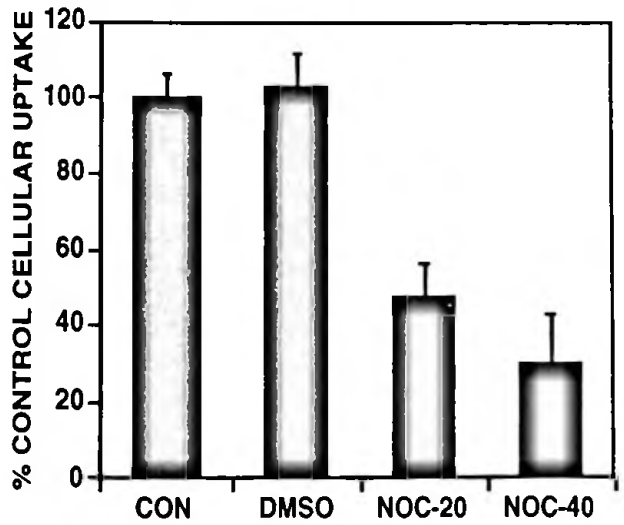


Figure 1. Time course of 0.5 µM NBD-NCT uptake by skate hepatocyte clusters. Each point represents the mean value for 8 clusters; when large enough the variability is given as SE bars.

Skate hepatocyte clusters were prepared by collagenase perfusion of isolated livers (Smith et al., J. Exp. Zool. 241:291, 1987) and then maintained on ice in an elasmobranch Ringer's solution (ER). For experiments, clusters were transferred to a microscope viewing chamber

(Bionique), warmed to 18°-20° C and incubated in ER containing 0.5-1.0 μM NBD-NCT. The floor of the chamber was a glass cover slip to which the clusters adhered; thus, incubation solutions could be changed and drugs added without disturbing the clusters. Cells in the chamber were viewed through an epi-fluorescence microscope (Nikon) fitted with a fluorescein filter set and a CCD video camera (Hamamatsu) connected to a MacIntosh IIcx computer. Frame-averaged images were stored on video disk for later analysis.

Initial experiments demonstrated that NBD-NCT was taken up rapidly by hepatocytes. Within about 30 min, cellular accumulation reached a plateau and a region of intense fluorescence corresponding to the bile canalicular space could be seen in many clusters (Fig. 1). At 60 min, the percentage of clusters showing intense canalicular dye accumulation varied with preparation from about 35 to 70%. NBD-NCT uptake into cells and canalicular spaces was inhibited by several organic anions, including, TC, bumetanide, bromosulphonphthalein, probenecid and p-aminohippurate. Of these, TC was the most potent, with 0.1-1.0 mM reducing NBD-NCT uptake by 60-80%. Uptake was also blocked by KCN and dinitrophenol, and by incubation at 4°C. Thus NBD-NCT uptake into hepatocytes was rapid, specific and energy dependent.



POSITIVE LUMENS	13/29	8/12	0/14	0/13

Figure 2. Effect of nocodazole exposure on the 60 min accumulation of 1 μM NBD-NCT by skate hepatocyte clusters. The graph shows the mean cellular accumulation; variability is given as SE bars. The table shows the number of clusters with intense fluorescence in the canalicular lumen over the total number studied. Treatments: CON, control; DMSO, 0.2% dimethylsulphoxide (solvent) control; NOC-20, 20 μM nocodazole (in 0.1% DMSO); NOC-40, 40 μM nocodazole (in 0.2% DMSO).

NBD-NCT uptake and secretion into the canalicular space was also dependent on an intact cytoskeleton. Hepatocyte clusters were fixed and immunofluorescently stained for tubulin using a monoclonal anti-α tubulin primary antibody and a goat anti-mouse IgG fluorescein-conjugated secondary antibody. Control clusters demonstrated well developed cytoplasmic arrays of microtubules, with groups appearing to radiate away from the apical membrane. Microtubules were depolymerized when clusters were exposed to 20-40 μM nocodazole for 3 h at 0° C. Preincubation (3 h at 0° C) of hepatocyte clusters with 10-40 μM nocodazole also reduced the cellular uptake of NBD-NCT and nearly abolished luminal dye accumulation (Fig. 2). However, nocodazole had no effects on hepatocyte tissue K levels, indicating that inhibition of bile acid transport was not secondary to

general toxicity.

Bile salt efflux from hepatocyte to canaliculus may be driven by the electrical potential difference (PD) across the canalicular membrane (Weinman et al., Amer. J. Physiol. 256:G826, 1989). To test this possibility, we incubated skate hepatocyte clusters in medium containing 140 mM KCl (isoosmotic replacement of NaCl) rather than 5 mM. This treatment causes a long-lasting depolarization of skate hepatocytes (microelectrode studies indicate that PD changes from about -60 to -40 mV when medium K is raised; Ballatori et al., Biochim. Biophys Acta 946:261, 1988). High K medium had no effects on the cellular accumulation of NBD-NCT. However, high K medium nearly abolished luminal accumulation of the dye. The effects of elevated K were rapidly reversed when clusters were returned to normal-K ER, a maneuver that also repolarizes the hepatocytes (Ballatori et al., op cit).

The present experiments with hepatocyte clusters from the little skate, a species that exhibits only Na-independent bile salt secretion (Smith et al., Amer. J. Physiol. 252:G479, 1987), show that a fluorescent taurocholate derivative is taken up and then secreted into a space that is functionally and morphologically equivalent to the bile canaliculus. Transport involves three steps: an uptake step that is specific and energy-dependent, an intracellular step that requires an intact microtubular network, and an efflux step that is at least partially PD-dependent. (Supported by a MDIBL Young Investigator Fellowship to G.F., the Deutsche Forschungsgemeinschaft SFB 154, a Pew Charitable Trusts Fellowship to T.W.S. and NIH Grants ES03828, DK34989, and DK25636. JHH acknowledges support from the Research Corporation, the Knight Foundation and the NSF ILI Program.)