IMMUNOLOGIC AND CATALYTIC ASSESSMENT OF HEPATIC XENOBIOTIC-METABOLIZING ENZYMES AND BIOTRANSFORMATION POTENTIAL IN BELUGA WHALE (*Delphinaterus leucas*) FROM TWO CANADIAN POPULATIONS

Melissa A. McKinney [1], Augustine Arukwe [1], Daniel Martineau [2], André Dallaire [2], Pierre Béland [3], Sylain De Guise [4] and Robert J. Letcher [1]

1 Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario, N9B 3P4 Canada
2 Départment de Microbiologie et Pathologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, J2S 7C6 Canada
3 Institute National d'Écotoxicologie du Saint-Laurent, Montréal, Québec, H2L 3M6 Canada
4 Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, Connecticut, 06269 U.S.A.

Introduction

Marine mammals, including the beluga whale (*Delphinapterus leucas*), are particularly susceptible to contamination by organohalogens due to their large lipid reservoirs, relatively long life spans and elevated positions in the aquatic food web. A small, isolated and endangered population of beluga inhabits the highly contaminated St. Lawrence (SL) Estuary in Québec, Canada. Organohalogen contaminants, particularly polychlorinated biphenyls (PCBs), p,p'-dichlorodiphenyltrichloroethane (DDT), polychlorinated dibenzo-dioxins/furans (PCDD/Fs), have been reported at high levels in SL beluga tissues [1,2]. Moreover, total PCB concentrations in brain, liver and muscle tissues of SL beluga were found to be 13, 11 and 11 times higher (lipid weight basis), respectively, than in beluga from the Canadian Arctic (CA) [3].

Metabolic capacity is an important determinant in the bioaccumulation, biomagnification, toxicokinetics and potential toxicity of lipophilic organohalogen contaminants. The cytochromes P450 (CYP) enzymes play a central role in the oxidative metabolism or biotransformation (Phase I) of a wide range of foreign compounds (xenobiotics), including environmental pollutants [4]. Phase II enzymes such as uridine diphosphoglucuronosyl transferase (UDPGT) catalyze conjugation reactions with Phase I intermediates. Together, Phase I and II enzymes transform a lipophilic compound into forms that facilitate elimination and excretion from the exposed organism. There are qualitative and quantitative differences in the level and inducibility of individual CYP iso-enzymes that mediate xenobiotic metabolism among species and populations. Therefore, it is important to determine metabolic capacity to assess the susceptibility of an exposed animal to environmental contamination. In mammals, CYP1A enzymes mediate the metabolism of planar polyhalogenated aromatic hydrocarbons, and CYP2B enzymes prefer nonplanar, liphophilic substrates [4]. CYP3A-like enzymes have been associated with the chlorobornane and PCB metabolism *in vitro* in seals [5,6]. Immunochemical characterization of CYP enzymes has been reported for a handful of whale species and populations [7-11]. In contrast to laboratory specimens, it is more often difficult to obtain well-preserved, enzymatically-viable tissue from stranded free-ranging animals. Therefore, catalytic

activity as a quantitative indicator of metabolic (biotransformation) potential is questionable in situations where adequate liver preservation cannot be assured.

The objectives of the present study are to immunologically profile and compare CYP1A, CYP2B, CYP3A, CYP2E, epoxide hydrolase (EH) and UDPGT enzymes in the hepatic microsomes from beluga whale, and assess the xenobiotic-metabolizing metabolic potential, in animals from the geographically distinct SL and western Hudson Bay (Canadian arctic, CA) populations. CYP1A (7-ethoxyresorufin-O-deethylase (EROD)) activity is also assessed to compare the effect of sample preservation on protein epitopic integrity and enzyme activity.

Materials and Methods

Liver tissues were obtained from four SL beluga stranded in the summer of 2002. The samples were excised 12-18 hours after discovery of the carcass and immediately stored in liquid nitrogen. Liver tissues from four CA beluga were obtained during subsistence hunts in August of 2002 and similarly stored. Microsomes were prepared by differential ultracentrifugation as described in McKinney *et al*. [12]. Briefly, liver tissues from the SL and CA beluga samples were thawed separately on ice. Four volumes of homogenization buffer (0.1 M K_2HPO_4, 0.15 M KCl, 1 mM Na_2EDTA, 1 mM DTT, 20% glycerol, pH 7.4) were added and each mixture was homogenized with an Ultra-Turrex. The homogenate was centrifuged at 12 500 g for 20 minutes at 4 °C. The obtained supernatent was centrifuged at 100 000 g for 60 minutes at 4 °C. One volume of resuspension buffer (0.1 M K_2HPO_4, 1 mM Na_2EDTA, 1 mM DTT, 20% glycerol, pH 7.4) was added to the resulting pellet containing the microsomes and this solution was stored at –80 °C until further use. Microsomal protein content was determined spectrophotometrically using bovine serum albumin (BSA) as the standard with a concentration range of 0.2 to 1.0 mg/ml. CYP catalytic activity was determined by measuring microsomal CYP1A activities using the EROD assay.

Separation and immunochemical characterization of microsomal enzyme proteins in the samples was performed by polyacrylamide gel electrophoresis (PAGE) in combination with Western (protein) immunoblotting for anticipated cross-reactivity with selected antibodies [12]. The primary antibody used to detect CYP1A1/2-like proteins was a polyclonal anti-rat CYP1A1 raised in goat. Detection of CYP2B1/2-like proteins employed a rabbit anti-rat primary antibody. A rabbit anti-rainbow trout polyclonal antibody (PAb), kindly donated by Dr. Malin Celander (University of Göteborg, Göteborg, Sweden), was used to detect CYP3A-like proteins. For the detection of a CYP2E homologue, a rabbit anti-human CYP2E1 PAb was employed. An EH-type enzyme protein was identified by cross-reaction with a goat anti-rabbit EH PAb. Finally, the UDPGT immunoblot utilized rabbit anti-human UDPGT1A PAb. Proceeding application of the primary antibody, a horseradish peroxidase (HRP)-labeled secondary antibody was incubated with the membranes, followed by an HRP-colour reagent (4-chloro-1-napthol plus hydrogen peroxide solution) to visualize the cross-reactive bands. The secondary antibody applied was donkey anti-goat or goat anti-rabbit antibody.

Results and Discussion

An immunologic comparison of CYP1A in individual beluga from SL and CA populations has yet to be reported until now. Two distinct CYP1A bands at 53 and 50 kDa were detected in both the SL and CA samples (Figure 1). A single previous study on hepatic CYP enzymes in beluga whale revealed the presence of one CYP1A band at 53 kDa using a monoclonal anti-scup antibody and a polylclonal anti-mouse antibody that detects CYP1A1 and 1A2 in mice and rats [7]. The expression of CYP1A1

and CYP1A2 proteins was highly variable among SL animals, compared to the generally higher and more consistent expression among the CA animals (Figure 1). This suggests that preservation of both catalytic activity and protein integrity in the CA individuals, whereas SL samples were compromised due to inadequate preservation. It is less likely that the differences in the SL animals can be accounted for by Ah receptor-mediated CYP1A induction. Band intensity is significantly less than for the CA animals who are exposed to lower levels of CYP1A-inducing contaminants [1-3]. Sampling variability was controlled and minimized for the CA animals suggesting that the minimal differences in the CYP1A band intensities among these individuals are likely a consequence of induction.

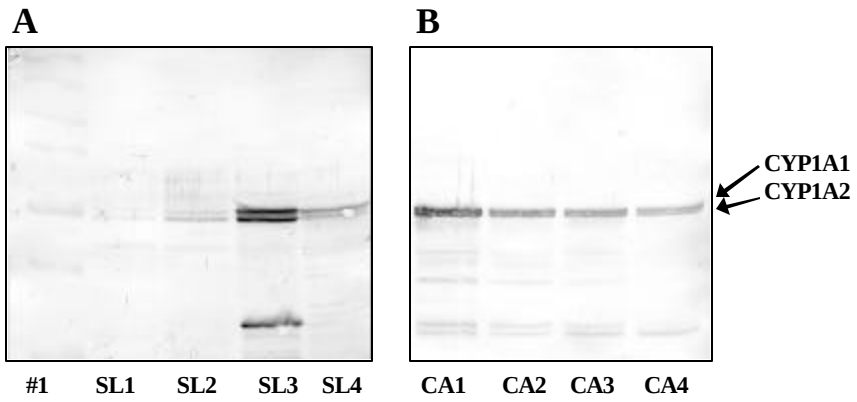

Figure 1. Immunoblots of proteins in the hepatic microsomes of beluga whale from **A)** St. Lawrence (SL) and **B)** western Hudson Bay (Canadian Arctic, CA) populations showing cross-reactivity with polyclonal goat antibody raised against rat CYP1A1. Lane 1 is loaded with 5 µl molecular weight standard. See Materials and Methods section.

Table 1 Microsomal protein content, CYP content and monooxygenase activity in the livers of stranded St. Lawrence and captured Canadian Arctic beluga whale.

	SL#1	SL#2	SL#3	SL#4	CA#1	CA#2	CA#3	CA#4
protein conc. (mg/g)	1.6 ±0.02	2.9 ±0.4	6.3 ±0.2	3.1 ±0.4	1.48 ±0.01	1.08 ±0.03	0.91 ±0.04	1.98 ±0.04
total CYP content (nmol/mg) *	ND	ND	ND	ND	0.27	0.16	0.12	0.26
EROD (pmol/mg/ min)	ND	ND	12.5 ± 0.6	ND	107 ± 6	51 ± 3	61 ± 2	107 ± 10

Means ± SD (n = 3 replicates). ND = Not detectable. *Limited tissue prevented replicate analysis.

Cross-reactive CYP2B1/2-like bands are not observed in the SL beluga samples, whereas two weak bands are present in the CA samples (not shown). It has been postulated that marine mammals are basically deficient in CYP2B-type proteins [10]. However, White *et al.* [7] reported a cross-reactive bands in liver microsomes from MacKenzie delta beluga using anti-rabbit CYP2B4 and anti-dog CYP2B11 PAbs. CYP3A-, CYP2E- and EH-like proteins of variable band intensity among animals were also observed in the SL and CA animals. A UDPGT-type protein was detected in two of the four SL beluga and all four of the CA beluga we studied. To our knowledge, this is the first report on the immunochemical characterization of EH and UDPGT proteins in cetaceans or pinnipeds.

EROD activity was below the detection limit in all but one of the SL samples, whereas EROD activity was detectable in the CA animals (Table 1). These results are indicative of loss of a majority of the hepatic enzyme viability at more than 12 hours after death of the animal. The lack of enzyme activity in the SL samples is likely due to degradation of the heme group on the CYP enzyme. P450 levels determined by the dithionite difference method, which measures the binding of CO to the iron center of the heme group, are below the detection limit (Table 1). Epitopic degradation may have been a factor in the reduction of enzyme activity as well, given that the bands on the SL Western blots are generally more faint (but nonetheless detectable, unlike catalytic activity) than for the CA blots. Studies of other marine mammal tissues collected immediately following death show similarly higher catalytic activities than those in the delayed sample collection studies [7,8].

Because the SL population is endangered, it is not possible to obtain the fresh liver tissues that are necessary for catalytic/immunologic biomarker assessments, whereas it is possible for arctic beluga, which are collected fresh as a consequence of regulated Inuit hunts. We have shown that liver samples from CA beluga have similar Phase I and II enzyme profiles as SL beluga, but are less epitopically degraded. Therefore, immunochemical and catalytic biomarkers of Phase I and II enzymes in liver from optimally preserved CA animals can be used as models in assessing the xenobiotic metabolism (biotransformation) potential of beluga whale from SL or other populations.

Acknowledgements
These studies are financially supported by the World Wildlife Fund Canada (to R.J. Letcher and P. Béland), and DFO Canada, NSERC and the Canada Research Chairs Program (to R.J. Letcher).

References

1. Hobbs K.E., Muir D.C.G., Michaud R., Béland P., Letcher R.J. and Norstrom R.J. (2003) Environ. Poll. 122, 291
2. Muir D.C.G., Ford C.A., Rosenberg B., Norstrom R.J., Simon M. and Béland, P. (1996) Environ. Pollut. 93, 219
3. Metcalfe C., Metcalfe T., Ray S., Paterson G. and Koenig B. (1999) Mar. Environ. Res. 47, 1
4. Lewis D.F.V., Eddershaw P.J., Dickins M., Tarbit M.H. and Goldfarb P.S. (1998) Chem. Biol. Interact. 115, 175
5. van Hezik C.M., Letcher R.J., de Geus H.-J., Wester P.G., Goksøyr A., Lewis W.E. and Boon, J.P. (2001) Aquat. Toxicol. 51, 319
6. Li H., Boon J.P., Lewis W.E., van den Berg M., Nyman M. and Letcher R.J. (2003) Environ. Toxicol. Chem. 22(3), 636
7. White R.D., Hahn M.E., Lockhart W.L. and Stegeman J.J. (1994) Toxicol. Appl. Pharmacol. 126, 45
8. White R.D., Shea D., Schlezinger J.J., Hahn M.E. and Stegeman J.J (2000) Comp. Biochem. Physiol. *C*. 126, 267
9. Celander M.C., Moore M.J. and Stegeman J.J. (2000) Environ. Toxicol. Pharmacol. 8, 245
10. Goksøyr A. 1995. in : Whales, Seals, Fish and Man (Blix, A.S., Walløe, L., Ulltang, O., Eds.), Elsevier Science, ISBN 0-444-82070-1
11. Boon J.P., Lewis W.E. and Goksøyr A. (2001) Aquat. Toxicol. 52, 297
12. McKinney, M.A., Arukwe A., Martineau D., Dallaire A.D., Béland P., De Guise S. and Letcher R.J. (2003) Aquat. Toxicol. Submitted