Published in final edited form as:

Comp Biochem Physiol C Toxicol Pharmacol. 2009 May ; 149(4): 566–571. doi:10.1016/j.cbpc.2008.12.009.

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

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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 7h). 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 $1.1 \pm 0.1 \mu\text{M}$ and $112 \pm 29 \mu\text{M}$. The K_m value for the high-affinity component was similar to the measured skate blood Mn concentration, $1.9 \pm 0.5 \mu\text{M}$. 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.

Keywords

manganese; transport; bile; little skate; liver

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

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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 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 (Klassen, 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, 1982b; 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.

Methods

Materials & and animals

Hepatocytes were isolated from male skates (*Leucoraja 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. $^{54}\text{MnCl}_2$ 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.

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 0.1g/L tricaine and 0.035g/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 hour, and at every subsequent hour. The perfusion was carried out for 7 h. After 1 h, $^{54}\text{MnCl}_2$ 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.

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 ($2\text{--}4 \times 10^6$ 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 LiCl₂, NaHCO₃ with choline bicarbonate, and Na₂SO₄ with MgSO₄, (MgCl₂ was decreased to adjust the Mg concentration), 3) sodium-free media prepared by isosmotic substitution of NaCl and NaHCO₃ with the respective potassium salts, 4) bicarbonate-free media in which NaHCO₃ 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.

⁵⁴MnCl₂ analysis

Skate hepatocytes in primary suspension were exposed to different concentrations of ⁵⁴MnCl₂ (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 Pasteur pipette. The residual fluid on the surface of the cell pellet was removed by gently touching the top of the pellet with a strip of filter paper (Ballatori and Boyer, 1988). The microfuge tubes were cut with a razor blade just above the cell pellets, and the pellets were transferred to plastic scintillation vials. The pellets were extracted by vigorous mixing in the presence of 0.2 mL of 4 % 5-sulfosalicylic acid and were dissolved in 5 mL of Opti-Fluor (Packard Instrument). Radioactivity in the pellet extracts and in aliquots of the incubation media and wash solutions was determined by scintillation spectrometry. For each experiment, quenching was estimated by adding an equivalent cell pellet containing no radioactivity (prepared at the beginning of the experiment) to an aliquot of the incubation solution. For the efflux studies, hepatocytes were preloaded with ⁵⁴Mn by incubating with 50 μM ⁵⁴MnCl₂ for 1.5–2.0 h at 15°C in normal Ringer. The cells were then centrifuged at 100 g for 2 min and resuspended in an appropriate Ringer solution also maintained at 15°C. This washing step was repeated so as to eliminate any residual extracellular ⁵⁴Mn. Manganese efflux measurements were started within 5–10 min of the final resuspension. To deplete ATP, cells were treated at the start of the efflux experiments with 2,4-dinitrophenol, with a final concentration of 0.5 mM, or with vehicle (DMSO) as a control (Ballatori et al., 1995).

Determination of blood Mn content by atomic absorption spectrometry

Whole blood samples were drawn from the tail vein using a syringe containing 50 μL heparin solution to a final volume of 1 mL. Samples were frozen at –80°C until the time of analysis. Blood manganese concentrations were determined using a Perkin-Elmer AAnalyst 600 atomic absorption spectrophotometer equipped with longitudinal Zeeman background correction and a transverse heated graphite furnace (Perkin-Elmer Life and Analytical Sciences, Shelton, CT, USA). The matrix modifiers, palladium nitrate (5 μg) and magnesium nitrate (3 μg) were used to stabilize manganese during the pyrolysis furnace step. Argon was used to protect and purge the end capped graphite tubes with an internal flow of 250 mL/min. A PerkinElmer Lumina hollow cathode lamp was used. The spectrometer settings were: wavelength, 279.5 nm; slit width, 0.2 nm; Cu lamp current, 20 mA; Background correction, Zeeman-effect; integration

time, 4 s; injection volume, 15 μ L; and matrix modifier volume, 5 μ L. The AA600 furnace thermal program was: Dry1, 100°C, ramp time 1 s, hold time 20 s; Dry2, 150°C, ramp time 15 s, hold time 30 s; Pyrolysis, 1300°C, ramp time 10 s, hold time 20 s; Atomization, 2000°C, ramp time 0 s, hold time 4 s; Clean out, 2450°C, ramp time 1 s, hold time 3 s. Blood samples were wet ashed with ultra pure nitric acid (SeaStar Chemicals Inc., Sidney, BC, Canada). The residue was dissolved in a small volume of concentrated nitric acid and then diluted to 2% nitric acid with 18 mOhm deionized water prior to analysis.

Statistical Analysis

Differences between treatments were analyzed by ANOVA, followed by Student's t-test. Statistical significance was assigned at $p < 0.05$.

Results

Mn was rapidly and efficiently removed from the recirculating perfusate of isolated perfused skate livers (Figure 1A). When livers were perfused with a ^{54}Mn concentration of 0.01 μM , approximately 55% of the dose was removed in the first 30 min of perfusion, and uptake reached an equilibrium value of around 80% of the dose after 2 h of perfusion. At a ^{54}Mn concentration of 1 μM , about 30% was taken up in the first 30 min, and uptake reached an equilibrium value of approximately 60% of the dose after 1 h of perfusion. Despite the high amount taken up by the liver, only a small fraction of the ^{54}Mn was excreted into bile over a 6-h period (Figure 1B). At the 0.01 μM concentration, less than 1% of the ^{54}Mn dose appeared in bile over this time interval, and at the 1 μM concentration about 2% was excreted into bile (Figure 1B).

To examine the concentration-dependence of Mn uptake and biliary excretion, skate livers were perfused for 6 h with Mn concentrations ranging from tracer levels to 1 mM. At the two lowest Mn concentrations, the livers removed 89 and 81% of the Mn dose from the perfusate, respectively (Figure 2A), but only a small fraction was released into bile (<0.8% of the dose) over this time period (Figure 2B). As the perfusate Mn concentration was increased to 1 μM and higher, a relatively constant percentage of the dose was removed from the perfusate. As shown in figure 2A, about 40% of the Mn remained in the perfusate at Mn concentrations from 1 μM to 1 mM. Of significance, the percent of the dose excreted into bile increased up to a perfusate concentration of 1 μM , and then declined at higher concentrations, suggesting that biliary Mn transport is saturable (Figure 2B).

Whole blood Mn concentration in skates was measured using atomic absorption spectrometry, and was found to be $1.9 \pm 0.5 \mu\text{M}$. This Mn concentration is higher than those reported for adult humans, 0.07–0.25 μM (ATSDR, 2001), but lower than those reported in freshwater carp (10.5–13.1 μM ; Field et al., 1943). As noted above, the fractional Mn removed from the perfusate by the skate livers decreased up to a Mn concentration of 1 μM (Figure 2A), indicating that this is most likely a physiologically relevant uptake mechanism. At these low perfusate Mn concentrations, uptake is most likely due to a high-affinity transport mechanism, whereas the constant fraction absorbed at higher Mn concentrations may be attributed to either a low-affinity transporter, or to some non-specific transport mechanism. Note that the Mn concentrations tested did not produce overt toxicity as measured by K^+ levels in the perfusate (data not shown), and thus the Mn uptake observed at the high Mn concentrations is most likely not simply due to non-specific cell damage.

To further characterize skate hepatocyte uptake and efflux mechanisms, additional studies utilized freshly isolated skate hepatocytes. As was the case with the perfused livers, Mn was quickly taken up by the isolated hepatocytes, with uptake approaching an equilibrium state in only about 15 min (Figure 3A). Interestingly, the uptake of 0.1 μM Mn was nearly completely inhibited by 200 μM cobalt (Figure 3A), suggesting that Mn and Co may share a common

uptake mechanism. To examine the effects of other metals on Mn uptake into isolated hepatocytes, 0.1 μM Mn uptake was measured in the presence of 10 μM of different metals. Uptake was inhibited by all divalent metals tested, but not by Cs, a monovalent cation (Figure 3B). Although the mechanism of inhibition was not examined, these results are consistent with the hypothesis that a multispecific divalent cation transporter is responsible for 0.1 μM Mn uptake into skate hepatocytes.

Analysis of the concentration dependence of Mn uptake revealed that uptake was saturable, consistent with a carrier-mediated transport process (Figure 4). Transformation of the initial rates of uptake into an Eadie-Hofstee plot indicates that uptake may be mediated by two components, with apparent K_m values of $1.1 \pm 0.1 \mu\text{M}$ and $112 \pm 29 \mu\text{M}$ (Figure 4). Interestingly, the K_m value for the high-affinity component is similar to the measured skate blood Mn concentration, suggesting that this may be the physiologically relevant mechanism of uptake, whereas the low affinity component may contribute to the uptake that was observed when the livers were perfused with Mn concentrations up to 1 mM (Figure 2).

There was no statistically significant effect on Mn uptake when Mg was removed from the Ringers solution (Figure 5A); however, removal of Ca from the media resulted in an increase in Mn uptake. In addition, Mn uptake was reduced by nearly half when bicarbonate was removed, suggesting that bicarbonate is somehow required for uptake. This effect of bicarbonate removal does not appear to be due to a change in pH, as varying the pH of the Ringers from 6.5 to 8.5 had no effect on Mn uptake (Figure 5B). Incubation in elasmobranch Ringers in which Li or K replaced Na had no significant effect on Mn uptake, indicating that Mn uptake is not mediated by a Na-dependent or membrane potential-sensitive transport process (Figure 5A).

Manganese was quickly released from isolated hepatocytes that had been pre-loaded with ^{54}Mn , and this release approached equilibrium after only about 15 min (Figure 6A). This time course for efflux parallels that for Mn uptake by the hepatocytes (Figure 3A). The amount of Mn found in the culture medium was enhanced by the addition of 200 μM Co to the media, suggesting either enhanced Mn release (e.g., *trans*-stimulation) or diminished re-uptake by the hepatocytes. Given the potent inhibitory effect of Co on Mn uptake (Figure 3A), this effect on Mn efflux is most likely due in part to cobalt's inhibition of Mn reuptake.

To examine the potential ATP requirement for Mn efflux from the skate hepatocytes, cellular ATP was depleted using 0.5 mM 2,4-dinitrophenol, immediately after loading the cells with Mn. Previous studies with skate hepatocytes have shown that this concentration of 2,4-dinitrophenol can deplete ATP levels to about 40–50% of control levels (Ballatori et al., 1995). The results demonstrate that the addition of 2,4-dinitrophenol led to a reduction in Mn efflux starting at 15 minutes, and this inhibition was sustained for the duration of the experiment, suggesting that Mn efflux from hepatocytes may be partially due to an ATP-sensitive mechanism (Figure 6B).

Discussion

The present results demonstrate that the skate can efficiently clear physiological concentrations of Mn from the sinusoidal circulation of the isolated perfused skate liver, but that only a small amount appears in bile. The decrease in fractional biliary excretion observed at the higher Mn concentrations suggests that biliary transport is saturable; however, additional studies are needed to identify the rate-limiting step in biliary secretion and to identify the transport mechanisms involved. Mn uptake and efflux by isolated skate hepatocytes was also rapid, and was inhibited by a number of divalent metals, suggesting the involvement of a multispecific

divalent cation transporter, whereas Mn efflux was inhibited after ATP depletion, suggesting an ATP-sensitive mechanism.

In mammals, the liver is the main organ responsible for the efflux of excess Mn into bile for eventual elimination in the feces: however, the mechanisms mediating hepatic uptake and biliary excretion are not well understood. Studies in rats have shown that the liver rapidly clears excess Mn and excretes it into bile, and that biliary secretion also appears to be saturable (Klaassen, 1974; Ballatori et al., 1987). Studies using human liver derived cell lines have also observed similar uptake and efflux characteristics (Finley, 1998). Taken together, it appears that the general mechanism(s) responsible for Mn excretion are conserved, and that the skate may be a good model for studying Mn excretion. The low biliary Mn excretion rates in skates as compared to rodent models can be explained in part by the exceedingly low bile flow rates in the skate, which are only about 1% of those in rodents (Boyer et al., 1976; Ballatori et al., 1987). In the perfused skate liver, a large fraction of the Mn was removed when the metal was added at low concentrations, but a smaller fraction was removed at higher concentrations, suggesting that there is a high affinity component of uptake that appears saturable at physiologically relevant Mn concentrations, along with either a low-affinity or non-saturable component of uptake. These findings were confirmed in the experiments with isolated hepatocytes. Mn was efficiently taken up by isolated skate hepatocytes, and uptake was saturable and inhibited by several divalent metal cations. Kinetic analysis also revealed two components of Mn uptake by isolated skate hepatocytes. The first component having a K_m of $1.1 \pm 0.1 \mu\text{M}$, and the second, low-affinity component having a K_m of $112 \pm 29 \mu\text{M}$. The low affinity component may represent a composite of diffusion and other Mn uptake pathways, given that Mn transport has also been demonstrated to occur via Zn transporters, Ca channels, and by receptor mediated endocytosis (Roth, 2006). Mn uptake did not appear to be dependent on the Na gradient or the membrane potential, as isosmotic substitution of Na with Li or K had no effect on Mn uptake. Taken together, these results are consistent with a Dmt1-like Mn uptake mechanism in skate hepatocytes. As noted earlier, Dmt1 is a multi-specific divalent metal uptake transporter with a K_m for Mn of around $1 \mu\text{M}$ (Garrrick et al., 2006). Mammalian Dmt1 transport is proton-coupled, with a pH optimum around 5.5 (Gunshin et al., 1997). Therefore, it is not surprising that varying the pH from 6.5 to 8.5 did not have any effect. It is likely that lowering the pH to 5.5 in our experiments would have stimulated Mn uptake into the isolated hepatocytes. However, it is doubtful that hepatocytes in the liver would be exposed to such a low pH *in vivo*. Interestingly, mammalian Dmt1 has been shown to also function at a nearly neutral pH (7.4), which supports our conclusion of a Dmt1-like uptake system in the skate (Mackenzie et al., 2006). As noted above, the high K_m component may be mediated in part by Zn transporters, Ca channels, and/or by receptor mediated endocytosis. Indeed, this may explain in part why removal of Ca from the culture media enhanced Mn uptake, as this would facilitate additional Mn uptake via Ca pathways. In addition, removal of bicarbonate may have inhibited uptake via bicarbonate-dependent Mn uptake mechanisms such as Zip8 and Zip14 (He et al., 2006; Girijashanker et al., 2008). However, additional studies are needed to identify the transport mechanism(s) that are involved and to fully characterize how bicarbonate may contribute to Mn uptake.

When preloaded with $^{54}\text{MnCl}_2$, isolated skate hepatocytes also quickly released around 15% of their Mn by 1 h, and this release was enhanced by the addition of $200 \mu\text{M}$ Co to the medium. This enhanced release is probably explained both by Co *trans*-stimulated efflux and a Co inhibited Mn re-uptake. Interestingly, skate hepatocyte Mn efflux was inhibited when ATP was depleted via the addition of 2,4-DNP, suggesting that Mn efflux is at least partially ATP-sensitive. However, there are other possible interpretations for this effect, and additional studies are needed to identify and characterize the ATP requirement for Mn efflux, and to establish if it is responsible for biliary secretion.

Acknowledgements

This work was supported in part by NIH/NIEHS ES03828, ES01247, and ES07026, NIH/NIDDK DK34989, DK25636, DK48823, and DK067214, and by NSF DBI-0453391. We thank Robert Gelein for assistance with the atomic absorption spectrometry.

NON-STANDARD ABBREVIATIONS

Mn	manganese
DMT1	divalent metal transporter-1/NRAMP2
FPN1	ferroportin/SLC40A1
DMSO	dimethyl sulfoxide

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Figure 1.

Time course of manganese removal from skate liver perfusate (A) and cumulative biliary manganese excretion (B) over 6 h. Isolated adult male skate livers were perfused in a recirculating system with complete elasmobranch Ringers containing 0.01 or 1 μM ^{54}Mn and supplemented with 10 $\mu\text{mol/h}$ of taurocholate to stimulate bile flow. Bile was collected into tared tubes in 60-min intervals. (A) Percent of ^{54}Mn remaining in perfusate at the indicated times. (B) Cumulative ^{54}Mn excretion into bile relative to the total ^{54}Mn added to the perfusate at time zero. Values are means $\pm$ SE, $n=3-4$.

Figure 2.

Manganese remaining in perfusate (A) and cumulative biliary manganese excretion (B) at 6 h. Isolated adult male skate livers were perfused in a recirculating system with complete elasmobranch Ringers containing various concentrations of ^{54}Mn and supplemented with 10 $\mu\text{mol/h}$ of taurocholate to stimulate bile flow. Bile was collected into tared tubes in 60-min intervals. Values are means $\pm$ SE, $n=3-4$. a $p < 0.05$, b $p < 0.06$.

Figure 3.

Time course and effect of other metals on manganese uptake in isolated skate hepatocytes. (A) $^{54}\text{MnCl}_2$ was added at a concentration of 0.1 μM in the presence (open circles) or absence (closed circles) of 200 μM cobalt in normal elasmobranch Ringer solution. (B) $^{54}\text{MnCl}_2$ was added at a concentration of 0.1 μM in the presence or absence of 10 μM concentrations of different metals in normal elasmobranch Ringer solution for 1 min. Values are means $\pm$ SE, $n=3-4$. * $p < 0.05$.

Figure 4. Concentration dependence of Mn uptake in isolated skate hepatocytes. (A) $^{54}\text{MnCl}_2$ uptake for 1 min in the presence of 0.01 to 300 μM Mn. (B) Eadie-Hofstee plot of the concentration dependence data. Values are means $\pm$ SE, $n=3-4$. * $p < 0.05$.

Figure 5.

Effects of Ringers components and pH on Mn uptake in isolated skate hepatocytes. (A) $^{54}\text{MnCl}_2$ was added at a concentration of $0.1 \mu\text{M}$ in normal elasmobranch Ringer solution; Ringers without calcium (-Ca), magnesium (-Mg), calcium and magnesium (-Ca/Mg), bicarbonate ($-\text{HCO}_3$); or with sodium replaced by lithium (Li) or potassium (K). (B) The pH of normal elasmobranch Ringers was adjusted from 7.5 to 6.5 or 8.5 via the addition of HCl or NaOH, respectively. Values are means $\pm$ SE, $n=3-4$. * $p < 0.05$.

Figure 6.

Effects of cobalt and 2,4-dinitrophenol (2,4-DNP) on Mn efflux in isolated skate hepatocytes. $^{54}\text{MnCl}_2$ was preloaded into isolated hepatocytes for 1.5–2 hours. Cells were washed and resuspended in fresh elasmobranch Ringers (A) in the presence (square) or absence (triangle) of 200 μ M cobalt, or (B) in the presence (square) or absence (triangle) of 500 μ M 2,4-DNP. Values are means $\pm$ SE, $n=3-4$. * $p < 0.05$.