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Immunohistochemical analysis of the vitellogenin response in the liver of Atlantic salmon exposed to environmental oestrogens

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Induction of vitellogenin (Vtg) in oviparous vertebrates has been used as a biomarker of response for environmental oestrogens. This study reports the cellular localization of oestrogen- and xenoestrogen-induced Vtg synthesis in the liver of juvenile Atlantic salmon (*Salmo salar*). Paraffin-embedded liver sections were incubated with homologous monoclonal antibody against Atlantic salmon Vtg. Following intraperitoneal (ip) exposure of fish to estradiol-17β [E_2; 5 mg kg^{-1}] or 4-nonylphenol [NP; 125 mg kg^{-1}], Vtg induction was primarily demonstrated immunohistochemically in the cytoplasm of hepatocytes, endothelial cells and within hepatic sinusoids. Vtg staining of hepatocytes was not evenly distributed, as there was a high degree of polarization toward the sinusoid. The intensity of positive Vtg staining was stronger in the liver sections of E_2-treated fish, compared with NP-treated fish. Hepatocytes of E_2-, NP- and vehicle (control)-treated fish showed normal cellular structures, thus showing no evidence of histopathological changes. In parallel, indirect enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis of plasma Vtg levels show significant induction of Vtg in E_2- and NP-treated fish, as compared with untreated (control) fish. The present study demonstrates the applicability of immunohistochemistry in studies of cellular structures, processes and responses of fish exposure to oestrogen and oestrogen-mimicking compounds.

Introduction

The increasing awareness of the scale of aquatic environmental problems has focused attention on the urgent need for sensitive and specific diagnostic tools (biomarkers) with predictive capability in the assessment of the impact of endocrine disruptors. In this regard, new cellular and molecular tools are enabling researchers to study the basic mechanisms of the action of natural reproductive hormones, and of endocrine disruptors such as synthetic and anthropogenic oestrogens (xenoestrogens). In addition, these tools also reveal how damaged or mutated genetic systems interact with synthetic and naturally occurring hormones and the role such hormones play in initiating tumorigenesis, causing developmental abnormalities and impairing reproduction (Huggett *et al.* 1992, Peakall 1992). In developing a bioassay for oestrogenic effects, vitellogenin (Vtg) and *zona radiata* proteins (*Zr*p) induction in males and juveniles of oviparous vertebrates have been used as sensitive biomarkers for oestrogenicity (Palmer and Palmer 1995, Sumpter and Jobling 1995, Arukwe *et al.* 1997a).

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Accumulating human and wildlife data indicate that the reproductive system, including its associated endocrine and neural controls, can be very susceptible to alterations by clinical, occupational or environmental exposures to a variety of chemical and physical agents (McLachlan 1981, 1985, Colborn and Clement 1992). Chemical compounds known to mimic the effects of endogenous oestrogen (oestradiol-17β; E_2), in laboratory and field studies includes synthetic steroids such as those used in the contraceptive pill (Pelissero *et al.* 1994), many pesticides (Palmer and Palmer, 1995, Donohoe and Curtis, 1996), phytoestrogens (Pelissero *et al.* 1991a,b), and alkylphenol polyethoxylates (APEs) (Soto *et al.* 1991, Jobling and Sumpter 1993, White *et al.* 1994, Jobling *et al.* 1996, Arukwe *et al.* 1997a,b).

APEs are non-ionic surfactants that have been in use for over 40 years, with over 360 000 tons produced worldwide in 1988 (Talmage 1994). They are widely used as detergents, emulsifiers, wetting and dispersing agents, and also in plastic products for industrial, agricultural and domestic use (Ahel *et al.* 1994, Nimrod and Benson 1996). As a result of their widespread use and water dispersal qualities, discharge to the environment occurs from industrial effluents, public treatment works and septic tanks. The induction of Vtg and *Zr*p by these compounds in oviparous vertebrates has recently received great attention (e.g. Palmer and Palmer 1995, Sumpter and Jobling 1995, Arukwe *et al.* 1997a).

Vitellogenesis is defined as the E_2-induced hepatic synthesis of the egg yolk protein precursor Vtg, its secretion and transport in blood to the ovary and its uptake into maturing oocytes (Mommsen and Walsh 1988). The vitellogenic process is an integral aspect of fish oogenesis. In the ovary, the phospholipoglycoprotein Vtg (250–600 kDa) is incorporated as yolk proteins and serves as a nutrient reserve (Mommsen and Walsh 1988). Changes in hepatic morphology, such as proliferation of the rough endoplasmic reticulum and Golgi apparatus, also accompany E_2 stimulation during vitellogenesis. Xenoestrogens possess the ability to mimic natural oestrogens and therefore initiate precocious or unscheduled vitellogenesis.

The present study was undertaken in order to investigate the potential use of immunohistochemical analysis as a diagnostic test for oestrogen- and xenoestrogen-induced Vtg synthesis in oviparous vertebrates. This approach may be particularly useful for studies of small-sized species where blood plasma samples might be difficult to collect. Immunohistochemical detection of Vtg in the liver of untreated (control), E_2- and NP-treated fish was performed using a specific homologous monoclonal antibody against Atlantic salmon Vtg. In parallel, plasma levels of Vtg were also investigated using ELISA and Western blotting with the same antibody.

Materials and methods

Chemicals and reagents

4-Nonylphenol (NP; 85 % of *p*-isomers) was purchased from Fluka Chemie AG (Buchs, Switzerland). The impurities in 4-nonylphenol consist mainly of phenol (8–13 %), tripropylene (~1 %) and 2,4-dinonylphenol (~1 %). E_2 and *o*-phenylenediamine dihydrochloride (OPD) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Histostain SP Kit for immunochemical/peroxidase staining of tissues and cells was obtained from Zymed Laboratories (San Francisco, CA, USA). Goat anti-rabbit/mouse-horseradish peroxidase (GAR/GAM-HRP) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Microtitre plates (MaxiSorp) were purchased from Nunc (Roskilde, Denmark). Mouse anti-salmon Vtg antibodies (BN-5 and KB-1) were obtained from Biosense Laboratories (Bergen, Norway).

Fish and treatment

Juvenile Atlantic salmon (*Salmo salar,* approximately 1 year old) were purchased from Sævereid smolt producer (Fusa, Norway). The fish belonged to the Norwegian salmon strain (NLA-stamme). They were maintained under natural photoperiod (60° N) and continuously running sea water of 35‰ salinity (parts per thousand, ppt) at 10 (± 0.4) °C at the Industrial Laboratory (ILAB), Bergen High Technology Center (HIB). They were divided into three groups and each group of juvenile salmon received a single intraperitoneal (ip) injection of either E_2 (5 mg kg^{-1}) or NP (125 mg kg^{-1}), or the carrier-vehicle for NP (control). NP was dissolved in 1:1 acetone:Alkamuls (carrier-vehicle: Rhone-Poulenc, Paris), while E_2 was dissolved in soybean oil. Each group of treated fish was kept in a separate 150-l tank. Fish were sacrificed 1 week after exposure and blood was collected from the caudal vein in heparinized precooled syringes and immediately centrifuged (5000 rpm for 5 min). Liver samples were excised and weighed in order to calculate the hepatosomatic index (HSI; liver weight × 100/body weight) and 0.5 g liver was immediately fixed in 10 % neutral buffered formalin for sectioning.

Immunohistochemical analysis

Fixed liver tissues were processed for paraffin embedding according to standard procedures. The immunohistochemical analysis was performed essentially as described by Husøy *et al.* (1994) using Histostain SP Kit for immunochemical/peroxidase staining of tissues and cells (Zymed). Before staining, hydrated 5 μm sections were incubated with 1 % hydrogen peroxide (H_2O_2) in methanol for 10 min to block endogenous peroxidase activity. Sections were then incubated with 10 % non-immune goat serum for 30 min to block non-specific reaction of the secondary antibodies (goat anti-mouse IgG), washed (1 × 10 min) with phosphate-buffered saline with 0.05 % Tween (PBS–Tween; pH 7.4), and incubated overnight at 4 °C with mouse monoclonal antibodies (BN-5 or KB-1) against salmon Vtg (Nilsen *et al.* 1998) diluted 1:500 in PBS. Sections were washed (3 × 5 min) in PBS–Tween, incubated with biotinylated secondary goat anti-mouse IgG (Zymed) for 30 min at room temperature, washed again (3 × 5 min) and incubated with streptavidin peroxidase conjugate (Zymed) for 30 min at room temperature. Colour visualization in sections was performed by 10 min incubation with liquid 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Zymed), with haematoxylin as counterstain. Finally, sections were rinsed, dehydrated and mounted in a synthetic resin. Control stainings were performed by omission of the primary antibody or by incubation with pre-immune sera. Relative intensity and occurrence of Vtg staining were evaluated using light microscopy.

Immunochemical analysis

Indirect non-competitive ELISA analysis of plasma samples was performed essentially as earlier described (Arukwe *et al.* 1997a). In Western blotting, proteins were separated using 4% stacking and 9% separating sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli (1970) before blotting as described by Towbin *et al.* (1979). Vtg was detected in both ELISA and Western blotting after reaction with the homologous monoclonal anti-salmon Vtg antibody [BN-5: diluted 1:1000 (ELISA) and 1:500 (Western blotting)]. Peroxidase-conjugated GAM-HRP (Bio-Rad) diluted 1:3000 and H_2O_2/*o*-phenylenediamine dihydrochloride (OPD; ELISA) or 4-chloro-1-naphthol (Western blotting; HRP colour development reagent, Bio-Rad) were used as detection systems.

Results

Using homologous monoclonal antibodies against Atlantic salmon Vtg, the localization of vitellogenic cells was studied in control, E_2- and NP-treated juvenile salmon using immunohistochemical assay. The comparison between signal and background staining obtained with this technique allowed for unambiguous identification of positive cells.

Validation of Vtg immunohistochemistry was performed by incubating liver sections of E_2-treated salmon with preimmune sera or by omission of the primary antibody (negative controls; figure 1(A)) and by incubating the antibody with sections from untreated fish. No background or non-specific staining was observed with the monoclonal antibody (BN-5). Similar analyses performed with a polyclonal antiserum against salmon Vtg showed some non-specific and artefactual cytoplasmic staining of endothelial cells and hepatocytes of treated (E_2 and NP) and untreated fish (results not shown). Using another monoclonal antibody (KB-1)

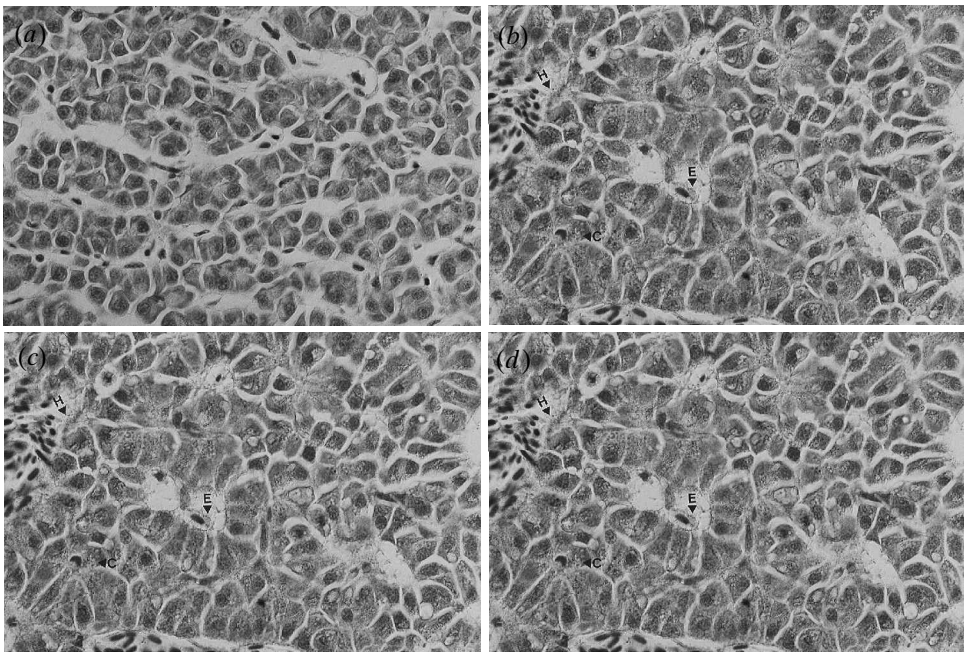

Figure 1. Localization of vitellogenin (Vtg) in liver sections of control (A), oestradiol-17β- (E_2: B) and nonylphenol-treated (NP: C) Atlantic salmon. Vtg was probed with mouse monoclonal antibody (BN-5) against salmon Vtg. D shows liver section of E_2-treated fish incubated by omission of the primary antibody. Strong Vtg-specific staining is demonstrated primarily in the endothelial cells (E), cytoplasm of hepatocytes (C) and hepatic sinusoids (H). Goat anti-mouse-horseradish peroxidase (GAM-HRP) was used as secondary antibody. Hepatocytes show normal cellular structures (x 60).

against salmon Vtg, we were not able to detect specific Vtg stainings in liver sections of E_2- and NP-treated salmon (results not shown).

In this study, no significant differences in the hepatosomatic index (HSI) of E_2- and NP-treated, compared with untreated (control) fish were observed (results not shown). However, hepatocytes of treated and control fish showed evidence of shrinking, probably caused by or during sample processing. Vtg synthesis was consistently observed in liver sections of both E_2- and NP-treated fish 1 week after exposure. Figure 1 shows the immunohistochemical staining of liver sections of control, E_2- and NP-treated fish. Specific stainings, as expected, were seen in the cytoplasm of hepatocytes, endothelial cells and hepatic sinusoids of E_2- and NP-treated fish (figure 1 (B and C, respectively)), while no positive reactions were observed in control fish (figure 1(D)).

Vtg staining intensity in E_2- and NP-treated fish, was stronger in the hepatocytes compared with the endothelial cells and hepatic sinusoids (figure 1(B and C)). Vtg staining in hepatocytes was not evenly distributed. In the cytoplasm, Vtg staining was highly polarized toward the sinusoids (figure 1(B and C)). Correlation between Vtg positive staining in the hepatocytes and endothelial cells surrounding the sinusoid walls was also evident. Non-specific Vtg staining was observed around the pancreas and in the cylindrical epithelial cells surrounding the bile ducts of treated (E_2 and NP) and untreated fish (results not shown). Generally, stronger staining intensity was detected in E_2-treated liver sections, compared with NP-treated liver sections.

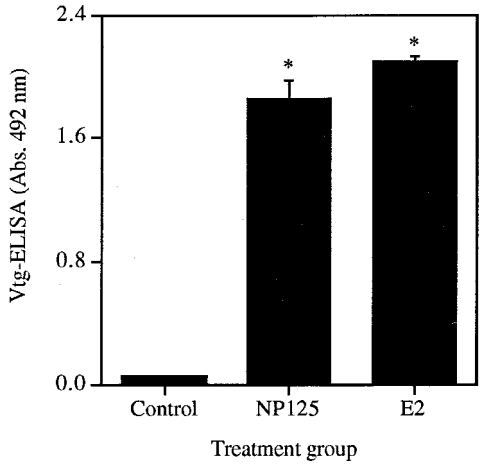

Figure 2. Indirect ELISA analysis of vitellogenin (Vtg) levels in plasma of control, oestradiol-17β- (E_2) and nonylphenol-treated (NP125) juvenile salmon. Vtg was probed with mouse monoclonal antibody (BN-5) against salmon Vtg. Goat anti-mouse horseradish peroxidase (GAM-HRP) was used as secondary antibody. Data are given as mean ELISA absorbance values (492 nm) ± standard error (SE; $n = 5$ per treatment group). Data were analysed statistically on log-transformed data using Dunnett's test with JMP software (version 3.1.6) for Statistical Visualization (SAS Institute, Cary, NC). *$p < 0.0001$.

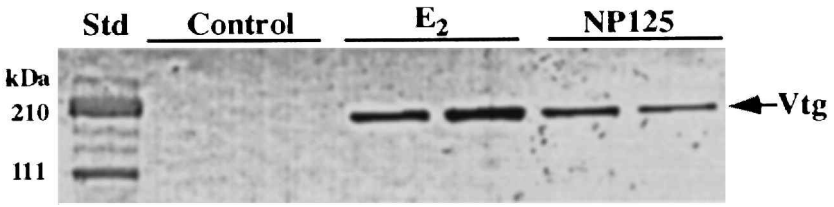

Figure 3. Immunoblot analysis of vitellogenin (Vtg) levels in plasma of control, oestradiol-17β- (E_2) and nonylphenol-treated (NP125) juvenile salmon. Vtg was probed with mouse monoclonal antibody (BN-5) against salmon Vtg. Goat anti-mouse-horseradish peroxidase (GAM-HRP) was used as secondary antibody. Reacting Vtg protein has a molecular weight of 170 kDa.

One week after injection, no Vtg was detected in plasma from untreated fish using either ELISA or immunoblot analyses with monoclonal Vtg antibody (figures 2 and 3, respectively). When compared with the untreated group, fish injected with E_2 or NP synthesized significant ($p < 0.0001$) amounts of Vtg (figure 2). In Figure 3, an immunoreactive Vtg protein (170 kDa) was detected in plasma from the group treated with E_2 and NP using Western blotting with the monoclonal antibody (BN-5). The monoclonal KB-1 antibody showed similar immunochemical reactivity with the Vtg protein in plasma samples of E_2- and NP-treated fish using ELISA and Western blotting (results not shown).

Discussion

In this study, we have investigated the possibility of using immunohistochemistry of liver sections as a method for detection of Vtg induction in fish after exposure to E_2 or oestrogen-mimicking compounds. Immunohistochemical methods may serve as a supplement to established methods such as radio-

immunoassay (RIA), ELISA and Western blotting where induction of Vtg is measured in plasma samples from exposed fish. Proteolytic degradation of Vtg which may be a problem and influence the results obtained from plasma samples may be more easily avoided when tissue sections are immediately fixed for immunohistochemistry. In addition, it is known that E_2 treatment induces ultrastructural changes in the hepatocytes of immature fish. These changes, which are similar to those found in vitellogenic female fish, include increased HSI (van Bohemen *et al.* 1982), proliferation of rough endoplasmic reticulum and Golgi apparatus, and increases in glycogen and lipid levels, all indicative of enhanced metabolic activity by the liver (Olivereau and Olivereau 1979, Ng *et al.* 1984). Immunohistochemistry may be a good tool for studying these cellular changes in the liver of fish exposed to oestrogen-mimicking compounds.

The specificity of the monoclonal antibody (BN-5) selected for this study has earlier been evaluated in ELISA and Western blotting (Nilsen *et al.* 1998). The results obtained showed that BN-5 specifically recognized Vtg from Atlantic salmon (and other species, see Nilsen *et al.* 1998) with no significant binding to other plasma proteins.

Immunoperoxidase staining methods are widely used in immunohistochemical staining and have been validated and used extensively in our laboratory for detection of xenobiotic-induced cytochrome P450 cellular levels (Husøy *et al.* 1994, Goksøyr and Husøy 1998). Immunocytochemical localization of Vtg has also been performed in liver and ovary sections of sexually mature English sole (*Pleuronectes vetulus*) (Roubal *et al.* 1997). However, both endogenous peroxidases, catalase and haemoglobin, which are present in variable levels in tissues, may give false positive reactions with chromogens in the immunoperoxidase staining methods. This problem may be avoided by preincubation with hydrogen peroxide, and non-specific background staining (usually due to highly charged collagen) can be eliminated after preincubation with non-immune serum. However, there are no reports in the literature on validated immunohistochemical analysis of Vtg levels in liver sections of xenoestrogen-treated oviparous vertebrates as a biomarker for oestrogen-mimicking compounds.

Our results demonstrate that the monoclonal antibody (BN-5) against salmon Vtg is suitable for immunohistochemical localization of Vtg in the salmon liver. Thus showing the induction of liver Vtg levels after 1 week's exposure to E_2 or the xenoestrogen nonylphenol. As expected, there were some differences in the staining pattern seen after exposure to E_2 compared with nonylphenol, with the former showing higher staining intensity. The pattern of Vtg staining in salmon shows a strong induction in the hepatocytes and endothelial cells, as well as diffuse induction throughout the liver. Localization of Vtg outside the hepatocytes is explained as a result of secreted or blood-borne Vtg rather than its synthesis in the appropriate cell, while polarization of positive Vtg staining toward the sinusoids is probably a result of early transitosis. Positively stained cells did not show any evidence of pathological changes, such as proliferation, vacuolization or hyperplasia. Evaluation of increases in hepatocyte numbers (hypertrophy) was not possible because of the shrinking observed in hepatocytes of both treated and control fish.

The effects of oestrogens and oestrogen-mimicking compounds can be measured at several levels of biological organization, from cellular changes to variations in the ecosystems. In order to give early warning signals, it is important

to investigate cellular changes and several biomarker proteins (Adams *et al.* 1989, McCarthy and Shugart 1990, Peakall 1992). The present study demonstrates the applicability of immunohistochemistry in studies of oestrogen- and xenoestrogen-induced Vtg response, as have been established for other xenobiotic-induced biological responses, such as CYP1A (e.g. Goksøyr and Husøy 1998).

Our immunohistochemical data are consistent with the ELISA and immunoblot results with pronounced E_2- and NP-induced Vtg levels. Generally, these results are consistent with earlier studies that have been performed using blood plasma samples from oviparous vertebrates exposed to oestrogen-mimicking compounds (Jobling and Sumpter 1993, Pelissero *et al.* 1993, White *et al.* 1994, Palmer and Palmer 1995, Donohoe and Curtis 1996, Arukwe *et al.* 1997a). The localization of Vtg in the cytoplasma of hepatocytes is consistent with the model that the liver is the origin of the increases in blood plasma levels of this protein normally seen in E_2- and NP-treated fish (Mommsen and Walsh 1988, Arukwe *et al.* 1997a).

In summary, the present study has demonstrated that immunohistochemistry can be a valuable tool in the studies of oestrogen and oestrogen-mimicking compound induced hepatic synthesis of Vtg in oviparous vertebrates, especially in situations where blood samples are difficult to collect, e.g. when studying small-sized species. Although it is a time-consuming method, localization of Vtg in liver sections may provide insight into responses of different cell types that are important for understanding the role and mechanisms of the oestrogens and oestrogen-mimicking compounds.

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