Comparative Biochemistry and Physiology, Part C 149 (2009) 566–571

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Hepatic uptake and biliary excretion of manganese in the little skate, *Leucoraja erinacea*

Michael S. Madejczyk [a,c], James L. Boyer [b,c], Nazzareno Ballatori [a,c,*]

[a] Department of Environmental Medicine, University of Rochester School of Medicine, Rochester, NY 14642, USA
[b] Department of Medicine and Liver Center, Yale University School of Medicine, New Haven, CT 06520, USA
[c] Mount Desert Island Biological Laboratory, Salisbury Cove, Maine 04672, USA

ARTICLE INFO

Article history:
Received 14 October 2008
Received in revised form 15 December 2008
Accepted 15 December 2008
Available online 24 December 2008

Keywords:
Manganese
Transport
Bile
Little skate
Liver

ABSTRACT

The liver is a major organ involved in regulating whole body manganese (Mn) homeostasis; however, the mechanisms of Mn transport across the hepatocyte basolateral and canalicular membranes remain poorly defined. To gain insight into these transport steps, the present study measured hepatic uptake and biliary excretion of Mn in an evolutionarily primitive marine vertebrate, the elasmobranch *Leucoraja erinacea*, the little skate. Mn was rapidly removed from the recirculating perfusate of isolated perfused skate livers in a dose-dependent fashion; however, only a small fraction was released into bile (<2% in 6 h). Mn was also rapidly taken up by freshly isolated skate hepatocytes in culture. Mn uptake was inhibited by a variety of divalent metals, but not by cesium. Analysis of the concentration-dependence of Mn uptake revealed of two components, with apparent K_m values 1.1 ± 0.1 μM and 112 ± 29 μM. The K_m value for the high-affinity component was similar to the measured skate blood Mn concentration, 1.9 ± 0.5 μM. Mn uptake was reduced by nearly half when bicarbonate was removed from the culture medium, but was unaffected by a change in pH from 6.5 to 8.5, or by substitution of Na with Li or K. Mn efflux from the hepatocytes was also rapid, and was inhibited when cells were treated with 0.5 mM 2,4-dinitrophenol to deplete ATP levels. These data indicate that skate liver has efficient mechanisms for removing Mn from the sinusoidal circulation, whereas overall biliary excretion is low and appears to be mediated in part by an ATP-sensitive mechanism.

1. Introduction

Manganese (Mn) is a ubiquitous trace element that is required for life; however, excess Mn can also be quite toxic. Chronic exposure to high levels can lead to Mn accumulation in the brain, specifically in the globus pallidus, and this accumulation is associated with a progressive neuronal loss that appears clinically as a Parkinson's-like disease known as manganism (Crossgrove and Zheng, 2004; Martin, 2006). The main organs involved in Mn homeostasis are the intestine and liver: the intestine acts as an initial control of Mn levels by absorbing a variable amount of the dietary Mn (1–5%), the main source of exposure (Aschner and Aschner, 2005), whereas hepatic biliary excretion serves as the major route of elimination for excess manganese (Papavasiliou et al., 1966). However, the specific mechanisms of Mn transport remain poorly defined.

Manganese appears to be taken up into cells, in part, via the same general mechanisms as iron. Mn^{2+} is a substrate for the divalent metal transporter DMT1 (Slc11A2/Nramp2), a highly conserved transporter that utilizes the proton gradient to drive the uptake of a number of divalent metals, including Fe, Mn, Cd, Co, Cu, Zn, and to a lesser extent Ni and Pb. Studies utilizing the microcytic anemia mouse and the Belgrade (*b*) rat, which have a defect in Dmt1 transport activity, have demonstrated the importance of Dmt1 in Fe and Mn homeostasis (Chua and Morgan, 1997; Fleming et al., 1998). However, a study by Crossgrove and Yokel (2004) using *in situ* brain perfusion of *b*/*b*, +/*b*, and +/+ rats, suggested that Dmt1 is not essential for Mn transport, and indicated the presence of other compensatory Mn transport mechanisms. Once it is taken up into cells, Mn is known to be sequestered in mitochondria (Gavin et al., 1999), and possibly in other intracellular compartments, and can bind to many intracellular ligands. Although Mn can induce the expression of metallothionein in the liver, it does not appear to bind to metallothionein.

The mechanism of cellular Mn efflux is also largely undefined. A hypothetical possibility is that efflux is mediated by ferroportin (Fpn1/Slc40a1), the only known iron efflux transporter. In polarized cells such as enterocytes, Fpn1 is localized to the basolateral membrane where it could theoretically function to release the absorbed Mn from the intestine into the splanchnic circulation. In the liver, excess Mn is

Abbreviations: Mn, manganese; DMT1, divalent metal transporter-1/NRAMP2; FPN1, ferroportin/SLC40A1; DMSO, dimethyl sulfoxide.

* Corresponding author. Department of Environmental Medicine, University of Rochester School of Medicine, 575 Elmwood Avenue, Box EHSC, Rochester, NY 14642, USA. Tel.: +1 585 275 0262; fax: +1 585 256 2591.
E-mail address: Ned_Ballatori@urmc.rochester.edu (N. Ballatori).

1532-0456/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2008.12.009

removed from the portal circulation and is excreted into the bile (Papavasiliou et al., 1966). Studies in rats have shown that the biliary excretion of Mn is saturable, indicating a carrier-mediated transport mechanism (Klaassen, 1974; Ballatori et al., 1987); however, the protein or proteins responsible for transporting Mn from hepatocytes into bile have not yet been identified. In hepatocytes, Fpn1 has been localized to the sinusoidal membrane (Abboud and Haile, 2000) where it functions to export iron, and possibly Mn, back into the circulation. However, there is currently no experimental evidence that Mn is a substrate for Fpn1.

To gain insight into potential mechanisms of hepatic basolateral Mn uptake and biliary Mn excretion, the present study characterized these processes in an evolutionarily primitive marine vertebrate, the elasmobranch *Leucoraja erinacea* (little skate). Skates evolved 200–400 million years ago, and express many of the same transporters found in the livers of mammals. Livers of the little skate are relatively large, easy to handle during surgery, and easily maintained in a perfusion system with minimal reagents due to their lower metabolic rate and temperature requirements. In addition, isolated hepatocytes maintain their polarity in culture (Ballatori et al., 2006). Bile formation and secretion in this model system has also been well characterized (Boyer et al., 1976; Reed et al., 1982a,b; Fricker et al., 1997; Ballatori et al., 2000). The present results demonstrate that skate liver has transport mechanisms for taking up Mn from the sinusoidal circulation and for excreting the metal into bile, and that the general Mn transport properties of skate hepatocytes are comparable to those in mammals. Thus, the skate may be a useful model for the further characterization and potential molecular identification of Mn transporters.

2. Methods

2.1. Materials and animals

Hepatocytes were isolated from male skates (*L. erinacea*; 0.7–1.2 kg body mass) that were caught off the coast of Maine and maintained for up to 4 days before use in large tanks equipped with flowing 15 °C sea water at the Mount Desert Island Biological Laboratory, Salisbury Cove, Maine. $^{54}MnCl_2$ was obtained from New England Nuclear, Boston, MA, USA. Collagenase (type I), deoxyribonuclease II, amino acids, and 2,4-dinitrophenol (2,4-DNP) were obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Skate liver perfusion

Livers were removed from the skates and perfused in an erythrocyte-free, recirculating perfusion system at 15 °C as previously described (Reed et al., 1982a; Simmons et al., 1991). In brief, skates were anesthetized in 6–8 °C seawater containing 0.1 g/L tricaine and 0.035 g/L of $NaHCO_3$. During surgery the animals' gills were superfused with running seawater. The bile duct was cannulated with a 17-cm segment of polyethylene tubing (PE-90). Because the proximal gallbladder and cystic duct are intrahepatic and cannot be ligated, the cystic duct was excluded by inserting a plug at the neck of the gallbladder through an incision at the gallbladder apex. The plug, which consisted of a plastic cap of an 18-G hypodermic needle covered with two layers of Parafilm, was secured into the gallbladder with sutures. Next, the collateral tributaries of the portal vein were ligated, and the portal vein was cannulated with a 2- to 3-cm segment of polyethylene tubing (PE-205) attached to an equal length of latex tubing. After the portal vein was cannulated, the liver was flushed with 40–50 mL of heparinized (2 U/mL) elasmobranch Ringer solution. The liver was then excised and perfused at a rate of 30 mL/min, which produced a perfusion pressure of 2–4 cm H_2O, which is optimal for bile production and O_2 consumption in the isolated perfused skate liver (Reed et al., 1982b). The perfusion medium consisted of well-oxygenated elasmobranch Ringer solution, containing (in mM) 270 NaCl, 4 KCl, 0.5 Na_2SO_4, 1 KH_2PO_4, 8 $NaHCO_3$, 350 urea, 2.5 $CaCl_2$, 3 $MgCl_2$, 5 D-glucose, and 5 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)-tris(hydroxymethyl)aminomethane (Tris) at pH 7.5. The first 150 mL of perfusate were discarded after a single passage; subsequently, a recirculating perfusion was performed with a reservoir containing 100 mL of perfusion medium. The medium was continuously filtered. The filter upstream from the perfusate reservoir consisted of two layers of cheesecloth stretched over a small funnel, whereas the downstream filter was a Millipore filter holder containing a prefilter (AP25-042-00). Bile was collected at 60-min intervals, whereas a 0.2-mL perfusate sample was taken at 30 min, 1 h, and at every subsequent hour. The perfusion was carried out for 7 h. After 1 h, $^{54}MnCl_2$ was added to the recirculating perfusate at concentrations ranging from tracer (i.e., essentially carrier free) to 1 mM, with an activity of 1 μCi per 100 mL perfusate. A 100 μL aliquot of 100 mM taurocholate was added to the recirculating medium every hour to stimulate bile flow. Bile volume was measured gravimetrically, assuming a density of one. In some experiments, radioactivity was omitted and perfusate samples were analyzed for K^+ content via an Instrumentation Laboratory (Lexington, MA, USA) model IL943 flame photometer.

2.3. Hepatocyte isolation

The methods for isolating skate hepatocytes, as well as their morphological and biochemical characterization are described in detail elsewhere (Smith et al., 1987; Ballatori and Boyer, 1988). In brief, skates were anesthetized and the animals' gills were superfused as mentioned above. A cannula was inserted into the portal vein, and the liver was removed from the abdominal cavity and placed in a water-cooled perfusion dish (15 °C). A non-recirculation perfusion with heparinized (2 U/mL) calcium and magnesium-free elasmobranch Ringer solution was then started immediately. After 10 min of single-pass perfusion, a recirculating perfusion was started with a collagenase solution containing 50–100 units of Sigma collagenase no. C-0130/mL of elasmobranch Ringer. The collagenase perfusion was continued for 5–10 min, at which time the liver was raked free of connective tissue in a solution of elasmobranch Ringer containing deoxyribonuclease II. The resulting cell suspension was filtered through cheesecloth and centrifuged at 100 g for 2 min. The cells were resuspended in either normal elasmobranch Ringer solution or a sodium-free medium (see below) and centrifuged at 100 *g*. The washed hepatocytes were resuspended at a concentration of 20–40 mg wet wt/mL (2–$4 \cdot 10^6$ cells/mL) in one of the following incubation buffers: 1) normal elasmobranch Ringer, 2) sodium-free media that were prepared by isosmotic substitution of NaCl with $LiCl_2$, $NaHCO_3$ with choline bicarbonate, and Na_2SO_4 with $MgSO_4$, ($MgCl_2$ was decreased to adjust the Mg concentration), 3) sodium-free media prepared by isosmotic substitution of NaCl and $NaHCO_3$ with the respective potassium salts, 4) bicarbonate-free media in which $NaHCO_3$ was replaced with additional NaCl, and 5) Ca, Mg, and Ca and Mg dropout media, in which these ions were omitted individually from the final solutions. The cells were preincubated at 15 °C in 50 mL polypropylene tubes for 20–30 min before the start of each experiment.

2.4. $^{54}MnCl_2$ analysis

Skate hepatocytes in primary suspension were exposed to different concentrations of $^{54}MnCl_2$ (tracer to 1 mM; 0.1 μCi/mL) at 15°C. In experiments in which the effects of competing metals were examined, all metals were added simultaneously. The 50-mL polypropylene incubation tubes were agitated by hand, so as to maintain the cells in suspension. Aliquots of the cell suspensions (0.3 mL) were removed at appropriate time intervals and were added to 1.5-mL polypropylene microfuge tubes containing 1.2 mL of ice-cold elasmobranch Ringer solution. This suspension was immediately centrifuged at room temperature for 5 s at 10,000 *g*. An aliquot of the supernatant was removed for scintillation counting, and the rest was aspirated with a