Cellular localization of CYP3A proteins in various tissues from pilot whale (*Globicephala melas*)

Malin C. Celander [a,b,*], Michael J. Moore [b], John J. Stegeman [b]

[a] *Göteborg University, Department of Zoology, Zoophysiology, Box 463, SE-405 30 Göteborg, Sweden*
[b] *Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA*

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Abstract

The in situ expression of cytochrome P450 3A- (CYP3A) like proteins in hepatic and extrahepatic tissues from a marine mammal, pilot whale (*Globicephala melas*), was investigated. Polyclonal antibodies (PAb) raised against either rat CYP3A1 or trout CYP3A27 both recognized a microsomal protein band in liver, lung, kidney and heart. The protein band observed in liver and lung had slightly lower molecular weight than that observed in kidney and heart, suggesting the existence of two CYP3A forms in pilot whale. Immunohistochemical analyses showed strong CYP3A-staining in hepatocytes, bile duct epithelial cells, bronchial epithelial cells, in primordial- and primary follicles and their surrounding zona glomerulosa. Moderate to strong CYP3A staining was seen in smooth muscle-like cells of large arteries and arterioles in all organs examined. Mild to moderate staining was evident in alveolar epithelial cells and in kidney tubular epithelial cells. Weak staining was seen in glomerular epithelial cells and in seminiferous tubular epithelial cells. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cytochrome P450 3A (CYP3A) is the dominant CYP subfamily in the liver and the gastrointestinal tract of human and rat (De Waziers et al., 1990; Kolars et al., 1992). CYP3A enzymes are believed to be important in steroid hormone homeostasis, being the major steroid 6β-hydroxylase catalysts (Waxman et al., 1988). CYP3A enzymes also metabolize a wide variety of structurally diverse non-steroidal molecules that are natural products, including therapeutic drugs, food-additives, pro-carcinogens, as well as anthropogenic substances such as pesticides and organic pollutants (reviewed by Maurel, 1996). Thus, the CYP3A subfamily also takes part in the biochemical defense against foreign chemicals. Drug–drug interactions as a result of CYP3A metabolism also have been reported (Pichard et al., 1992; Halpert et al., 1994; Kostrubsky et al., 1995, 1997). Many of the substrates to the CYP3A

enzymes induce the expression of *CYP3A* genes (reviewed by Maurel, 1996). Expression of *CYP3A* genes can be induced in response to treatment with steroids and numerous therapeutic drugs. Recently identified nuclear orphan receptors have been suggested to regulate CYP3A gene expression in humans (Bertilsson et al., 1998; Lehmann et al., 1998; Pascussi et al., 1999).

Knowledge of the CYP3A subfamily derives predominantly from studies performed in humans and traditional laboratory animals and cell cultures from these organisms. Relatively little is known about CYP3A in wildlife species. Proteins functionally and immunochemically related to mammalian CYP3A have been isolated from fish, and distinct similarities seem to exist between the mammalian and piscine CYP3A immunoreactive proteins (Celander et al., 1989; Miranda et al., 1991; Husøy et al., 1994; Celander et al., 1996). Cloning of piscine *CYP3A* genes and phylogenetic analysis of *CYP3A* subfamily further indicates that these genes have been conserved during vertebrate evolution (Celander and Stegeman, 1997; Lee et al., 1998).

Marine mammals live and reproduce in areas where they are continuously exposed to CYP substrates/in-

* Corresponding author. Tel.: +46-31-7733693; fax: +46-31-7733807.

E-mail address: malin.celander@zool.gu.se (M.C. Celander).

hibitors, such as natural products and athropogenic compounds, as a result of food preferences and human activities. There have been detailed studies of the polycyclic aromatic hydrocarbon and planar halogenated aromatic hydrocarbon inducible CYP1A in beluga whale (*Delphinapterus leucas*; White et al., 1994) and in minke whale (*Balaenoptera acutorostrata*; Goksøyr et al., 1988). Information concerning other CYP proteins in cetaceans is scant, although CYP2 immunoreactive proteins could be indicated in beluga whales (White et al., 1994). The existence of CYP3A-like proteins in cetaceans also is suggested by the occurrence of steroid 6β-hydroxylase activity in liver microsomes from minke whale (Goksøyr et al., 1988), and by immunoblot analysis using monoclonal antibodies to rabbit CYP3A6 and polyclonal antibodies to trout CYP3A27 (Goksøyr et al., 1989). From an evolutionary point of view, the CYP system in marine mammals is intriguing. The *CYP3* gene family is believed to have appeared between 800 and 1100 million years ago (Nebert and Gonzalez, 1987; Nelson and Strobel, 1987; Nebert et al., 1989; Gonzalez, 1990). Cetaceans diverged from the terrestrial ungulate line some 65 million years ago, and thus their *CYP3* genes could have evolved in separate directions than that in terrestrial mammals.

A result of a wide substrate specificity, CYP3A enzymes have significant impact on physiology, pharmacology as well as toxicology. To better understand functions and possible endogenous roles of these CYP enzymes, it is necessary to identify the various organs and cell types that express these genes. Furthermore, CYP3A enzymes activate several pro-carcinogens and thus are believed to take part in chemical carcinogenesis (Guengerich and Schimada, 1991; Gonzalez and Gelboin, 1994; Parkinson, 1996). Information about tissue specific expression of CYP3A could identify cell types that conceivably are targets for in situ bioactivation of carcinogens through CYP3A metabolism.

The present study was undertaken to investigate the presence and cellular localization of CYP3A immunoreactive proteins in a marine mammal; the pilot whale (*Globicephala melas*). We here present evidence of the presence of CYP3A immunoreactive proteins in liver, lung, kidney, gonads and in the cardiovasculature in adult pilot whales.

2. Materials and methods

2.1. Chemicals

Polyclonal antibodies (PAb) against rat CYP3A1 was a gift from Dr David J. Waxman, Boston University. PAb raised against a CYP3A-like protein (presumably CYP3A27) isolated from rainbow trout (*Oncorhynchus mykiss*) livers are described elsewhere (Celander et al.,

1989, 1996). Whole rabbit serum was purchased from Cappel Research Products (Durham, NC, USA). Crystal/Mount was from Biømeda (Foster City, CA, USA). The Universal Immunoperoxidase Staining Kit (Rabbit) and the aminoethylcarbazole (AEC) chromogen system for immunohistochemistry were obtained from Signet Laboratories (Dedham, MA, USA). Nylon sheets, blocking powder and alkaline phosphatase-conjugated goat anti-rabbit IgG for immunoblot analyses were purchased from Schleicher and Schuell (Keene, NH, USA). The CSPD chemiluminescent alkaline phosphatase substrate was obtained from Tropix (Bedford, MA, USA). All other chemicals were of the highest purity available in the US from BioRad, Curtin Matheson Inc., EM Science, ICN Biochemicals, and Sigma.

2.2. Animals and dissection

Tissues were obtained from pilot whales stranded on Cape Cod, MA during November 1990 and December 1991. The animals sampled were unable to be rescued and were euthanazed by authorized personnel. Pieces of tissue from liver, lung, heart and kidney from two sexually immature females and from two sexually immature males stranded in 1990 were dissected at the stranding site, within 1 h of death (for details see Moore and Stegeman, 1996) and immediately frozen in liquid nitrogen for preparation of the microsomal fractions. Liver tissues from the whales stranded in 1991 were removed within 1 h after death and frozen in liquid nitrogen. Slices of liver, lung, kidney, heart, adrenal gland, spleen and gonads from animals stranded in 1990 were fixed in 10% neutral buffered formalin (pH 7.4). The tissues were embedded in paraffin using standard methods (Luna, 1968). Microsomes from lung, kidney and heart from animals stranded in 1990 and from livers from animals stranded in 1991 were prepared according to the protocol described by White et al. (1994).

2.3. Immunoblot analysis

Microsomes from liver, lung, heart and kidney were solubilized by adding sample treatment buffer (0.25 M Tris:HCl, pH 6.8, 40% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 4% (w/v) sodium dodecyl sulfate (SDS), 0.008% (w/v) bromphenol blue) to a final concentration of 4 µg microsomal protein/µl. The solubilized samples were boiled for 5 min and then immediately placed on ice. Solubilized microsomal protein (20 or 40 µg) was resolved on 12% (w/v) continuous acrylamide using a Mighty Small Gel Electrophoresis Unit from Hoefer Scientific Instruments. Purified CYP3A (1 pmol) from rainbow trout liver was used as a positive control. This CYP3A-like protein from trout is immunochemically

related to mammalian CYP3A proteins (Celander et al., 1996), and it is probably identical to the gene product of trout CYP3A27 (Lee et al., 1998). It is denoted CYP3A27 throughout this paper. SDS-polyacrylamide gel electrophoresis and immunoblotting onto nylon membranes were performed using standard procedures. Remaining protein binding sites on the nylon membranes were blocked with 1% (w/v) Schleicher and Schuell blocking powder in 10 mM Tris:HCl, pH 7.4, including 0.9% (w/v) NaCl (blocking solution). PAb raised against rat CYP3A1 (diluted 1:3000 in blocking solution) or PAb raised against trout CYP3A27 (diluted 1:5000 in blocking solution) were used as primary antibodies. Alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:10 000 in blocking solution) was used as secondary antibody. Protein bands were detected using the chemiluminescent substrate CSPD, according to the protocol provided by the manufacturer. The protein bands were visualized by fluorography on Kodak X-OMAT AR films. Fluorograms were photographed with a Kodak DCS 200 digital camera, collated using the Adobe Photoshop software version 3.0.4 and printed on a Kodak XL7700 dye sublimation printer. Images were not digitally enhanced beyond contrast adjustment.
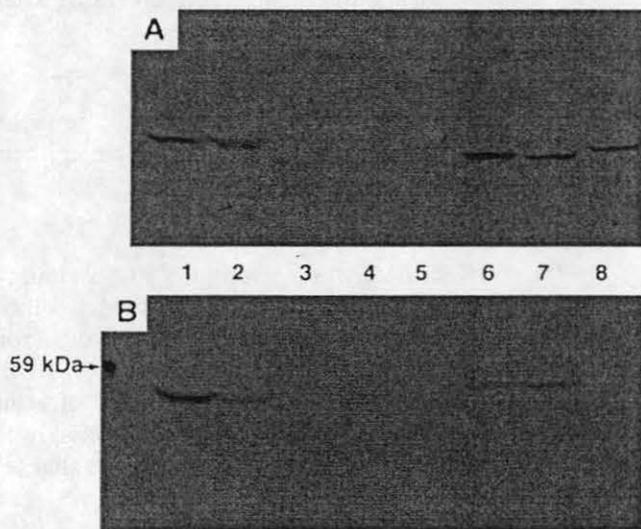

Fig. 1. Western blot analyses of microsomal fractions of various tissues from pilot whale using (A) polyclonal antibodies (PAb) against rat CYP3A1 (20 µg microsomal protein/lane) and (B) PAb against rainbow trout CYP3A27 (40 µg microsomal protein/lane). Purified trout CYP3A27 protein (1 pmol/lane; Mr 59 kDa) was included for comparison in panel B. Lanes 1–2: liver microsomes; lanes 3–5: heart microsomes; lanes 6–7: lung microsomes and lane 8: kidney microsomes. The immunoreactive proteins in heart microsomes in blot A and B and that of kidney microsomes, with 40 µg microsomal protein, in blot B was visible only on the fluorograms and not on the scanned image.

2.4. Immunohistochemistry

Serial 5-µm sections of liver, lung, heart, kidney, adrenal, spleen, ovary and testis were mounted on super frost(+) slides and were rehydrated and stained for CYP3A, basically according to the procedure described for CYP1A (Smolowitz et al., 1991). PAb raised in rabbits against rat CYP3A1 or trout CYP3A27, each diluted 1:500 in 10 mM phosphate buffer saline (PBS; pH 7.4) containing 1% (w/v) bovine serum albumin (BSA), were used as primary antibodies. Whole serum from non-immunized rabbits, diluted 1:500 in PBS/BSA, was used as a negative control. Positive reaction was detected using the Universal Immunoperoxidase Staining Kit from Signet Laboratories and was visualized using the AEC chromogen.

2.5. Photomicrography

Microscopic images were made on 35-mm color slides (Kodak EPY 64), scanned in monochrome using a Kodak 2500 slide scanner, collated using the Adope Photoshop software version 4.0 and printed on a Kodak XL7700 dye sublimation printer. Images were not digitally enhanced beyond contrast adjustment.

3. Results

3.1. Immunoblot analysis of CYP3A

Immunoblot analysis of microsomes from liver, lung, heart and kidney is shown in Fig. 1. Polyclonal antibodies (PAb) raised against rat CYP3A1 strongly recognized a single protein band within the P450 molecular size range (46–60 kDa) in microsomes from liver, lung, heart and kidney that were run on the same gel (Fig. 1(A)). Immunoblot analysis revealed strong expression of CYP3A-like proteins in liver, moderate to strong expression in lung and kidney and weak expression of CYP3A-like proteins in heart. However, the protein band in heart microsomes was visible only on the original fluorogram and not on the scanned image. An identical pattern was observed when blots were probed with PAb raised against rainbow trout CYP3A27 (Fig. 1(B)). Interestingly, although only one immunoreactive protein band was visible in each lane, there was a difference in apparent molecular weight between the band seen in liver or lung, compared to that observed in heart or kidney (Fig. 1).

3.2. Cellular localization of CYP3A

Immunohistochemical (IHC) analyses were carried out using either PAb to rat CYP3A1 or trout CYP3A27. Results with various tissue sections, showing

Table 1
Cellular localization of CYP3A proteins in various tissues from pilot whale

Organ	Cell type	CYP3A-staining
Liver	Hepatocytes	Strong
	Bile duct epithelial cells	Strong
	Vascular smooth muscle-like cells	Moderate
	Vascular endothelium	None
Adrenal	Adrenal cortex, medulla and capsule cells	None
	Vascular smooth muscle-like cells	Strong
	Vascular endothelium	None
Kidney	Tubular epithelial cells	Mild to moderate
	Glomerular epithelial cells	Weak
	Vascular smooth muscle-like cells	Strong
	Vascular endothelium	None
Lung	Bronchial epithelial cells	Strong
	Alveolar epithelial cells	Mild
	Vascular smooth muscle-like cells	Moderate to strong
	Vascular endothelium	None
Ovary	Primordial follicles	Strong
	Primary follicles	Strong
	Zona glomerulosa	Strong
	Vascular smooth muscle-like cells	Moderate to strong
	Vascular endothelium	None
Testis	Seminiferous epithelial cells	Weak
	Vascular smooth muscle-like cells	Moderate to strong
	Vascular endothelium	None
Heart	Cardiomyocytes	Weak
	Vascular smooth muscle-like cells	Strong
	Vascular endothelium	None
Spleen	Hematopoietic cells	None
	Macrophages	None
	Vascular smooth muscle-like cells	Moderate
	Vascular endothelium	None

the staining obtained using PAb against trout CYP3A27, are summarized in Table 1 and illustrated for some tissues in Figs. 2–4. In all tissues, similar results were observed using either PAb to rat CYP3A1 or trout CYP3A27. However, the PAbs differed in obtaining a positive signal in vascular smooth muscle-like cells. Thus, only the PAb raised against trout CYP3A27 recognized immunoreactive proteins in smooth muscle-like cells of arteries and arterioles, whereas no staining was seen in these cells using PAb against rat CYP3A1. Apart from this difference in vascular smooth muscle-like cells, using either of these antibodies resulted in identical patterns of cellular localization of CYP3A immunoreactive proteins in all other cell types investigated. None of the tissues examined showed any staining with whole serum from non-

immunized rabbits; a representative image of a negative control slide is provided in Fig. 2(B).

3.2.1. Cardiovasculature
Moderate to strong CYP3A staining was observed in smooth muscle-like cells of arteries and arterioles in all organs examined using PAb against trout CYP3A27. No CYP3A staining was observed in the vascular endothelium in any organ examined (Table 1).

3.2.2. Liver
Strong CYP3A staining was observed in hepatocytes. Strong CYP3A staining also was seen in arteriolar smooth muscle cells and in bile duct epithelial cells (Fig. 2(A)). Moderate staining was observed in smooth muscle-like cells of large arteries. No CYP3A staining was detected in the vascular endothelium in the liver (Fig. 2(C)).

3.2.3. Adrenal
There was no CYP3A staining in adrenal cortex, medulla and capsule cells. Strong CYP3A staining was observed in the smooth muscle-like cells of arterioles (Fig. 3(A)).

3.2.4. Kidney
Mild to moderate CYP3A staining was observed in epithelial cells in kidney tubules, whereas staining in the glomerular epithelial cells was weak. Strong CYP3A staining was seen in smooth muscle-like cells of arterioles in the kidneys (Fig. 3(B)).

3.2.5. Lung
Strong CYP3A staining was observed in bronchial epithelial cells and mild staining in alveolar epithelial cells (Fig. 3(C)).

3.2.6. Gonads
In the ovaries, there was a strong CYP3A staining in the ovarian primordial follicles and in primary follicles and the single layer of squamous follicular cells (zona glomerulosa) surrounding the oogonium. Strong staining also was seen in smooth muscle-like cells of arterioles in the ovaries (Fig. 4(A)). In the testis, weak staining was observed in the seminiferous epithelial cells (Fig. 4(B)).

3.2.7. Heart
Weak CYP3A staining was seen in cardiomyocytes. Strong staining was seen in smooth muscle-like cells of arterioles in the heart (Table 1).

3.2.8. Spleen
There was no CYP3A staining in hematopoietic cells nor in macrophages. Moderate CYP3A staining was observed in smooth muscle-like cells of arterioles (Table 1).

There were slight differences in the intensity of CYP3A staining in primordial follicles and primary follicles and their surrounding zona glomerulosa between the two females examined and in that of the seminiferous tubule cells between the two males examined. Apart from these results in gonads, there were no individual differences in CYP3A staining intensity and cellular localization in the other organs examined.

4. Discussion

Marine mammals play important roles in the ecosystem. Information about CYP gene families other than CYP1A in this vertebrate group is limited. Conceivable, these animals also are exposed to CYP3A inducers/substrates of natural or anthropogenic origin. In this study we describe the expression of CYP3A in situ in hepatic and extrahepatic tissues from adult pilot whales and we also provide new insights concerning the cellular localization of CYP3A proteins generally.

The presence of CYP3A genes in whales has been shown by recent cloning of partial CYP3A cDNA sequences from two cetacean species, Dall's porpoise (*Phocoenoides dalli*) and minke whale (Shoichi Fujita,

personal communication). In this study, the presence of CYP3A-like proteins in pilot whale was indicated by immunoblot analysis, using PAb raised against either rat CYP3A1 or rainbow trout CYP3A27, both of which recognized single protein bands, within the CYP molecular weight range, in microsomes from four different organs from pilot whale. The apparent molecular size of the band in liver and lung differed from that in heart and kidney, which implies that there could be at least two CYP3A isoforms one expressed in liver/lung and another expressed in heart/kidney in pilot whale. However, sequence data are required to unequivocally determine whether these two proteins are products of two different *CYP3A* genes.

Organ distribution of various xenobiotic metabolizing enzymes using immunoblotting was described earlier in human and rat. Typically, there is strong expression of CYP3A in the liver, weak to moderate expression of CYP3A in the gastrointestinal tract and detectable, albeit not quantifiable, expression of CYP3A in the kidneys (De Waziers et al., 1990). CYP3A activities and mRNA expression were described in another study in rat kidneys (Ghosh et al., 1995). In pilot whale, IHC analysis revealed strong expression of CYP3A in liver and weak to moderate
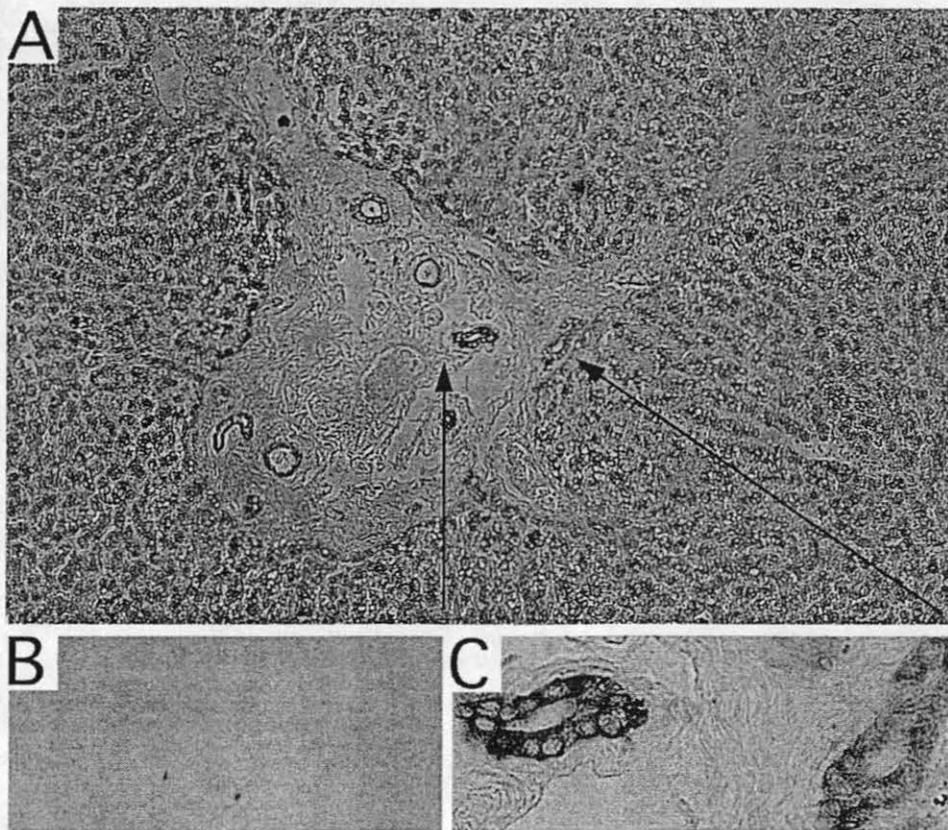

Fig. 2. Immunohistochemical staining of CYP3A in liver from pilot whale. (A) Liver (100 ×) probed with PAb to trout CYP3A27. (B) Liver (200 ×) probed with whole serum from non-immunized rabbits. (C) Liver (400 ×) probed with PAb to trout CYP3A27. Arrows indicate strong positive staining in bile duct epithelial cell and mild staining in smooth muscle-like cells.
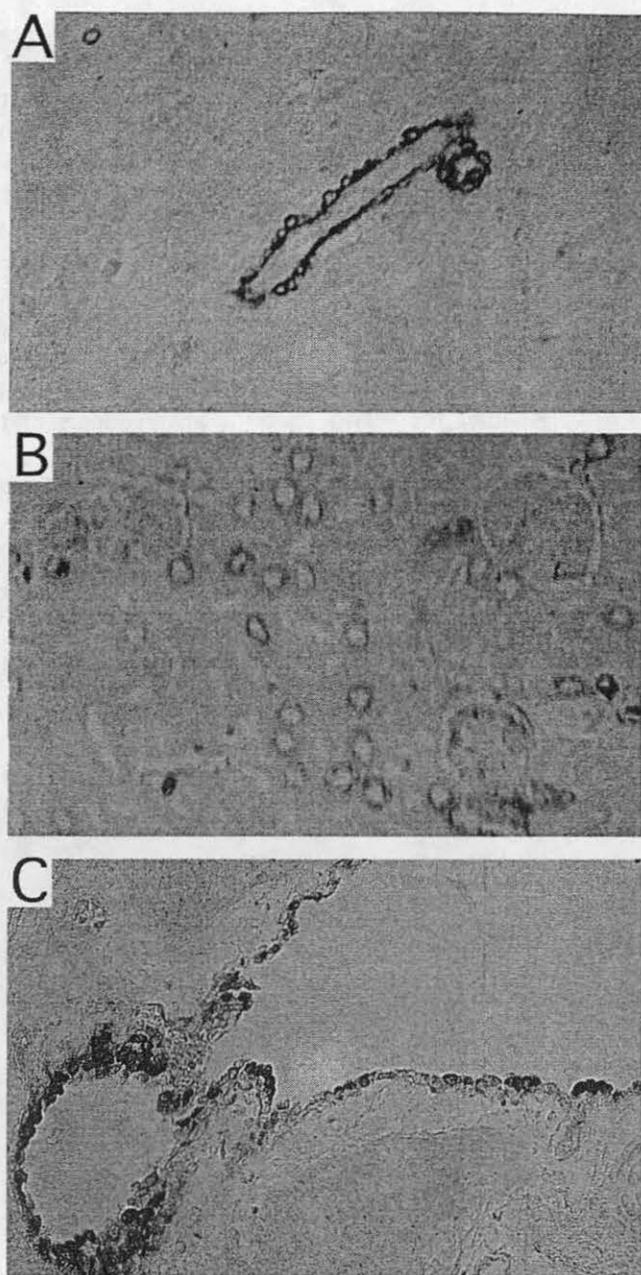
Fig. 3. Immunohistochemical staining of CYP3A in adrenal, kidney and lung from pilot whale. (A) Adrenal (400 ×) probed with PAb to trout CYP3A27. Strong staining was evident in smooth muscle-like cells. (B) Kidney (400 ×) probed with PAb to trout CYP3A. Mild to moderate positive staining was evident in tubular epithelial cells and mild staining was evident in glomerular epithelial cells. (C) Lung (400 ×) probed with PAb to trout CYP3A27. Strong staining was seen in bronchial epithelial cells whereas mild staining was evident in alveolar epithelial cells.

expression of CYP3A in kidney epithelial cells. Furthermore, moderate to strong staining also was observed in vascular smooth muscle-like cells in both liver and kidneys from these animals. Previously, IHC analysis was used to demonstrate cellular localization of CYP3A proteins in the digestive tract of rats, showing

strong expression of CYP3A in mature enterocytes and epithelial cells throughout the gastrointestinal tract (Kolars et al., 1992). Unfortunately, no tissue samples from the gastrointestinal tract of pilot whale were available, and consequently comparisons of the gastrointestinal tract to other organs in this species could not be made in this study.

In addition to the digestive tract, the presence of CYP3A protein and/or mRNA recently was shown in human lung (Anttila et al., 1997). In pilot whale lung, prominent CYP3A staining was seen in pulmonary epithelial cells, implying a role of these enzymes in metabolizing airborne compounds. Overall, CYP3A expression is significant in tissues such as liver, kidney, lung or gills and the gastrointestinal tract of various vertebrate species (De Waziers et al., 1990; Husøy et al., 1994; Ghosh et al., 1995; Anttila et al., 1997). Thus, CYP3A is expressed in tissues that are important sites of exposure, as well as sites of excretion of lipophilic compounds. CYP3A enzymes metabolize a wide variety of compounds, many of which are natural products or anthropogenic chemicals (Schuetz et al., 1984; Waxman et al., 1988; Berthou et al., 1994; Ronis et al., 1994; Gautier et al., 1996; Kostrubsky et al., 1997). Thus, it seems likely that one role of the CYP3A enzymes is to metabolize inhaled and ingested foreign lipophilic compounds.

In addition to high levels of CYP3A in liver, lung and kidney of pilot whale, we also found high levels of CYP3A expression in the gonads of these animals. To our knowledge, this is the first report of CYP3A expression in mammalian reproductive organs. In rainbow trout, Northern blot analysis revealed expression of *CYP3A27* in the gonads, with considerably higher CYP3A27 mRNA levels in the ovary than that in the testis (Lee et al., 1998). In pilot whale, strong CYP3A staining was seen in the primordial follicles and the primary follicles and their surrounding zona glomerulosa in the ovaries. Weak CYP3A staining was observed in the seminiferous tubule cells of the testes. The roles of CYP3A in the gonads are not known. However, CYP3A enzymes metabolize steroids and thus could be important in providing further regulation by fine tuning levels of steroid hormones in situ in these steroidogenic organs.

We also observed strong CYP3A expression in arterioles and moderate staining in large arteries in all organs of pilot whale examined using PAb against trout CYP3A27. This staining was restricted to what is probably smooth muscle cells and are tentatively named smooth muscle-like cells in this paper. However, whether these cells are smooth muscle cells or some other cell type still remains to be verified. To our knowledge this would be the first report on CYP3A expression in smooth muscle cells, although recently CYP3A-mediated bioactivation of nitroglycerin was

demonstrated in rat aorta microsomes (Yuan et al., 1997), supporting the expression of *CYP3A* genes in blood vessels. In contrast to smooth muscle-like cells in pilot whale, no CYP3A staining was seen in the endothelial cell layer, a cell layer that has been shown to express CYP1A at high levels upon exposure to CYP1A inducers (Dees et al., 1982; Stegeman et al., 1989). Lack of expression of CYP3A-like protein in the intact vascular endothelium is consistent with our finding of a lack of CYP3A immunoreactive proteins in cultured

porcine aorta endothelial cells, even when treated with CYP3A inducers (Celander et al., 1997). However, by using reverse transcriptase polymerase chain reaction (RT-PCR), constitutive levels of CYP3A have been detected in cultured endothelial cells from human umbilical vein (Farin et al., 1994). In addition to certain blood vessels, we observed a weak CYP3A staining in cardiomyocytes. The function of CYP3A enzymes in the cardiovasculature is not known and at this stage we only can speculate. Renal CYP3A activity (corticos-
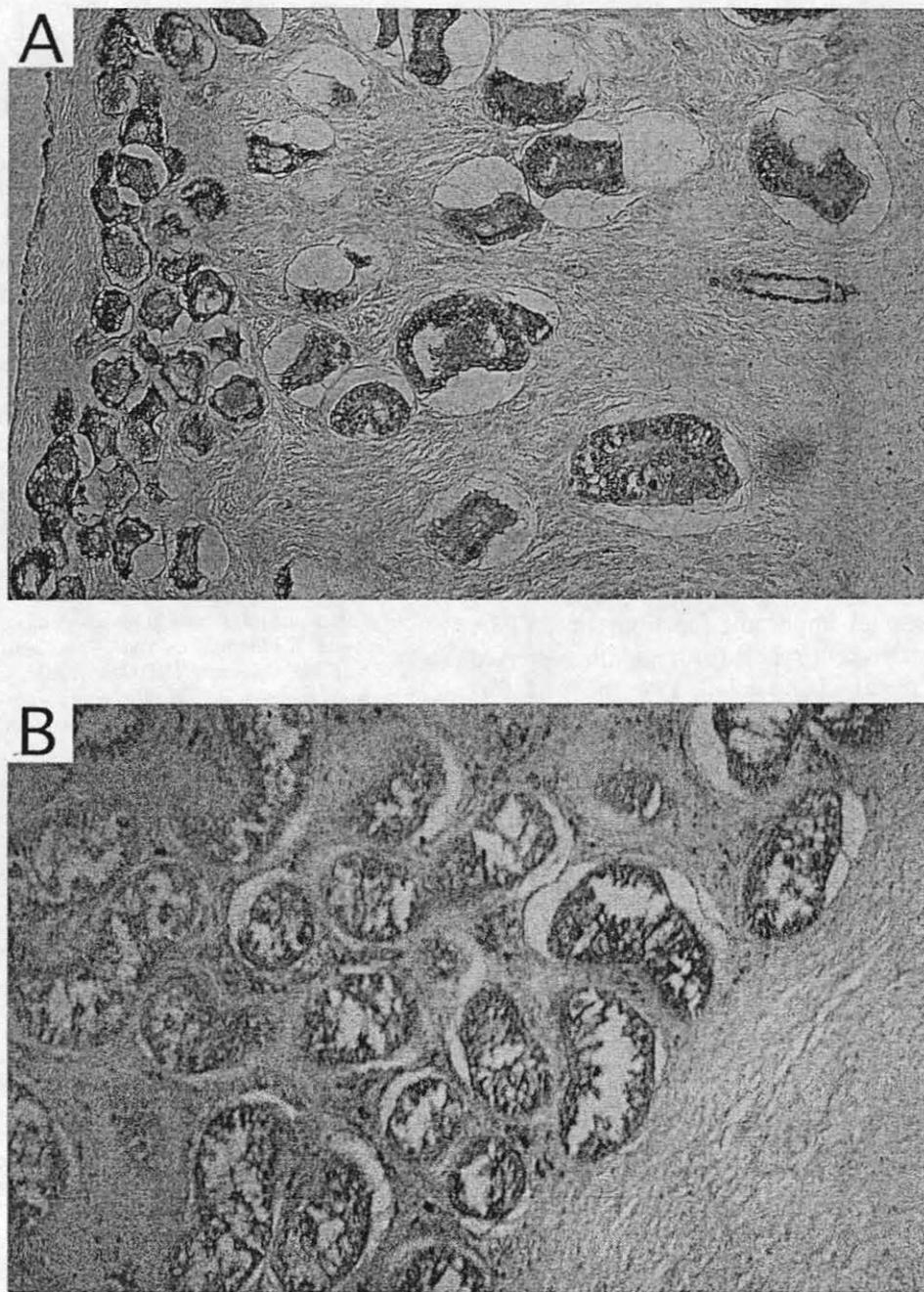

Fig. 4. Immunohistochemical staining of CYP3A in gonads from pilot whale. Ovary and tested probed with PAb to trout CYP3A27. (A) Ovary (200 ×) probed with PAb to trout CYP3A27. Strong staining was evident in primordial follicles, primary follicles and zona glomerulosa. (B) Testis (400 ×) probed with PAb to trout CYP3A27. Mild staining was evident in seminiferous tubule cells.

terone 6β-hydroxylation) was earlier shown to be positively correlated to systolic blood pressure in spontaneously hypertensive rats (Ghosh et al., 1995). Thus, it is possible that CYP3A activity is involved in blood-pressure regulation.

In summary, the present study showed that expression of CYP3A immunoreactive proteins in hepatic and extrahepatic tissues is prominent in pilot whales, implying functional similarities of this CYP subfamily between marine and terrestrial mammals. In this study, we extend the analyses to, in addition to the liver and lung also includes other organs such as gonads, adrenal, spleen and the cardiovasculature. Strong staining for CYP3A immunoreactive proteins in pilot whale was demonstrated in hepatocytes, bile duct epithelial cells in the liver, in bronchial epithelial cells in the lung and in primordial follicles and primary follicles and their surrounding zona glomerulosa in the ovaries. Weak to moderate CYP3A staining was seen in alveolar epithelial cells, kidney tubules and glomerular epithelial cells in the kidney and in seminiferous tubule cells of the testes. Moderate to strong staining in smooth muscle-like cells of large arteries and arterioles in all organs examined. Furthermore, weak staining was observed in cardiomyocytes. The results from the present study show cell specific expression of CYP3A that conceivable could have consequences in regulation of levels of, e.g. hormones, vasoactive compounds, pro-carcinogens and environmental pollutants. The strategic localization of these proteins implies important functions of CYP3A enzymes in certain cell types for regulating steroid hormones and metabolizing inhaled or ingested foreign compounds in situ.

Acknowledgements

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