

Induction of cetacean cytochrome P4501A1 by  $\beta$ -naphthoflavone exposure of skin biopsy slices

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## ABSTRACT

Marine mammals can accumulate environmental contaminants in their blubber at concentrations harmful to laboratory animals. Induction of the cytochrome P450 1A1 (CYP1A1) enzyme is widely used as a biomarker of exposure and molecular effects in animal species, yet the validity of this biomarker has not been established in marine mammals. *In vivo* studies are generally precluded in these protected species but skin biopsies (epidermis and dermis) can be collected in a minimally invasive way. We developed an *in vitro* assay using skin biopsy slices to examine CYP1A1 protein induction in marine mammals in response to chemical exposure. Skin biopsies from sperm whale (*Physeter macrocephalus*) were exposed for 24 hr to  $\beta$ -naphthoflavone (BNF), a prototypical CYP1A1 inducer and CYP1A1 induction was detected by immunochemical staining in endothelial cells, smooth muscle cells and fibroblasts. Biopsy slices were exposed to a range of BNF concentrations (0.6 -600  $\mu$ M) and a significant concentration-effect relationship was observed in both endothelial and smooth muscle cells ( $p=0.05$ ). This is the first study using skin biopsy slices to examine exposure of cetacean tissue to a CYP1A1 inducer. It demonstrates a causal relationship between chemical exposure and CYP1A1 induction and therefore validates the use of CYP1A1 expression in skin biopsies as a biomarker in cetaceans. Our protocol can be adapted to the investigation of chemicals, mixtures, concentrations, incubation times or biological endpoints of choice. This should prove particularly relevant for these and other protected species that cannot be studied in the laboratory.

Keywords: marine mammal, CYP1A1, skin,  $\beta$ -naphthoflavone, sperm whale, endothelium, dose-response

## INTRODUCTION

The oceans are the final sink for many toxicants and there is growing concern for the health of marine mammals as they are known to accumulate high levels of polyhalogenated aromatic hydrocarbons, pesticides and other lipophilic contaminants in their blubber (Aguilar and Borrell 1994; Colborn and Smolen 1996; Kannan *et al.* 1993; Martineau *et al.* 1987; Ross *et al.* 2000; Tanabe *et al.* 1981). Marine mammals can bioaccumulate and biomagnify these lipophilic marine contaminants due to a high position in marine food chains, large fatty tissue reserves and longer life spans than many other marine organisms (Boon *et al.* 1992).

High concentrations of organochlorine pollutants deleteriously affect the endocrine, reproductive, immune and nervous systems of laboratory animals and elicit adverse responses such as skin and liver damage, thymic atrophy, weight loss and neurobehavioral problems (Geyer *et al.* 1984). Contaminant tissue burdens equal to or above levels found harmful in laboratory animals have been reported in several cetaceans and pinnipeds, including beluga whales of the Saint Lawrence Estuary, long-finned pilot whales from the Faroe Islands, killer whales from the North Pacific and animals involved in recent mass stranding events (Colborn and Smolen 1996; Kannan *et al.* 2000; Kuehl *et al.* 1991; Martineau *et al.* 1987; Ross *et al.* 2000). While a direct link between contaminant burden and cetacean epizootics or mass stranding has not been established, some of the highest PCB concentrations found in wildlife have been reported in these animals (Aguilar and Borrell 1994; Kannan *et al.* 1993). However, the concentrations of chemicals present in marine mammal tissues can provide only a partial insight as to the actual toxicity to the animal.

Linking biological effects with exposure to organochlorines and other pollutants is particularly challenging in marine mammals because of their legal status as protected species, the

complex logistics involved in studying them in their natural habitat, the impracticality of laboratory studies, and the complex ethical issues involved. To our knowledge, the only *in-vivo* exposure experiments involving organic contaminants reported in the literature for cetaceans are those of Geraci and St Aubin (1982) in the late 1960s, when three bottlenose dolphins and one Risso's dolphin (*Grampus griseus*) were exposed topically to crude oil or orally to machine oil. Topical exposure resulted in transient cell damage in the epidermis while the extensive hepatic and pancreatic fibrosis observed after oral exposure were attributed to trematode parasites.

To date, the effects of chemicals in cetaceans have been inferred largely from correlations between high body burdens and pathologies and by extrapolation from dose-response relationships for both toxicities and molecular effects in other species. Molecular effects correlated with toxicity include the induction of cytochrome P450 enzymes (CYP) and particularly of CYP1A1 by chemicals such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins and furans via the aryl hydrocarbon receptor (AHR) signaling pathway (Poland and Knutson 1982). CYP1A1 induction is widely used as a biomarker of exposure and molecular effects in animal species (Stegeman *et al.* 1992). Among the few studies of cytochrome P450s in cetaceans, several have examined the metabolism of foreign chemicals in hepatic microsomes or cell cultures (Boon *et al.* 1998; Goksøyr *et al.* 1986; Murk *et al.* 1994; White *et al.* 1994; White *et al.* 2000) and a CYP1A1 has been identified in several species (Teramitsu *et al.* 2000). Correlations between non-*ortho* and mono-*ortho* PCB burdens in blubber and hepatic CYP1A1 content and activity were observed in beluga whales (White *et al.* 1994). Such correlations generally support the use of CYP1A1 induction as a biomarker of exposure to AHR agonists in cetaceans, but data to directly demonstrate the concentration-dependence of induction is critically absent (Angell *et al.* *In Press*). We employed skin biopsy

slices to show directly a link between chemical exposure and CYP1A1 induction in cetacean tissues. The use of skin biopsy for measuring CYP1A1 activity in marine mammals has been advocated as a valid non-destructive method since the early 1990s (Fossi *et al.* 1992; Fossi *et al.* 2003). We treated sperm whale skin biopsy sections with various concentrations (0-600  $\mu$ M) of  $\beta$ -naphthoflavone (BNF), a prototypical CYP1A1 inducer. Levels of CYP1A1 induction in the endothelium (a prominent site of induction in vertebrates), in smooth muscle cells and in fibroblasts were then determined using immunohistochemical staining.

## METHODS

### *Biopsy collection*

Sperm whale biopsies were obtained in the Sea of Cortez, Mexico in the summer and fall of 1999 (August-October) between 24°41.6N and 28°31.0N latitude and 110°02.0W and 112°41.7W longitude. The biopsy cruise was part of the Voyage of the Odyssey, a 5-year research program headed by Ocean Alliance and designed to gather baseline data on the levels and effects of synthetic contaminants in the marine environment worldwide, using sperm whales as an indicator species. The skin biopsies were collected using a 150 lb draw weight compound crossbow (Barnett RC-150). The skin biopsies collected included epidermis and dermis layers. Biopsy arrows with 40 mm by 7 mm internal diameter tips were designed and fabricated by Finn Larsen of the Danish Institute for Fisheries Research, Charlottenlund, Denmark. Samples were obtained under US National Marine Fisheries Service permit N° 1004 to Ocean Alliance, and Mexico Secretaria de Medio Ambiente Recursos Naturales y Pesca permit N° 4903 to Dr. Jorge Urban Ramirez of the Universidad Autonoma de Baja California Sur, Mexico.

### *Biopsy treatment*

Immediately after collection, we manually cut two thin (about 2 mm thick) slices spanning the epidermis and dermis from each of 50 biopsies. We incubated one of the two slices (treated slice) for 24 hr in cell culture media with BNF. Incubation was carried at the ambient temperature of the air conditioned pilot house. Temperature logs indicate the ambient temperature in the pilot house to be maintained at about 32°C when air conditioned. Treatment groups were 0, 0.6, 6, 60, or 600 µM BNF prepared in dimethylsulfoxide (DMSO) as carrier, with 10 animals per treatment group. The 0 µM BNF corresponded to DMSO alone and allowed us to test for carrier effect. For each biopsy, we incubated the other slice (untreated slice) for 24

hr in media alone. After the 24-hour incubation in media, untreated and treated slices were placed in 10% neutral buffered formalin until embedding in paraffin to ensure protein preservation.

### ***Media and chemicals***

DMEM (Dulbecco's Modified Eagle's Medium, Sigma, St. Louis, MO, USA) medium was prepared with 5.96 g HEPES free acid (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; 4-[2-Hydroxyethyl]piperazine-1-[2-ethanesulfonic acid],Sigma), 2.2g NaHCO<sub>3</sub> (sodium bicarbonate, Sigma) and 0.58g NaCl (sodium chloride, Fisher Scientific, Pittsburgh, PA, USA) per liter. Medium was adjusted to pH 7.5, filter-sterilized and refrigerated before use. BNF was purchased from Aldrich (Milwaukee, WI, USA) and DMSO from Fisher Scientific. BNF was chosen for its low toxicity to humans, which allowed for safety protocols compatible with our field work.

### ***Immunohistochemical (IHC) analysis***

Biopsy slices were prepared for immunohistochemical staining of cytochrome P4501A1. Slices fixed in 10% neutral buffered formalin were embedded in paraffin. Serial microtome sections (5 µm thick) were obtained from within the 0.2 mm outer layers and then stained using a peroxidase anti-peroxidase detection system (Signet Laboratories, Dedham, MA) with either a monoclonal antibody against scup CYP1A (MAb 1-12-3, 0.3 µg/ml) or a purified mouse myeloma protein non-specific antibody (MOPC31, 0.3 µg/ml, Sigma, St. Louis MO USA), as previously described (Smolowitz *et al.* 1991). MAb 1-12-3 is highly specific for CYP1A1 in mammals (Drahushuk *et al.* 1998), and the epitope recognized is a CYP1A1 specific epitope (unpubl. info). CYP1A1 staining was evaluated under light microscopy after incubation with amino-9-ethylcarbazole as chromogenic substrate (AEC, Signet Laboratories) and

counterstaining with Mayer's hematoxylin (Sigma). For each section, CYP1A1 staining scores (scale of 0-15) were determined as the products of the staining occurrence (scale of 0-3) and the staining intensity (scale of 0-5) in each cell type. A staining occurrence of 0 corresponds to no staining while a staining occurrence of 3 corresponds to staining in all cells. The staining intensity represents the average intensity observed for each cell type throughout a section. A staining intensity of 0 corresponds to an absence of staining or to a staining equal to that observed with the control MOPC antibody. A staining intensity of 5 corresponds to a very strong staining equal to that observed in a highly CYP1A-induced liver section of scup treated with 3,3',4,4' tetrachlorobiphenyl (TCB). Serial liver sections of this TCB-induced scup were used as controls for staining intensity among IHC runs. IHC staining scores have been shown to reflect accurately the content of CYP1A1 protein measured by immunoblotting techniques (Woodin *et al.* 1997). A treatment-specific staining score was determined for each animal as the difference between the staining scores of the treated (with DMSO or BNF) and untreated (media alone) biopsy sections. For each treatment group, two biopsies were randomly selected for hematoxylin and eosin staining of both treated and untreated sections (H and E, Richard Allen Scientific, Kalamazoo, MI USA) according to standard protocols (Allen 1992). We evaluated tissue integrity (nuclear stain intensity, nucleus shape, eosinophilia) in all sections using IHC and hematoxylin- and eosin-treated slides.

### ***Statistical analyses***

Differences among treatment-specific CYP1A staining scores for endothelial and smooth muscle cells were statistically analyzed by one-way ANOVA using Fisher's Protected LSD test for equal sample size (N=10) using the SuperANOVA (Abacus Concepts). One untreated biopsy sample in the DMSO group could not be scored for fibroblasts due to the faintness of the

counterstain. The differences among treatment-specific CYP1A1 staining scores for fibroblasts were therefore statistically analyzed by one-way ANOVA using the Tukey HSD Compromise test for unequal sample size (N=9 for DMSO group and N=10 for all other groups) using the SuperANOVA (Abacus Concepts). The  $\alpha = 0.05$  level was considered significant.

## RESULTS

### *Biopsy collection and treatment of tissue slices*

Biopsies from 50 sperm whales were treated with BNF in DMSO or with DMSO alone for 24 hour, with 10 animals per treatment group (0, 0.6, 6, 60, or 600  $\mu$ M BNF). We examined all biopsy sections for tissue integrity. Some samples exhibited perinuclear vacuolation in the epithelium and partial cell junction loss between epithelium and dermis but no apparent alteration was observed in the dermal cells, the focus of our IHC study. Immunohistochemical analysis carried out to evaluate the expression of CYP1A1 in skin biopsy sections showed CYP1A1 staining in three different cell types: the endothelial cells comprising the lining of all blood vessels including capillaries, the smooth muscle cells present in the larger blood vessels, and fibroblasts. In all 50 sperm whales studied, no staining was observed in the antibody-control slides (incubated with the non-specific antibody MOPC) prepared for each untreated and treated biopsy section, indicating that the staining observed in sections incubated with the monoclonal antibody to CYP1A1 is specific. The specificity of the CYP1A1 staining is illustrated in Figure 1.

### *Inducibility of cetacean CYP1A1*

Table 1 presents the average CYP1A1 IHC staining scores for the untreated and treated biopsy sections, along with the average treatment-specific staining scores (i.e., the difference between untreated and either BNF or DMSO treated sections). Faint staining can be observed in all untreated sections (with 91% of the staining scores below 5), probably reflecting environmental exposure of the whales to CYP1A1 inducers. Average staining scores for endothelial cell, smooth muscle cell and fibroblast staining in all untreated biopsies were 2.5, 2.3, and 2.9 respectively (standard deviation STD = 1.4, 1.3, and 2.0; range = 1-6, 1-4.5, and 0.5-

9). For all cell types, the average BNF-specific staining scores were significantly different from the DMSO-specific average staining scores, consistent with induction of CYP1A1 in sperm whale endothelial cells, smooth muscle cells and fibroblasts. This is illustrated in Figure 2, which shows CYP1A1 staining in untreated and treated biopsies from 3 animals (PM99-346, PM99-336 and PM99-352). Untreated sections showed only a faint staining (average staining score of 2.2, STD of 1.6) while there was an increase in staining intensity with BNF treatment in all three cell types. For the three animals illustrated in Figure 2, the treatment-specific staining scores for endothelial cells, smooth muscle cells and fibroblasts were, respectively, 2.5, 2.5, and 3 in the DMSO group; 7, 7, and 7 in the 0.6  $\mu$ M BNF group; and 13.5, 10.5, and 13 in the 600  $\mu$ M BNF group. It is important to note that the staining intensity for any particular section reflects the average intensity observed for all vessels in that biopsy section and that at times both low and high intensity staining vessels were observed within the biopsies treated with BNF.

#### ***Concentration-effect in cetacean CYP1A1 inducibility***

As illustrated in Figure 3, statistical analyses showed a concentration-dependent relationship for cetacean CYP1A1 inducibility in endothelial and smooth muscle cells. In fibroblasts, all treatment groups had CYP1A1 scores statistically different from the control (DMSO) group, but staining did not statistically differ between doses of BNF. In both endothelial and smooth muscle cells there were three statistically different levels of CYP1A1 staining. Treatment groups were assigned randomly but, interestingly we observed higher CYP1A1 expression in endothelium and smooth muscle cells from untreated biopsy sections than in the matched sections treated with DMSO alone. However, these higher CYP1A1 expression scores were not statistically different from the CYP1A1 expression scores observed in untreated biopsies matched to the BNF treatment groups. To further ensure that the higher

CYP1A1 expressions in the untreated biopsy sections matched with DMSO-treated sections did not bias our results, we also calculated treatment-specific staining scores in two other ways. Thus, treatment-specific staining scores were calculated by subtracting either the mean staining score of all untreated sections or the mean staining score of the untreated biopsies matched to the DMSO treatment group, from the BNF-treated biopsy staining score for each animal (data not shown). Regardless of the statistical method used, we detected significant concentration-dependent increases in CYP1A1 staining in response to BNF exposure in endothelium and smooth muscle cells.

## DISCUSSION

A higher overall incidence of mass mortalities, epizootics, pathologies, and reproductive failures has been reported in marine mammal populations in the last 30 years (Colborn and Smolen 1996). It has been hypothesized that this elevated incidence may be related to the mass production of organochlorines that started in the 1940s and the subsequent effects of long-term chronic exposure in the first generation of animals exposed, and/or of developmental and early postnatal exposure in their offspring (Colborn and Smolen 1996). Numerous studies have established that marine mammals can accumulate high levels of organochlorines and other lipophilic contaminants (Martineau *et al.* 1987; Ross *et al.* 2000) but studies reporting on the molecular or physiological effects of these compounds are scarce. Minimally invasive methods for biomarkers of exposure and effect are critically needed to investigate the impact of pollution on marine mammals (Fossi *et al.* 1992). CYP1A1 induction is widely used in animal species as a biomarker of exposure to AHR agonists. Because the induction reflects a change in gene or protein expression levels or enzymatic activity, it is also considered a biomarker of molecular effects. Correlations between CYP activity and levels of some organochlorines (PCBs, dichlorodiphenyltrichloroethanes) in skin biopsies of common dolphins (*Delphinus delphis*) have been reported (Fossi *et al.* 2003), indicating that CYP1A1 induction may be a valid biomarker of exposure in these animals. However, the validity of this biomarker, i.e. demonstrating that it responds in a dose-dependent manner to toxicant exposure, remains to be directly established in cetacean species.

To specifically address this issue we adapted a tissue slice protocol for use with cetacean skin biopsies. In contrast with *in vitro* exposure studies that rely on cell culture, the normal tissue architecture (including cell heterogeneity and cell-cell interactions) is maintained in this

protocol. We collected skin biopsies from 50 sperm whales in a minimally invasive manner and exposed biopsy sections to 0, 0.6, 6, 60, or 600  $\mu\text{M}$  BNF, a prototypical CYP1A1 inducer. We selected this wide range (0.6 to 600  $\mu\text{M}$  BNF) of BNF concentrations to increase the likelihood of detecting changes in CYP1A1 induction. 600  $\mu\text{M}$  BNF was selected because it neared the highest concentration of BNF that could conveniently be prepared in DMSO. We used 0.6  $\mu\text{M}$  BNF as the lowest concentration based on a previous study on porcine endothelial cells (Stegeman *et al.* 1995). The use of tissue slices for studies of cytochrome P450 activities and inducibility by chemicals such as BNF, TCDD and Aroclor® 1254 (commercial PCB mixture) has been validated by comparisons with *in vivo* experiments (Drahushuk *et al.* 1996; Lake *et al.* 1993). In mammals, precision-cut tissue slices and outer layers of generally thicker manually-cut slices have been shown to retain viability and metabolic capacity for at least 24 hr (Parrish *et al.* 1995). Similarly, we did not observe any apparent alteration of the dermal cells in all biopsy sections after 24hr, treated or untreated.

The faint staining observed in all untreated slices (91% of these slides had a staining score below 5) probably reflects environmental exposure of the sperm whales to CYP1A1 inducers; such environmental induction has been suggested in biopsies from numerous cetacean species (Miller *et al.*). A lesser staining was seen in DMSO-treated sections, DMSO has been reported to have a protective action on CYP enzymes *in vitro* that may be due to the scavenging of hydroxyl radicals while its effects *in vivo* are unclear with both increased and decreased monooxygenation rates having been reported (Glockner and Muller 1995). However, the average DMSO treatment specific scores for all three cell types examined were not statistically different from zero, indicating the suitability of this compound as a carrier in our experiments. In BNF-treated slices, statistically significant induction of CYP1A1 was detected in endothelial

cells, smooth muscle cells and fibroblasts, and at all four concentrations tested (Table 1, Fig. 2). The results showed a concentration-dependent relationship for cetacean CYP1A1 inducibility in endothelial and smooth muscle cells, with three statistically different levels of CYP1A1 induction observed in each cell type (Fig. 3). In rat liver slices, CYP1A1 induction has been detected at the protein, mRNA and enzyme activity levels after incubation with 25  $\mu$ M BNF for 24 hr (Lupp *et al.* 2001; Muller *et al.* 1996) and a concentration-dependent induction was also detected enzymatically after both 48 hr and 72 hr incubation with 0-50  $\mu$ M BNF (Lake *et al.* 1993). Therefore, our observation of CYP1A1 protein induction in sperm whale skin biopsies occurred at concentrations comparable to those known to produce induction, and to show a concentration-dependent effect in rat liver slices. For endothelial cells, smooth muscle cells and fibroblasts, the 0.6  $\mu$ M BNF treatment group resulted in the lowest observed effect level in our study but this could be an overestimation since lesser concentrations were not tested.

Endothelial cells are in immediate contact with blood-borne xenobiotics and may play an important toxicological role in their transfer and metabolism. Both *in vitro* and *in vivo* studies have shown CYP1A1 to be catalytically active and inducible in endothelium of terrestrial mammals (Hennig *et al.* 2002; Stegeman *et al.* 1995). Our results confirm that CYP1A1 is also inducible in cetacean endothelium. Chlorinated dioxins and coplanar PCBs can generate oxidative stress and an inflammatory response in mammalian endothelial cells after being activated by CYP1A1 *in vitro* (Hennig *et al.* 2002). Organochlorines that are not rapidly metabolized by CYP1A1 also may produce oxidative stress or radical-induced damage, resulting from uncoupling of CYP1A1 (Schlezinger *et al.* 1999). While contaminant-induced toxic effects in endothelial cells are still to be characterized in cetaceans, our findings underline the

importance of examining the endothelial tissue when assessing exposure to and potential effects of environmental contamination in these animals.

CYP1A1 mRNA expression and inducibility have been reported in smooth muscle cells of laboratory animals and humans (Kerzee and Ramos 2001; Zhao *et al.* 1998). However, some studies suggest the existence of a labile repressor preventing a basal transcriptional activation in vascular smooth muscle cells of adult rodents (Giachelli *et al.* 1991; Kerzee and Ramos 2001). The sperm whales sampled for our study are of unknown age but likely included immature and mature animals. While our results demonstrate CYP1A1 protein was inducible by BNF in cetacean smooth muscle cells, CYP1A1 basal expression could not be assessed since environmental exposure in our untreated samples cannot be ruled out. In fibroblasts, we did not detect a statistically significant concentration-effect, possibly because of a relatively high variability of response in this cell type, or because the range of BNF concentrations used caused maximum induction or was otherwise inadequate to reveal a concentration effect. In terrestrial mammals, CYP1A1 expression and inducibility in fibroblasts appear to vary widely in both primary cultures and cell lines (Gradin *et al.* 1999; Kim *et al.* 1997). Based on our findings, CYP1A1 is inducible in dermal smooth muscle cells and fibroblasts and the toxicological significance of induction in these two cell types in cetaceans needs to be established.

In human and rodents, skin CYP1A1 is known to play a significant role in xenobiotic metabolism: it is inducible after topical or systemic treatment by BNF and other AHR agonists and activity levels can reach 27% of that of the liver, the major organ of xenobiotic metabolism (Ahmad *et al.* 1996). In aquatic mammals, few studies have examined how changes in skin tissue may relate to overall systemic effects of environmental chemicals. Significant correlations between certain planar PCB blubber burdens and hepatic CYP1A1 content and activity have

been observed in beluga whales (White *et al.* 1994). A recent study on captive river otter (*Lontra canadensis*) chronically fed crude oil demonstrated a dose-dependent induction of dermal endothelial CYP1A1 (Ben-David *et al.* 2001). That study illustrates the validity of using skin tissues for contaminant exposure through the oral route, generally the most important source of exposure in marine mammals. However, there still remains a need for further research investigating and modeling the relationships among contaminant concentrations, toxicokinetics in blubber and whole body, dermal CYP1A1 expression and biological effects in marine mammals.

This report provides the first direct demonstration that an AHR agonist can induce CYP1A1 in cetacean tissue in a concentration-dependent manner. In the case of endangered or protected species, studies using incubation or culture of skin biopsies may indeed be the sole avenue for investigating responses to contaminant exposure in live tissues. The protocol presented here could be adapted to investigate experimental exposure to specific chemicals or chemical mixtures, at selected concentrations and incubation times and with specific biological endpoints of interest. It also could be modified in order to preserve exposed tissue sections for enzyme activity or mRNA analyses. In future studies, the use of precision-cut slices would ensure standardization of slice dimensions and enhance viability in culture (by creating thinner slices) and therefore could provide opportunities for detailed metabolic studies.

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## FIGURE LEGENDS

**Fig. 1.** Specificity of the staining for CYP1A1. Serial sections of sperm whale PM99-371 skin biopsy slice treated with 600  $\mu$ M BNF for 24 hr were stained with specific (MAb 1-12-3) or non-specific (MOPC31) antibodies. Magnification is 600X. Panel A shows blood vessels (v) after staining with non specific MOPC31 antibody and panel B shows same vessels after CYP1A1 specific staining (staining is red).

**Fig. 2.** CYP1A1 expression in untreated and BNF-treated sperm whale biopsy slices. Biopsy slices shown are from three whales: animal PM99-346 (DMSO group), animal PM99-336 (0.6  $\mu$ M BNF group) and animal PM99-352 (600  $\mu$ M BNF group). Biopsy sections were immunohistochemically stained with MAb 1-12-3. CYP1A1-specific staining is red. Magnification is 600X. Symbols are: v for blood vessel, c for capillary, r for red blood cell, and f for fibroblast.

**Fig. 3.** CYP1A1 is inducible in multiple cell types of cetacean skin. Levels of CYP1A1 expression due to BNF treatment are given as treatment-specific staining scores. Treatment-specific staining scores were determined for each animal as the difference between the staining scores of the untreated and treated biopsy sections. N = 10 animals for each treatment group. Within each panel, scores with different letters are statistically different at  $\alpha = 0.05$ . Panel A: endothelial cells. Panel B: smooth muscle cells. Panel C: fibroblasts.

**TABLE 1.**

Cytochrome P450 1A1 expression in sperm whale endothelial cells, smooth muscle cells and fibroblasts as determined by immunohistochemistry in untreated and  $\beta$ -naphthoflavone-treated skin biopsy slices.

[BNF] ( $\mu$ M)	Endothelial cells staining scores			Smooth muscle cells staining scores			Fibroblasts staining scores		
	Untreated slices	Treated slices	Treatment specific	Untreated slices	Treated slices	Treatment specific	Untreated Slices <sup>a</sup>	Treated slices	Treatment specific <sup>a</sup>
0	3.8 (1.8)	3.9 (1.8)	0.1 (2.3)	3.5 (1.5)	3.8 (2.1)	0.3 (2.1)	3.0 (1.6)	3.0 (1.4)	-0.2 (2.0)
0.6	2.2 (1.0)	10.3 (2.5)	8.2 (2.8)	2.1 (0.8)	8.2 (1.8)	6.2 (2)	2.0 (1.1)	8.0 (4.2)	6.1 (4.1)
6	2.1 (1.3)	11.8 (2.1)	9.7 (1.8)	2.3 (1.2)	9.4 (1.4)	7.2 (1)	2.8 (1.9)	9.9 (4.4)	7.1 (4.2)
60	1.9 (0.8)	13.1 (2.5)	11.2 (2.8)	1.6 (0.8)	10.9 (2.9)	9.4 (3.2)	2.2 (1.3)	11.1 (3.8)	8.9 (3.6)
600	2.7 (1.2)	14.0 (2.4)	11.3 (3.1)	2.3 (1.2)	12.7 (1.7)	10.5 (1.9)	4.7 (2.7)	12.0 (3.4)	7.4 (3.9)

Note. For each animal, the treatment specific CYP1A1 expression was calculated as the difference between treated and untreated staining scores. All values correspond to average CYP1A1 staining scores (standard deviation) within each treatment group.

<sup>a</sup>N=10 in each group except in untreated and treatment specific fibroblasts scores corresponding to the 0  $\mu$ M BNF (DMSO) group (N=9).

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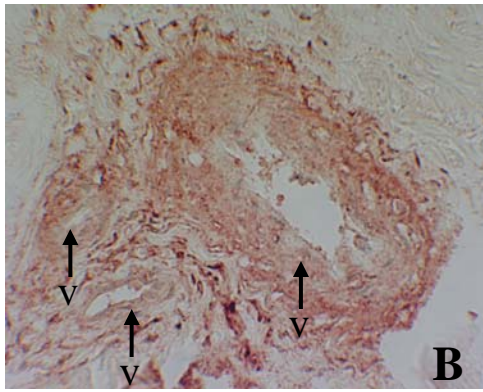
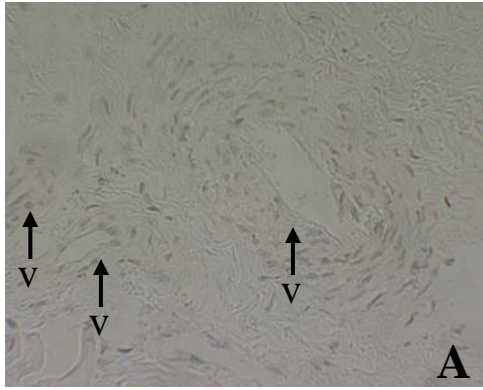
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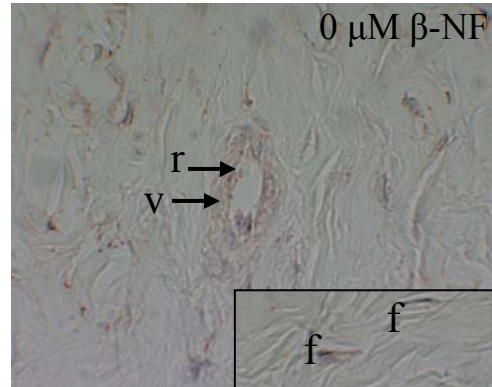
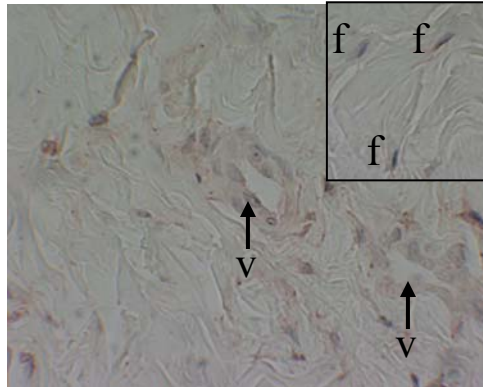
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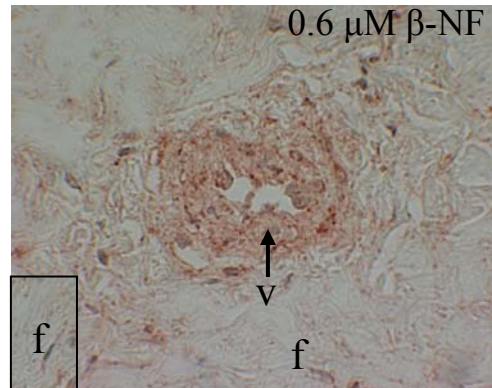
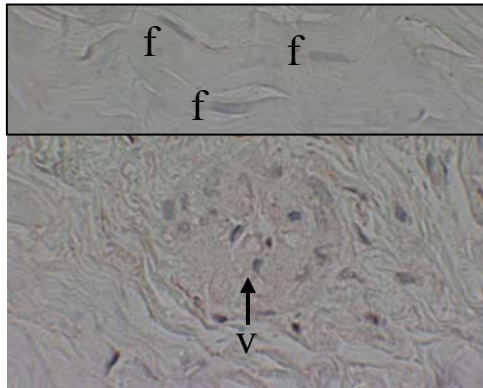
# Untreated

# Treated

PM 99-346



PM 99-336



PM 99-352

