HEPATIC TRANSPORT AND TOXICITY OF THE CYANOBACTERIAL TOXIN MICROCYSTIN IN THE LITTLE SKATE *RAJA ERINACEA*

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Aquatic wildlife as well as farm raised fish and shellfish are exposed to a variety of natural and man made toxins. Aquatic cyanobacteria produce a number of compounds that are either neurotoxic or hepatotoxic (Carmichael, Scientific American (January), 78-86, 1994). The effects of these compounds have been studied mainly in mammals but it is clear that their toxic effects are not limited to this group but in fact apply to all vertebrates and in some cases to invertebrates as well. Microcystins are amongst the most potent hepatotoxins produced by aquatic cyanobacteria. Consumption of water from a source contaminated with microcystins produced by natural blooms (scums) of *Microcystis aeruginosa* can have lethal consequences: including a recent report of human fatalities (Barreto et al. Hepatology 24:127A, 1996) to many accounts of animal deaths including fish.

The mode of action of microcystins has been well established by several groups. Microcystins are potent specific inhibitors of serine/threonine protein phosphatases 1 and 2A (Mackintosh et al. FEBS Letters 264:187-192, 1990). These enzymes are very conserved proteins present in all eukaryotic cells where they are essential components of the machinery that controls and regulates normal cell function and replication. Microcystins are cell impermeant cyclic heptapeptides that are hepatotoxic because hepatocytes have unique transporters that mediate the entry of the toxin in the cell. This results in protein phosphatase inhibition causing metabolic and cytoskeletal changes that lead to the demise of the cell, disruption of the liver and death of the animal. The toxicity of microcystins is therefore defined by transport; only cells capable of accumulating the peptide will be primary targets of the toxin.

The overall goal of this research project is to characterize the toxicity of microcystin in the little skate (*Raja erinacea)* and to determine the role that hepatic transport plays in the process.

Microcystin-YM (tyrosine, methionine variant) was radiolabelled with ^{125}I (5-10 Ci/mmol) in order to quantitate uptake of microcystin in hepatocytes. Tracer amounts (2-3 nM) of ^{125}I-microcystin together with varying amounts of unlabelled microcystin were added to suspensions of skate hepatocytes (isolated by collagenase perfusion of the liver). Incubations were carried out at 15^0C and 5^0C. Cell associated microcystin was determined by gamma counting of the cell pellets separated by centrifugation. Protein phosphatase 1 and 2A activity of cell lysates was determined by measuring the release of inorganic ^{32}P-phosphate from ^{32}P-phosphorylase A at 30^oC. One unit of activity equals the release of 1 nmol phosphate/min. Results are shown as mean ± SEM of n, the number of cell preparations. Raw values were analyzed across preparations by one-way ANOVA followed by Fisher's test. A p value of ≤ 0.05 was considered statistically significant (*). To determine the in vivo toxicity of microcystin to the skate, microcystin was injected in the tail vein of skates and, after sacrifice or death, the liver, kidneys and rectal glands were taken for histology and determination of protein phosphatase activity.

Using ^{125}I-YM we found that uptake of microcystin in skate hepatocytes was temperature dependent. Apparent activation energies calculated from initial rates of uptake at 15^{0}C and 5^{0}C were 51 kJ/mol for 2 nM microcystin (label only) incubations and 67 kJ/mol for 10 µM microcystin incubations (n=3), consistent with carrier mediated transport, and higher than values usually obtained for simple diffusion.

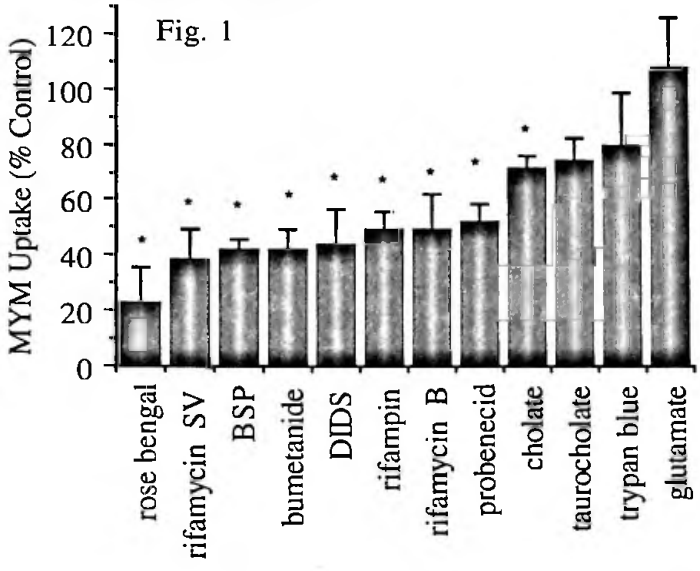

Figure 1. Effect of organic anions and bile acids (100 µM) on the initial rate of uptake of microcystin (MYM, 10 µM) by skate hepatocytes

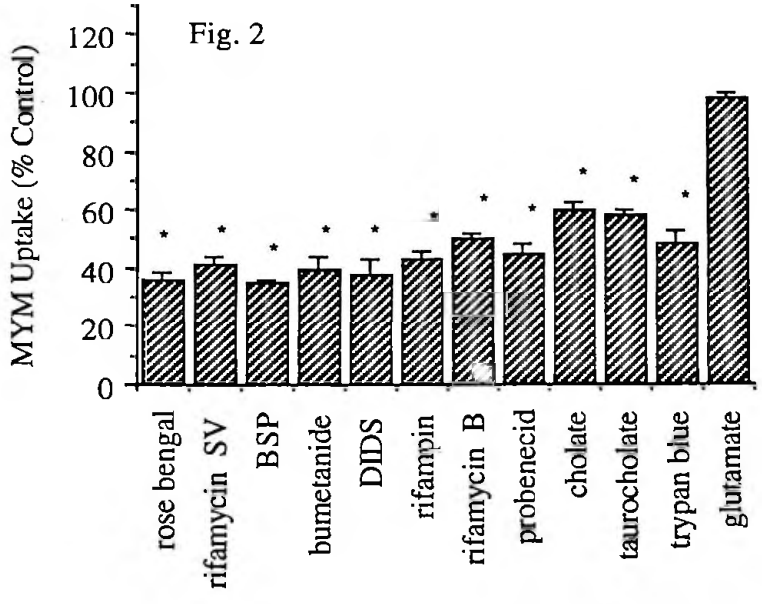

Figure 2. Effect of organic anions and bile acids (100 µM) on the accumulation in 30 min of microcystin (MYM, 10 µM) by skate hepatocytes.

A series of dyes (all 100 µM) inhibited the intial rate of uptake of 10 µM microcystin by skate hepatocytes when added 1 min before microcystin, n=4-5 (Fig. 1). Uptake of 10 µM microcystin without inhibitors was 131±21 pmol min^{-1}ml^{-1} at 15^{0}C. The inhibition persisted when the accumulation of microcystin in skate hepatocytes was determined after 30 min incubation with 10 µM microcystin (Fig.2). At 30 min cell associated microcystin in incubations without inhibitors was 580±23 pmol/ml, n=4-5. The pattern of inhibition observed is consistent with uptake of microcystin being mediated by one of (the) organic anion transporter(s) present in skate liver.

The finding that bile acids are less potent than other organic anions and dyes argues that the uptake of microcystin is unlikely to be through the bile acid carrier present in skate hepatocytes (Fricker et al. Biochem. J. 29:665-670, 1994). No clear saturation of the initial rate of microcystin uptake could be shown up to 300 µM microcystin. It is therefore difficult to determine what is the relative contribution of binding or diffusion to the cell associated microcystin but it is probably of the order of 20-30% in 10 µM microcystin incubations at 30 min, from a comparison of the degree of inhibition observed at 5^{0}C and 15^{0}C.

10 μM microcystin inhibited protein phosphatase 1 and 2A activity of skate hepatocytes that had been incubated for 30 min at 15^{0}C. Activity decreased from 2.49±0.14 units/mg protein to 0.28±0.15 units/mg of protein (n=3). Lower doses of microcystin tested, namely 100 nM, 500 nM and 1 μM, had no inhibitory effect on protein phosphatase activity of hepatocytes (n=3). In mammalian hepatocytes inhibition of protein phosphatase activity causes cytoskeletal changes that result in characteristic blebbing of the cells (Runnegar, Berndt and Kaplowitz, Toxicol Appl. Pharmacol. 134:264-272, 1995). We found that similar changes were seen in skate hepatocytes that had been incubated with 10 μM microcystin (5-10^{0}C, n=2 for 14 hrs). Parallel incubations of skate hepatocytes with 1 μM okadaic acid or 500 nM calyculin A resulted in similar blebbing. Okadaic acid and calyculin A, although cell permeant and chemically different from microcystin, inhibit protein phosphatase 1 and 2A and cause cytoskeletal changes similar to microcystin. These common responses to protein phosphatase inhibitors indicate that the cytoskeleton of skate hepatocytes is regulated by phosphorylation and dephosphorylation events on serine/threonine residues in a manner analogous to that described in mammalian cells (Runnegar et al. Toxicon 29:43-51, 1991; Runnegar et al. J.Pharmacol. Exp. Ther. 273:545-553, 1995; Runnegar et al. Toxicol. Appl. Pharmacol. 134:264-272, 1995; Hamm-Alvarez et al. Am. J. Physiol. 271:C929-C943, 1996).

Pilot studies of microcystin in vivo toxicity to skates showed severe liver injury and death between two and three days after iv dosing. At death or sacrifice liver, kidneys and rectal glands were taken for histology and protein phosphatase activity measurements. For normal controls three skates not injected with microcystin were processed the same way. A total of seven skates were injected with 2-4 mg/kg of microcystin. Four were sacrificed 2-4 days after dosing and three died between 2-3 days after dosing. Preliminary histology of all the liver samples for microcystin dosed skates showed severe lesions. We hope to complete the histological analysis of the kidneys and rectal glands in the forthcoming months.

Protein phosphatase activity of the liver of the microcystin treated skates that survived was totally inhibited (Table 1) while protein phosphatase activity of kidney was also inhibited but to a much lesser degree (55% of controls). Protein phosphatase activity of rectal gland was not significantly affected by microcystin (79% of controls).

Table 1. Effect of microcystin on protein phosphatase activity of skate liver, kidney and rectal gland. (protein phosphatase activity as units/mg protein)

Treatment	Liver	Kidney	Rectal Gland
Controls (n=3)	7.29±1.54	5.42±0.33	6.36±0.85
Microcystin dosed (n=4)	0.02±0.01*	3.00±0.48*	5.00±0.56

Until the histological analysis is complete, it is not possible to state whether the partial inhibition of protein phosphatase activity in the kidney results in any significant lesions as has been reported for rainbow trout (Kotak et al. Toxicon 34:517-525, 1996). In mammals microcystin causes no kidney lesions, and in mice given toxic doses of microcystin there was no inhibition of kidney protein phosphatase (Runnegar, Kong and Berndt, Am. J. Physiol. 265:G224-G230, 1993). These differences between mammals and fish might reflect differences in specificity of the carriers for organic anions in the kidney: with no transport of microcystins in mammals and some transport in fish, particularly if the capacity of the hepatic tranporter(s) is considerably less than that of mammals. An alternative or additional contributing factor could be the lower body temperature of

fish resulting not only in lower metabolic rates but also in lower rates of hepatic uptake leaving significant levels of microcystin in the circulation for much longer periods.

The effect of microcystin in skate needs to be further investigated to determine the temporal relationship between protein phosphatase inhibition, histological changes in liver and possibly kidney at sacrifice. With isolated skate hepatocytes it will be possible to correlate directly cell associated microcystin with protein phosphatase inhibition particularly at longer times of exposure and doses of microcystin less than 10 μM. The spectrum of inhibitors of microcystin uptake in skate are similar to those seen in the rat, indicating common features of the carrier(s) in these mammals and fish.

This work was supported by a New Investigator Award from the Center for Membrane Toxicity Studies (P30 ES03828) (to MR), and in part by NIEHS grant ES05678 and NIDDK grant DK51788 (to MR).