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Induction of cytochrome P450 1A1 expression in captive river otters fed Prudhoe Bay crude oil: evaluation by immunohistochemistry and quantitative RT-PCR

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Numerous studies have explored the relationships between exposure to a variety of environmental contaminants, such as polycyclic aromatic hydrocarbons, and induction of cytochrome P450 1A (CYP1A) in different vertebrates. Few controlled studies, however, simulated chronic long-term exposure with repeated non-lethal sampling of the same individuals, which should better represent repeated exposure incidents in animals inhabiting polluted areas. In this study, we investigated the effects of chronic exposure to crude oil on levels of CYP1A1 in endothelial cells of skin biopsies and peripheral mononuclear blood cells in captive river otters (*Lontra canadensis*) using repeated sampling of the same individuals. We hypothesized that ingestion of oil would result in an increase in levels of CYP1A1 in both targets, and predicted that the relationship between prolonged exposure and expression of CYP1A1 would reach a plateau indicative of continuous detoxification of hydrocarbons. Fifteen wild-caught male otters were acclimated to captivity, and then fed diets containing no oil (control) or diets containing weathered crude oil at 5 mg day^{-1} kg^{-1} body weight (low-dose) and 50 mg day^{-1} kg^{-1} body weight (high-dose), at the Alaska Sealife Center in Seward, Alaska, USA. Expression of CYP1A1 was assessed with immunohistochemical analysis of CYP1A1 protein in skin biopsies and by quantitative RT-PCR analysis of CYP1A1 mRNA in mononuclear blood cells. Both assays revealed a decrease between capture and the transfer to captivity, indicating that the enclosure at the Alaska Sealife Center, and the food we offered to the otters were free of potential inducers of CYP1A1. During the exposure period, increases in CYP1A1 expression were registered by both techniques, followed by a decline in CYP1A1 after oil administration ended. Levels of endothelial CYP1A1 in the high-dose group were comparable to those recorded for wild river otters in PWS in 1996 and 1997. Levels of CPY1A1 mRNA in mononuclear blood cells, however, were well below levels recorded for river otters in Prince William Sound, and no correlation was detected between values obtained from the two methods. Thus, our results from this longitudinal study with repeated sampling of the same individuals provide support for the use of cytochrome P450 1A1 as a biomarker for hydrocarbon exposure. Nonetheless, our results also suggest that the induction process of CYP1A1 may be complicated and interacting with other processes *in vivo*. Such interactions may obscure our ability to describe specific, quantitative, predictable, dose–response relationships between exposure to hydrocarbons and induction of CYP1A1, which are required of reliable biomarkers. Evaluations of such interactions based on theoretical physiological models in live-animals merit further investigation.

Keywords: Alaska, biomarkers, chronic exposure, CYP1A, dose–response, hydrocarbons, *Lontra canadensis*, mononuclear blood cells, mono-oxygenases, skin samples.

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Introduction

Many studies assessing the risk of pollutant exposure in wildlife populations involve the measurement of chemical residues in sediments, water, or soil. Because chemical residue analysis can be expensive, laborious, and often inadequately measure bioavailability, biomarker analyses have become widely used in ecotoxicological studies (Vanden Heuvel and Davis 1999). Members of the cytochrome P450 (CYP) family of haemoproteins that catalyse oxidative transformation of organic chemicals are important in the detoxification of environmental contaminants (Marks 1985, Stegeman and Lech 1991, Bucheli and Fent 1995). Evaluations of the mRNA, protein or catalytic activity of CYP1A have been used as a biomarker for exposure to a variety of contaminants such as polycyclic aromatic hydrocarbons (PAH), naphthoflavones, dioxins, planar polyhalogenated biphenyls (i.e. 3-methylcholanthrene (MC) type agents), which induce CYP1A via the aryl hydrocarbon receptor (AhR; Miranda *et al.* 1987, Stegeman *et al.* 1988, 1992, Kloepper-Sams and Stegeman 1989, Juchau 1990, Goksyür and Husoy 1992, Rattner *et al.* 1993, Willet *et al.* 1997, Woodin *et al.* 1997). CYP1A1 is broadly distributed in different species and tissues, and is not readily induced by steroids and other lipids (Juchau 1990, Vanden Heuvel and Davis 1999). These characteristics make CYP1A1 a good indicator of exposure to MC-type agents in vertebrates (Vanden Heuvel and Davis 1999).

Petroleum, including that spilled by the *Exxon Valdez* oil spill (*EVOS*), contains numerous PAH compounds (Short *et al.* 1996) that potentially induce the expression of CYP1A1 in vertebrates. Early investigations in Prince William Sound (PWS), following *EVOS*, revealed that coastal river otters (*Lontra canadensis*) on oiled shores had lower body mass and elevated levels of liver enzymes (ALT, AST), haptoglobin, interleukin-6, and faecal porphyrins than animals living in non-oiled areas (Duffy *et al.* 1993, 1994a, b, 1996, Blajeski *et al.* 1996, Bowyer *et al.*, submitted). In addition, otters from oiled areas selected different habitats, had larger home ranges, and less diverse diets than those in non-oiled areas (Bowyer *et al.* 1994, 1995). These differences between river otters from oiled shores and those from non-oiled areas suggest that oil contamination had an effect on physiological and behavioural processes in otters. Continued exposure could have the potential to impede recovery of populations of river otter inhabiting areas affected by *EVOS*. Later studies of river otters in PWS between 1996 and 1998 employed two different techniques for evaluating expression of CYP1A1 in an attempt to determine the occurrence and level of exposure to hydrocarbons in these animals. The first technique involved evaluation of immunohistochemical (IHC) staining of CYP1A1 haemoprotein in endothelial cells from skin tissue. The second employed a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) to measure CYP1A1 mRNA content in peripheral blood lymphocytes and monocytes (i.e. mononuclear blood cells; Ballachy *et al.*, submitted). Endothelial CYP1A1 content was significantly higher in river otters captured in oiled compared with non-oiled areas in 1996, but values of CYP1A1 levels in samples collected in 1997 and 1998, were nearly identical for otters living in oiled versus non-oiled areas (Bowyer *et al.*, submitted). Similarly, expression of CYP1A1 mRNA in lymphocytes, measured by quantitative RT-PCR, revealed no differences in river otters from oiled and non-oiled areas in 1998 (Ballachy *et al.*, submitted). To adequately interpret these findings it became necessary to establish the relationship between exposure to hydrocarbons, dose levels, and expression of CYP1A1 in these mustelids under controlled conditions.

Although numerous studies explored the relationship between hydrocarbon exposure and expression of CYP1A1 in different vertebrates (Payne *et al.* 1987, Stegeman *et al.* 1988, Juchau 1990, Bucheli and Fent 1995), few controlled studies simulated chronic long-term exposure with repeated non-lethal sampling of the same individuals. Such controlled chronic-exposure studies would probably provide better representation of conditions experienced by wild animals inhabiting polluted areas and a better frame for interpreting data collected from free-ranging exposed-animals. In this study, we investigated the effects of chronic exposure to crude oil on levels of CYP1A1 in endothelial cells of skin biopsies and lymphocytes in captive river otters using repeated sampling of the same individuals. We hypothesized that ingestion of oil would result in an increase in levels of CYP1A1 in both targets, as both mononuclear blood cells and endothelial cells would be in immediate contact with the assimilated hydrocarbons transported in the blood. We predicted that the relationship between prolonged exposure (i.e. cumulative dose) and expression of CYP1A1 would reach a plateau, suggesting continuous detoxification of the ingested hydrocarbons. In addition, we expected a correlated response in the two target cells indicative of a systemic response to exposure.

Materials and methods

General

Fifteen wild male river otters were captured in northwestern PWS using No. 11 Sleepy Creek® leghold traps (Blundell *et al.* 1999). Traps were placed on trails at latrine sites and monitored by trap transmitters (Telonics, Mesa, Arizona, USA) that signal when a trap is sprung (Bowyer *et al.*, submitted). Processing of otters began within 1–2 h from capture and lasted between 0.5 (regular processing) and 5 h (processing involved implanting the individual with radio transmitter). Otters were anaesthetized with Telazol (9 mg kg^{-1}; A. H. Robins, Richmond, Virginia, USA) administered using Telinject® darts and a blowgun. Blood and tissues were sampled from each individual otter within 15 min of sedation.

The river otters were then transferred under sedation via air to the Alaska Sealife Center in Seward, Alaska (ASLC). The otters were held in captivity at ASLC from May 1998 to March 1999. The animals were housed as one large group in an area of 90 m^2 surrounding six pools (one large salt-water pool—4.5 m diameter by 3 m depth; four small salt-water pools—$2 \times 1.5 \times 1.5$ m^3; and one small fresh-water tote—$1 \times 1 \times 1$ m^3). Otters were fed twice daily *ad libitum* on a diet of frozen fish of the following species: pink salmon (*Oncorhynchus gorbuscha*), capelin (*Mallotus villosus*), Pacific herring (*Clupea pallasii*), walleye pollock (*Theragra chalcogramma*), prawns (*Pandalus platyceros*), and eulachon (*Thaleichthys pacificus*). These fishes were purchased from commercial harvest and approved for human consumption. Two to three times per week, the diet was supplemented with live prey (pink salmon, kelp greenling (*Hexagrammos decagrammus*), and rockfish (*Sebastes* sp.)) captured in Resurrection Bay, Alaska (ADFG permit CF 98-024). Initially, vitamins (HiVite™, EVSCO Pharmaceuticals, IGI, Inc., Buena, NJ) were provided with the food, but because otters seemed reluctant to consume those fishes, we injected Butler B-complex vitamins (0.5 ml; Phoenix Scientific, St Louis, MO) intramuscularly during the blood-sampling sessions every 3 weeks. Minerals were provided as a standard cattle mineral block to which otters had continuous access.

Experiments began in August after 2.5 months of acclimation to the enclosure, feeding regimes, and handling. During that time, the average daily food intake of the otters was monitored to quantify the amount of oil required for achieving each oiling level. Daily food intake per animal averaged at 1000 g per day over the acclimation period. Average body weight of the otters at the end of the acclimation period was 10.7 kg (± 0.3, SE). At the end of that period, otters were randomly assigned to three experimental groups of five individuals each. Group assignment was accomplished with a randomized complete block design. Original assignment of otter identification numbers were done based on body mass at capture to control for potential differences in age and size. For the complete block design otter identification numbers were randomly permutated within blocks (T. L. McDonald, West, Inc. Cheyenne, WY). The three treatment groups were: a control group that received no oil; a low dose group that received 100 mg of oil every other day (i.e. 5 mg day^{-1} kg^{-1} body weight); and a high dose group which received 1000 mg every other day (i.e. 50 mg day^{-1} kg^{-1} body weight). The exposure level for the low-dose group was determined based on levels of Prudhoe Bay crude oil (PBCO) found in mussel beds in PWS in 1995 (Short *et al.* 1996), in an attempt to simulate conditions of chronic exposure

in the wild. The high-dose level was selected to simulate conditions in PWS immediately following *EVOS*.

Prudhoe Bay crude oil (PBCO; obtained from Williams Inc. Fairbanks, Alaska, USA) was mixed in seawater and stirred continuously for 10 days at 25 ¡C. Two batches of oil were weathered separately and a sample from each batch was sent for analysis at Auke Bay Laboratory (J. Short, NOAA, Juneau, Alaska). The two batches differed slightly in composition, but both were comparable to the oil profile of *EVOS* shortly after landfall in 1989 (table 1; Short *et al*. 1996). Weathered oil was separated from water and administered to otters in gel capsules hidden in fishes every other day. The quantity of oil placed in the gel was measured with a micro-pipette (Rainin Instruments Co., Emeryville, CA) and weighed on a micro-balance (nearest 0.01 g). Occasionally the otters bit into the capsule when feeding and subsequently dropped the fish. On such occasions, oil was administered again during the following

Table 1. Concentrations of hydrocarbons in the two batches of weathered Prudhoe Bay Crude oil fed to river otters at the Alaska Sealife Center in Seward Alaska from 21 August to 28 November 1998.

	Analyte concentrations: (μg/g)	
Compound	Batch 1	Batch 2
Naphthalene	283.14	60.16
2-Methylnaphthalene	904.43	616.81
1-Methylnaphthalene	753.80	531.44
2,6-Dimethylnaphthalene	641.37	577.26
C-2 Naphthalenes	2601.33	2359.91
2,3,5-Trimethylnaphthalene	268.37	278.51
C-3 Naphthalenes	2284.48	2305.84
C-4 Naphthalenes	609.96	593.84
Biphenyl	155.55	120.85
Acenaphthylene	0.00	0.00
Acenaphthene	13.42	12.43
Fluorene	97.31	100.48
C-1 Fluorenes	228.37	247.95
C-2 Fluorenes	273.45	309.82
C-3 Fluorenes	169.23	183.75
Dibenzothiophene	177.90	188.40
C-1 Dibenzothiophenes	359.69	378.64
C-2 Dibenzothiophenes	473.14	511.84
C-3 Dibenzothiophenes	371.25	387.41
Phenanthrene	244.29	263.38
1-Methylphenanthrene	182.57	196.85
C-1 Phenanthrenes/anthracenes	759.29	820.86
C-2 Phenanthrenes/anthracenes	874.31	940.14
C-3 Phenanthrenes/anthracenes	485.21	540.15
C-4 Phenanthrenes/anthracenes	80.23	85.26
Anthracene	7.72	3.41
Fluoranthene	4.34	4.46
Pyrene	11.70	12.34
C-1 Fluoranthenes/pyrenes	66.05	644.75
Benz-*a*-anthracene	6.64	4.28
Chrysene	44.48	45.27
C-1 Chrysenes	66.13	67.56
C-2 Chrysenes	66.78	71.22
C-3 Chrysenes	15.75	33.08
C-4 Chrysenes	2.76	3.25
Benzo-*b*-fluoranthene	14.73	15.66
Benzo-*k*-fluoranthene	0.00	0.00
Benzo-*e*-pyrene	9.89	11.76
Benzo-*a*-pyrene	2.90	2.21
Perylene	1.03	11.09
Indeno-123-*cd*-pyrene	1.03	0.58
Dibenzo-*a*,*h*-anthracene	1.31	1.07
Benzo-*g*,*h*,*i*-perylene	2.50	2.97

feeding to ensure that each otter ingested the amount of oil required for the experiment. Careful notes on oil feeding were kept throughout the experiment. Oil feeding lasted 100 days, from 21 August to 28 November 1998. The first batch of oil was fed to the otters between August 21 and October 19, as well as between November 19 and November 28. The second batch was fed to the otters between October 20 and November 18 (table 1). Data collection continued for an additional 100 days of rehabilitation. Cumulative dose of oil provided in food throughout the period of exposure was determined for each individual otter and used in later analyses. Analysis of passage rate and assimilation efficiency indicated that some of the ingested oil was occasionally deposited in faeces (Ormseth and Ben-David 2000). At the end of the rehabilitation period, animals were fitted with radio transmitters and released at the site of their capture in PWS. Animals are currently being monitored to determine post-release survival (Ben-David, unpublished data).

Collection of blood and tissues

Prior to the exposure to oil (29–30 June and 15–16 August 1998), blood and tissues were sampled from each individual otter. Blood and tissue sampling continued every 3 weeks from 15 August 1998 until 12 January 1999. An additional sampling session occurred on 22–24 February 1999 in conjunction with implanting of radio transmitters.

Otters were anaesthetized with a combination of ketamine hydrochloride (100 mg ml^{-1}, Ketaset®, Aveco Co., Fort Dodge, Ia., USA) at a dosage of 10 mg kg^{-1}, and midazolam hydrochloride (5 mg ml^{-1}, Versed®, Hoffman-LaRoche, Nutley, NJ, USA) at a dosage of 0.25 mg kg^{-1} (Spelman *et al.* 1993). The dosage was mixed in the same syringe and administered intramuscularly with Telinject® darts and a blowgun or hand-injected while the otter was immobilized in a squeeze-box. Before beginning to dart the otter, each individual received one fish containing 0.5 ml Versed®. This calmed the animals and reduced the stress associated with handling.

A 3-mm disposable skin-biopsy punch was used to obtain a tissue sample from each river otter for analysis of endothelial cytochrome P450 1A1. This amount of tissue was a maximum approved by the ASLC veterinarian and the Independent Animal Care and Use Committee (IACUC) given the repeated nature of tissue sampling. Prior to collecting the sample, we clipped hair on the medial surface of the triceps on the left front limb, and a surgical scrub was performed. The tissue specimen was preserved in 10% neutral-buffered formalin immediately after collection, and sent to Woods Hole Oceanographic Institution for analysis. Samples were coded and analysis conducted in a blind study (i.e. analyst had no knowledge of group assignment of samples).

A total of 10 ml of heparinized (preservative free heparin, Sigma Chemical Co., St Louis, MO) blood was collected by vena-puncture from each animal for the isolation of peripheral blood mononuclear cells. An additional 12 ml of blood was collected for other analyses (Ben-David *et al.,* in press). Blood was centrifuged at 2000 rpm for 10 min and plasma was siphoned off and frozen. Cells were re-suspended with RPMI 1640 (Cellgro™, Mediatech; Fischer MT-10-041-LV) medium supplemented with 10% CPSR-3 (Sigma C-9030) to produce 10 ml of solution. The solution was combined with 10 ml of Hank´s balanced salt solution (HBSS; Cellgro™, Mediatech; Fischer MT-21-021-LV) with 1% antibiotic/antimycotic solution added (Sigma A-5955) and carefully layered on 4 ml of histopaque (Sigma). Samples were centrifuged at 1300 rpm for 25 min after which HBSS was removed and leukocytes were re-suspended and washed with 10 ml HBSS. After centrifugation at 1500 rpm for 10 min, HBSS was removed and cells were re-suspended in 3 ml freezing medium (i.e. a solution of 1:1 RPMI and CPSR-3 and 0.3 ml of 10% DMSO). Samples were aliquoted into two 1.5 ml nalgene cryovials and placed in a cryo-freezing container (Nalgene) with 70% alcohol. Samples were frozen and stored at –70 ¡C, and shipped to Purdue University in liquid nitrogen.

Laboratory procedures for CYP1A assays

Immunohistochemistry. For estimation of CYP1A1 in skin biopsies, preserved biopsy samples were embedded in paraffin tissue and were sectioned and examined with procedures modified from Smolowitz *et al.* (1991). Prior to immunochemical staining, standard 5 μm sections were deparaffinated and hydrated in 1% bovine serum albumin/phosphate buffered saline (BSA/PBS). During the hydration process, sections were incubated in 0.5% H_2O_2 in methanol for 45 min to block endogenous peroxidase. Hydrated sections were immunochemically stained using an indirect peroxidase stain (Universal Immunoperoxidase Staining Kit (Murine), Signet Laboratories, Inc., Dedham, MA) with the monoclonal antibody MAb 1-12-3 (Park *et al.* 1986) and peroxidase-labelled goat anti-mouse IgG secondary antibody. MAb 1-12-3 is highly specific for cytochrome P450 1A proteins in vertebrates (Drahushuk *et al.* 1998). The specificity of monoclonal antibody CYP1A1 in mammals has been determined by evaluating cross-reactivity with proteins in liver microsomes and with heterologously expressed CYP1A1 and CYP1A2 from mice, rabbits and humans. The antibody recognizes only CYP1A1 (e.g. Drahushuk *et al.* 1998). Moreover, the epitope recognized by MAb 1-12-3 has been identified and it is a sequence that is present only in mammalian CYP1A1, not in CYP1A2 nor in any other mammalian P450 (J. J. Stegeman, unpublished observations). Specific staining by MAb 1-12-3

was evaluated by light microscopic examination of the stained sections (figure 1). Cell types that stain and their associated occurrence and staining intensity were recorded for each tissue section examined. At least two immunochemically stained sections were examined from each sample. Staining of CYP1A1 was scored for intensity of staining (0 = no stain, 1 = mild stain, 2 = moderate stain, 3 = strong stain, 4 = very strong stain) and for occurrence of staining (0 = no cells, 1 = rare cells, 2 = some cells, 3 = many cells, 4 = all cells staining). A stain index was computed by multiplying the intensity and occurrence for scale from 0 to 16. In other studies, this stain index compared well with CYP1A content measured by quantitative immunoblot (Woodin *et al*. 1997).

Quantative RT-PCR. For analysis of CYP1A1 mRNA expression in peripheral blood mononuclear cells, frozen cells were thawed rapidly in a 37 ¡C water bath and immediately placed on ice. Samples were then transferred to a 15 ml centrifuge tube and diluted to 10 ml with HBSS, centrifuged and pelleted. Cell viability was determined by trypan blue exclusion. The RT-PCR assay was performed on total RNA isolated from peripheral blood mononuclear cells (PBMC). The messenger RNA (mRNA) that codes for the CYP1A1 protein was quantified, using specific cDNA primers and a quantitative reverse transcriptase-polymerase chain reaction (Vanden Heuvel *et al*. 1993, 1994). Initially, the RT-PCR assays required the isolation, cloning and sequencing of the PCR product, and the development of river otter specific primers for CYP1A (Ballachy *et al*., submitted). Since mustelid CYP1A1 had not been previously described, we designed primers based on a comparison of several known CYP1A1 cDNA sequences: sheep, human, mouse, guinea pig, hamster and rat. We aligned these sequences using a clustal MegAlign method and chose primers from highly conserved areas. The primers were then utilized to amplify mustelid CYP1A from liver RNA isolated from sea otters (*Enhydra lutris*) exposed to high concentrations of oil (Ballachy *et al*., submitted). The PCR product was cloned, into T7Blue T-vector, and sequenced. Using Primer Select (DNA Star, Madison, WI) and Prettyplot methods we obtained more efficient primers for mustelid CYP1A1 cDNA. Because these primers are very short it is difficult to run them in BLAST even for epitope identification. Nonetheless, we have examined the PCR product sequence in BLAST and the sequence homology is highest with other known CYP1A1 sequences.

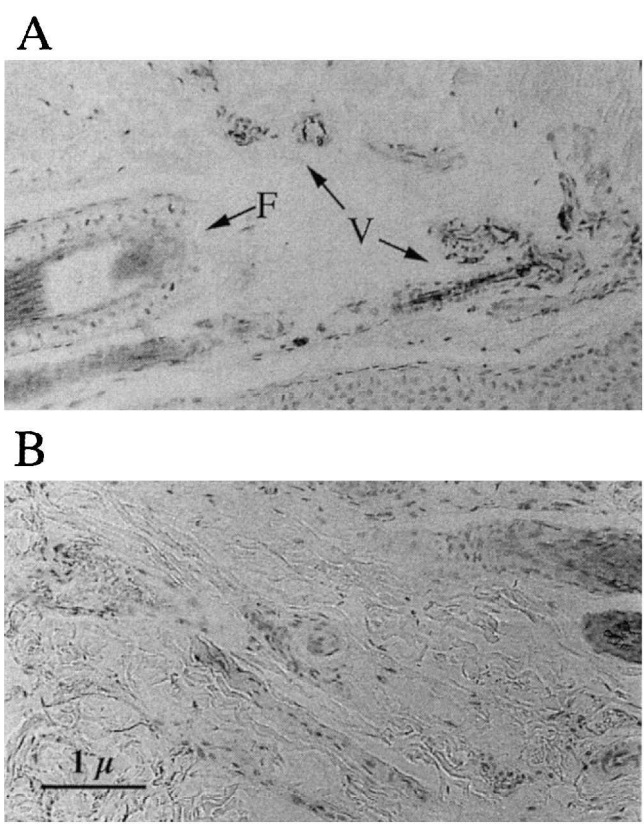

Figure 1. Immunohistochemical staining of CYP1A1 in endothelium from skin biopsy of river otters. (A) An MAb 1-12-3 staining in captive river otter skin plug; F denotes follicle and V represents vascular endothelial staining. (B) An UPC10 control MAb staining of same tissue.

The primers were optimized for Mg, annealing temperature, pH, and number of cycles. The optimized conditions were: 4mM Mg, 54 ¡C annealing temperature, pH 8.8, and 30 cycles. The PCR products were cloned into T7Blue T-vector according to the manufacturer's instructions (Novagen, Madison, WI). Following isolation of plasmid DNA, fluorescence dideoxynucleotide sequencing was performed at the Purdue University DNA Facility. The sequence information was used to obtain more efficient primers specific for river otter cDNA using PrimerSelect (DNAStar, Madison, WI).

River otter 1A forward primer, 5´-TGGTCAATTTTCTGTTCCTAG-3´
River otter 1A reverse primer, 5´-AGGTCAGCTCAACCTTGAGA-3´

The use of an internal standard that contains target (i.e. river otter CYP1A1) primer sequences negates tube-to-tube variability in PCR amplification and is essential to quantifying mRNA expression by RT-PCR. We generated recombinant RNA (rcRNA) internal standards as described by Vanden Heuvel *et al.* (1993). Using this method, a rcRNA was generated that upon amplification with river otter CYP1A1 primers results in a product that is easily resolved from target product following agarose gel electrophoresis.

Competitive PCR was performed as described by Gilliland *et al.* (1990a,b) and modified by Vanden Heuvel *et al.* (1994). For each sample eight to ten aliquots of total RNA (0.1 mg) were prepared, and a dilution series of the rcRNA internal standard was spiked into these aliquots. Reverse transcription of RNA was performed in a final volume of 20 µl containing 25 mM Tris–HCl (pH 8.3 at 25 ¡C), 50 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.1 mg ml^{-1} bovine serum albumin, 5 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 1 unit RNase inhibitor, 2.5 units M-MLV Reverse Transcriptase (Life Technologies, Inc.), 2.5 mM oligo$(dT)_{16}$, 0.1 µg total RNA, and varying amounts of rcRNA internal standard. The samples were incubated at 42 ¡C for 15 min and reverse transcriptase was inactivated by heating at 99 ¡C for 5 min. PCR reaction mixture contained 3 mM $MgCl_2$, 2.5 units Taq polymerase, and 6 pmol of forward and reverse primers. The reactions were heated to 94 ¡C for 3 min and cycled 30 times through a 30 s denaturing step at 94 ¡C, a 30 s annealing step at 54 ¡C, and a 30 s elongation step at 72 ¡C. Following the final cycle, a 5 min elongation step at 72 ¡C was included.

Aliquots of the PCR reaction were electrophoresed on 2.5% NuSieve® 3:1 agarose (FMC Bio Products, Rockland, ME) gels, and PCR fragments were visualized with ethidium bromide staining (figure 2). A photographic negative was prepared and densitometry was performed using a LKB Gel Scan II laser densitometer (LKB, Piscataway, NJ). Quantification of the amount of target mRNA present was determined as described by Gilliland *et al.* (1990a). The actual number of molecules of CYP1A1 were determined by comparing the ratio of the volume of the internal standard to CYP1A1 mRNA PCR products were plotted against the amount of internal standard added to individual tubes as previously described by Gilliland *et al.* (1990a). Linear regression analysis was used to define the equation for the line through the data points. The amount of CYP1A1 mRNA present for individual animals was defined as the amount of rcRNA present where the volume ratio was equal to 1.

Statistical analyses

Of 150 samples collected through the experiment for the quantitative RT-PCR, three were contaminated during processing. Missing values for those samples were replaced by means of near points approach (Johnson and Wichern 1992). To determine the effects of oil ingestion on levels of CYP1A1 we used repeated

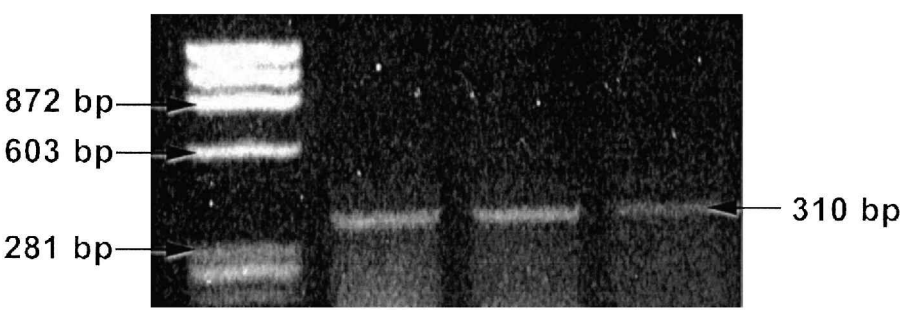

Figure 2. Gel electrophoresis of CYP1A1 mRNA product from lymphocytes of river otters. Ethidium bromide-stained agarose gel containing PCR products resulting from amplification of river otter lymphocytes CYP1A cDNA (310 bp). Left lane represents standards for molecular weight markers.

measures ANOVA with oiling group (i.e. control, low-dose, and high-dose) and session as factors (Johnson and Wichern 1992; SPSS for Windows). Analysis was followed by Tukey's multiple comparisons to establish where significant differences occurred for those models in which either group or session effects were significant. We used non-linear regression curve estimation to describe the relations between staining index of endothelial CYP1A1 as the dependent variable and cumulative dose levels as the independent one to determine the effects of cumulative effects of chronic exposure on this parameter (Zar 1984). In addition, we used correlation analysis (Pearson's ρ; Zar 1984) to determine the relationship between the endothelial and mononuclear blood cells CYP1A1.

Results

Expression of CYP1A1 in endothelial cells in skin biopsies as determined by IHC was similar for all 15 otters at capture (figure 3; repeated measures ANOVA, $P > 0.05$). Significant changes occurred in levels of the index throughout the experimental period (figure 3; repeated measures ANOVA, $P < 0.001$; group effect $P = 0.282$; session effect $P = 0.001$). The index value declined significantly between capture and the first sampling in captivity in June (prior to oil administration) for otters in the control and low-dose groups ($P < 0.05$). A similar non-significant trend was observed for the high-dose group (figure 3; $P = 0.09$).

A significant increase in levels of the index occurred in all groups between the June sampling session and the first exposure sampling session 16 days after the beginning of oil administration (Sept I; figure 3). The levels in the animals receiving the high dose were significantly higher than those in the other two groups (figure 3; Tukey's multiple comparisons, $P < 0.05$). The levels in this group declined somewhat by the October and November II sampling but remained high until the end of the oil administration. The CYP1A1 staining signal declined significantly in the high-dose group by the middle of the rehabilitation period (January; figure 3; Tukey's multiple comparisons, $P < 0.05$).

In the low-dose group the levels of endothelial CYP1A1 at the first sampling session were between those detected in the control and high-dose group. A significant decline in levels of the index occurred during the October sampling (figure 3). During rehabilitation, the levels in both the low- and high-dose groups were similar to those recorded during capture (figure 3; $P > 0.05$), but higher than those recorded before the start of the experiment (figure 3; $P < 0.05$).

Non-linear curve estimation detected no relationship between cumulative dose of ingested PBCO for the low-dose group (figure 4(A); non-linear curve estimation; $R^2 = 0.06$, $P = 0.311$). A significant near asymptotic relation was observed for the high-dose group (figure 4(B); non-linear curve estimation; $R^2 = 0.36$, $P = 0.005$; $y = 1.53 \times (x^{0.32})$).

QRT-PCR results showed that the CYP1A1 mRNA concentration in peripheral blood mononuclear cells (expressed as molecules of CYP1A1 per 100 ng of total RNA) was similar for all 15 otters at capture (figure 5; repeated measures ANOVA, $P > 0.05$). A significant decline in those levels occurred between capture and the experimental period (figure 5; repeated measures ANOVA, $P = 0.002$; group effect $P = 0.658$; session effect $P = 0.001$). As with the endothelial CYP1A1, there was a significant increase in CYP1A1 mRNA expression in all groups between the August sampling period and the second sampling after the beginning

Figure 3. Mean (± SE) values of staining index of CYP1A1 from endothelial cells for control, low-dose and high-dose groups ($n = 15$; 5 in each group) of river otters held in captivity at the Alaska Sealife Center in Seward, Alaska, USA. Oil was administered between 21 August and 28 November 1998. Significant changes occurred in levels of the index throughout the experimental period (repeated measures ANOVA, $P < 0.001$; group effect $P = 0.288$; session effect $P = 0.001$). Levels significantly decreased between capture and transfer to captivity and significantly increased during the oiling period for all groups.

of oil administration (Sept II; figure 5). Concentrations fluctuated through the period of oil administration and then significantly declined during rehabilitation (figure 5; Tukey's multiple comparisons, $P < 0.05$). Concentrations during the oil administration period were significantly lower than those recorded during capture for all except two of the control animals (UI02: capture level = 0.64, highest during oiling = 3.43; UI03: capture level = 1.72, highest during oiling = 4.01 molecules of CYP1A1 per 100 ng of total RNA).

No correlation was detected between values of the CYP1A1 staining index in endothelium and the concentration of CYP1A1 mRNA in lymphocytes, for the entire data set ($r = 0.006$, $P = 0.96$, $n = 75$).

Discussion

The development of effective biomarkers for environmental agents depends on the ability to describe quantitative, predictable, dose–response relationships between exposure and the biological indicator (Vanden Heuvel and Davis 1999). In addition, the biomarker examined should be specific to a particular chemical or

Figure 4. Relationship between cumulative dose of ingested weathered PBCO and values of staining index of CYP1A1 from endothelial cells of river otters from the low-dose (A) and high-dose (B) groups. No relationship was detected for the low-dose group, but a significant near asymptotic relation was observed for the high-dose group (non-linear curve estimation; $R^2 = 0.36$, $P = 0.005$; $y = 1.53 \times (x^{.32})$).

class of compounds, and relatively non-invasive (Vanden Heuvel and Davis 1999). Evaluations of the mRNA, protein or catalytic activity of CYP1A in a variety of vertebrates, have been widely used as a biomarker for exposure to MC-type environmental contaminants (Miranda *et al*. 1987, Payne *et al*. 1987, Stegeman *et al*. 1988, 1992, Kloepper-Sams and Stegeman 1989, Stegeman and Lech 1991, Juchau 1990, Goksùyr and Husoy 1992, Rattner *et al*. 1993, Bucheli and Fent 1995, Willet *et al*. 1997, Woodin *et al*. 1997). A prominent induction of CYP1A in endothelium of mammals and fish was identified in the 1980s (Stegeman *et al*. 1989). Those observations prompted studies to evaluate CYP1A in biopsy samples

Figure 5. Mean (± SE) values of number of CYP1A1 mRNA molecules 10^6 per 100 ng of total RNA in peripheral mononuclear blood cells of control, low-dose and high-dose groups ($n = 15$; 5 in each group) of river otters held in captivity at the Alaska Sealife Center in Seward, Alaska, USA. Oil was administered between 21 August and 28 November 1998. A significant decline in those levels occurred between capture and the experimental period (repeated measures ANOVA, $P = 0.002$; group effect $P = 0.681$; session effect $P < 0.001$). Note the change in values on axes. A significant increase occurred in all groups between the August sampling session and the second bleeding session in September (Sept II). Levels fiuctuated through the period of oil administration and then significantly declined during rehabilitation (Tukey's multiple comparisons, $P < 0.05$).

collected from marine mammals, as a marker of exposure to CYP1A inducers in these protected animals (Fossi *et al.* 1992, Goksùyr, 1995). Similarly, CYP1A1 mRNA has been successfully measured in peripheral mononuclear blood cells of humans, sea otters, and river otters (Vanden Heuvel and Davis 1999, Ballachy *et al.*, submitted). Thus, given the specificity of CYP1A1 to MC-type inducers (as reviewed by Juchau 1990), the evidence of dose–response in CYP1A1 expression (as reviewed by Vanden Heuvel and Davis 1999), and the ability to conduct non-destructive sampling for measurement of CYP1A1 expression in skin biopsies and peripheral blood cells (Fossi *et al.* 1992, Goksùyr 1995, Vanden Heuvel and Davis, 1999, Ballachy *et al.*, submitted), CYP1A1 should be considered a reliable biomarker.

Wild animals, inhabiting polluted areas, likely experience repeated exposures to CYP1A inducers. Nonetheless, chronic long-term exposure with repeated non-lethal sampling of the same individuals was rarely adopted in controlled studies designed to obtain a dose response in CYP1A expression. In this study, we simulated such chronic exposure in captive river otters fed weathered Prudoe Bay crude oil and

measured levels of CYP1A1 in endothelial cells of skin biopsies and peripheral blood mononuclear cells using repeated sampling of the same individuals. Despite the lack of correlation between those assays, both the IHC analysis and QRT-PCR exhibited a similar overall pattern in relation to exposure to hydrocarbons. Our results indicated the occurrence of induction of CYP1A1 expression in both the peripheral vascular endothelium and in peripheral blood mononuclear cells. In addition, this induction was measurable throughout the exposure period and declined shortly following the cessation of oil administration (figures 3 and 5). That the values of IHC for the low-dose group were on average between those of the control group and the high-dose group shortly following the initiation of dosing indicates a dose–response expression of CYP1A1 in the skin endothelium.

Our expectation that a systemic response to exposure would result in a correlated response between the two target tissues was not met. This could be a result of differences in turnover rate of endothelial and blood mononuclear cells or different responses to circulating xenobiotics by the two tissues. Other studies on fish and mammals documented differences in temporal accumulation of MC type agents and differential induction of CYP1A1 in different tissues (Brunström 1992, Woodin *et al.* 1997). Because of these potential differences, it would have been beneficial to conduct an IHC examination on peripheral blood mononuclear cells and conduct RT-PCR on endothelial cells. Unfortunately, the repeated sampling design of this experiment, and the need to obtain relatively large quantities of blood for other assays (Ben-David *et al.,* in press) precluded the collection of additional samples for such a cross-check study. Such study, potentially conducted on larger animals, will enhance our ability to explore tissue specific responses to hydrocarbons as well as the applicability of both analytical methods.

Alternatively, the lack of correlation between endothelial and peripheral blood mononuclear cells CYP1A1 expression could be attributed to the occurrence of clinical anaemia in the oiled captive otters (Ben-David *et al.*, in press). In instances where toxins affect production of erythrocytes (anaemia), and cause a regenerative response with release of nucleated erythrocytes into circulation from the bone marrow, the RNA of the nucleated erythrocytes would dilute the quantity of total RNA that is being evaluated from lymphocytes and monocytes. The anaemia observed in our study animals was accompanied by the occurrence of nucleated erythrocytes suggesting a regenerative response (Ben-David *et al.*, in press). Therefore, it is possible that we harvested nucleated erythrocytes with the other peripheral blood mononuclear cells, thus reducing the overall measure of mRNA of CYP1A1. Furthermore, the observed reduction in white blood cells in the oiled animals (Ben-David *et al.*, in press) may be indicative of suppression of the bone marrow and abnormal cell release from that tissue. Under conditions of long-term chronic exposure, new mononuclear blood cells could potentially be produced from an abnormal bone marrow tissue. The ability of cells, produced by such tissue, to respond to MC-type agents, in circulation, is unknown and merits further investigation. Analysis of CYP1A1 mRNA in mononuclear blood cells may produce quantitative dose–response results in cases where exposure is low enough so suppression of bone marrow does not occur. Regardless, the lack of quantitative, dose–response expression of CYP1A1 in mononuclear blood cells under conditions of clinical anaemia in this study suggests that the use of this measure to describe exposure in free-ranging animals should be done with caution and determination of anaemia should be done concurrently with evaluation of CYP1A1. Many

controlled studies as well as field studies of wildlife exposed to hydrocarbons frequently documented anaemia in their subjects (Øristland *et al.* 1981, Leighton *et al.* 1983, Fry and Lowenstine 1985, Rebar *et al.* 1994, Williams *et al.* 1995).

As expected a significant near asymptotic relation between cumulative dose of ingested PBCO and values of IHC was observed for the high-dose group, indicative of induction of CYP1A1 and probable continuous oxidation of the hydrocarbons. It is important to note that the cumulative dose of ingested PCBO cannot be linearly translated into accumulation of hydrocarbons in the target tissue (i.e. skin endothelium). Cumulative dose in this study should be regarded as a surrogate for continuous chronic exposure rather than accumulation of hydrocarbons. The increased variability in values of IHC with time (i.e. cumulative dose; figure 4) suggests that long-term chronic exposure may elicit different responses in individuals experiencing similar conditions. In addition, the lack of similar asymptotic relation in the low-dose group indicates that under chronic low exposure levels, induction of CYP1A1 may be unpredictable.

The reduction in levels of CYP1A1 mRNA in mononuclear blood cells, in our captive animals during rehabilitation, was more pronounced than that of CYP1A1 protein in endothelial cells. This is not an unexpected result because the half-life of the CYP1A1 protein is likely longer than that of mRNA (see review by Vanden Heuvel and Davis 1999). Thus, studies employing IHC techniques on skin endothelium may be able to record the occurrence of exposure to MC-type agents for longer periods of time than those using QRT-PCR. In contrast, the persistent and diminished signal in IHC may complicate interpretations of field data. Without additional information, a lingering, lower signal, resulting from elapsing time between exposure and sampling may be interpreted mistakenly as a recent exposure to a lower dose.

During the oiling period induction of CYP1A1 was detected in animals from the control group. Although this induction mainly occurred in mononuclear blood cells, by the end of the oiling period CYP1A1 values measured with IHC in endothelial cells for the control group were similar to the diminishing values of the oiled animals. The high levels of CYP1A1 in peripheral mononuclear blood cells recorded in the control animals may represent the fact that these individuals did not suffer from clinical anaemia (Ben-David *et al.*, in press), thus the levels of CYP1A1 in these animals were not diluted by nucleated erythrocytes. It is also possible that animals in our experiment were unintentionally exposed to MC-type agents through the fish they consumed. Although we did not test the foods we offered the otters for hydrocarbons, PCBs or other xenobiotics, otters were fed the same diet throughout the experiment (i.e. the frozen fish offered to the otters came from the same shipment and the live fish were caught in the same location throughout the experimental period). That both analytical approaches exhibited an initial decline in expression of CYP1A1, between capture and the experimental period (figures 3 and 5) indicated that the enclosure at the Alaska Sealife Center, and the food we offered to the captive otters were free of potential inducers of CYP1A1.

Alternatively, it is possible that the control animals were indirectly exposed to hydrocarbons because all the otters in our study were housed in one enclosure. Oiled animals occasionally dropped a partially consumed fish that contained oil. Although we cleaned the contaminated area with paper towels it is likely that we were unable to remove the more volatile compounds from the enclosure (table 1). In addition, an experiment designed to investigate the effects of oil ingestion on passage rate in the

same individuals documented the excretion of hydrocarbons in faeces of the oiled animals (Ormseth and Ben-David 2000). River otters use a communal latrine site in the wild (Bowyer *et al.* 1995). Similarly, the experimental animals established communal latrine sites in the enclosure. Thus, although river otters do not consume faeces, animals from the control group could have been exposed to hydrocarbons in faeces of oiled animals while exploring the communal latrines. A study on captive koalas demonstrated that animals kept in separate cages experienced induction of cytochrome P450 in response to the odour of leaves from a certain eucalyptus tree without actually consuming the foliage (Cork and Foley 1997). Whether similar processes occur in river otters is unknown. That a measurable induction of CYP1A1 occurred in the control animals which may have experienced a low and indirect exposure, illuminates the sensitivity of this biomarker.

The levels of endothelial CYP1A1 in the high-dose group during the oiling period were within the range of values reported for wild river otters in Herring bay, Knight Island, PWS (oiled area) in 1996 and 1997 (3.2 ± 0.5, mean ± SE and 4.7 ± 0.6, respectively; Bowyer *et al.*, submitted,). In contrast, the levels of CYP1A1 in mononuclear blood cells in the experimental animals were much lower than those recorded in river otters sampled in oiled areas of the Sound in 1998 (36.9 ± 16.7, mean ± SE; Ballachy *et al.*, submitted,). The latter, however, may be a result of the quantification problems due to the anaemia discussed above. If indeed expression of CYP1A1 in skin endothelium in response to hydrocarbon exposure is dose-dependent as we observed, then wild river otters in oiled areas of the Sound would have been exposed to levels in excess of 50 mg day^{-1} kg^{-1} body weight 7–9 years following *EVOS*. This, however, is unlikely. First, no differences in body mass, other biomarkers, or differences in diet selection and home-range sizes were detected between otters inhabiting oiled vs non-oiled areas in 1996–1998 (Bowyer *et al.*, submitted). In addition, investigators did not record any signs of anaemia and reduction in white blood cell counts in wild otters in 1996–1998 (Bowyer *et al.*, submitted), which were the most pronounced effects of oiling in the captive experimental otters (Ben-David *et al.*, in press). Therefore, it is possible that other agents in addition to hydrocarbons may have elicited the induction of CYP1A1 in river otters in PWS. Although the nature of these agents has not been determined yet, PCBs, components of the diet, as well as secondary compounds from Sitka spruce (*Picea sitchensis*) and western hemlock (*Tsuga heterophylla*) trees have been suggested as potential inducers (for other examples see Förlin *et al.* 1985, Payne *et al.* 1987, Juchau 1990, Brunström 1992, Courtney *et al.* 1993, Lee *et al.* 1997). Evaluation of the occurrence of other potential inducers, as well as the relationship between diet, body composition, and levels of CYP1A1 in these mustelids merits further investigation.

Alternatively, the high levels in both IHC and QRT-PCR during capture compared with the levels immediately after acclimation to captivity in our experimental river otters (figures 3 and 5) could be associated with trapping stress. Ben-David *et al.* (in press) identified a group of variables (AST, LDH, glucose, and BUN) that exhibited a similar pattern of a significant decrease between capture and the June sampling session followed by stable values throughout the experiment for the same individual otters. Although no other studies documented an increase in expression of CYP1A in response to trapping stress, several studies identified AST, LDH, glucose, and BUN as indicators of capture stress in a variety of wild animals (Seal and Hoskinson 1978, Williams *et al.* 1992, Boonstra *et al.* 1998, Keech *et al.* 1998). Of these variables, only BUN and mononuclear blood cells CYP1A1 mRNA

were significantly correlated ($r = 0.32$, $P < 0.001$; Ben-David *et al.*, in press). Another anecdotal evidence suggesting that trapping stress may have been involved in induction of CYP1A is indicated by the observation that of 111 wild-caught river otters (Bowyer *et al.*, submitted,), those three with the highest staining index of endothelial CYP1A1 either died shortly after capture from an unassociated disease process, or were detained in the trap for an extended period of time (> 48 h) due to failure of the trap transmitter (G. M. Blundell, pers. comm.). Usually, the trapped otters spent less than 8 h in the trap (G. M. Blundell, pers. comm.). Therefore, an association between trapping stress and CYP1A1 expression will depend on the interval between the onset of stress (as may be transmitted by excretion of cortisol and/or other adernocorticosteroids) and the initiation of induction. Interestingly, Engelhardt (1982) documented that the half-life of cortisol was significantly reduced in ringed seals (*Phoca hispida*) exposed to hydrocarbons. Nonetheless, which of the CYP haemoproteins is involved in the degradation of cortisol in both seals and river otters is unclear and merits further investigation.

In conclusion, this is the first study to document induction of CYP1A1 expression under conditions of chronic exposure to hydrocarbons. We observed increases in expression of CYP1A1 in skin endothelium in the oil-exposed groups during the oil administration period, followed by a significant decrease during rehabilitation in the river otters exposed to PBCO in the diet. The observation that in a longitudinal study with repeated sampling of the same individuals, such a pattern was observed provides encouragement for the use of cytochrome P450 1A1 as a biomarker for chronic hydrocarbon exposure in these animals. Nonetheless, our results, especially those involving the RT-PCR on mononuclear blood cells, also suggest that the induction process of CYP1A1 may be complicated and interacting with other processes *in vivo*. Such interactions may obscure our ability to describe specific, quantitative, predictable, dose–response relationships between exposure to hydrocarbons and induction of CYP1A1, which are required of a reliable biomarker. Evaluations of such interactions based on theoretical physiological models in live animals merit further investigation and will assist in substantiating the potential utility of this approach of analysing CYP1A as a marker of exposure to MC-type compounds.

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