Paper VI

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Effects of alkylphenols on CYP1A and CYP3A expression in first spawning Atlantic cod (*Gadus morhua*)

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Abstract

Alkylphenols are continuously released into the ocean as a result of offshore oil production. Alkylphenols, including 4-*tert*-butylphenol (C_4), 4n-pentylphenol (C_5), 4n-hexylphenol (C_6), and 4n-heptylphenol (C_7), up to 237 ppb concentrations, have been detected in produced water from oil platforms. Previous studies have shown that alkylphenols induce vitellogenesis in fish. Atlantic cod (*Gadus morhua*) of both sexes were force-fed with various doses ranging between 0.02 and 80 ppm of a mixture of alkylphenols (C_4:C_5:C_6:C_7 ratio 1:1:1:1) or 5 ppm 17β-estradiol. We investigated effects on hepatic CYP1A and CYP3A protein expression in protein blots, using antibodies against scup (*Stenotomus chrysops*) CYP1A1 and rainbow trout (*Oncorhynchus mykiss*) CYP3A. There was a sexually dimorphic expression of CYP1A and CYP3A protein levels, with females expressing higher levels than males. Treatment of male Atlantic cod with 17β-estradiol resulted in increased CYP1A and CYP3A protein levels. Exposure to alkylphenols resulted in a dose-dependent increase of CYP1A and CYP3A protein expression in males, but not in females. However, this increase of CYP1A protein levels was not reflected on the CYP1A-mediated ethoxyresorufin-*O*-deethylase (EROD) activity, implying that alkylphenols inhibited the CYP1A enzyme activity in vivo. In vitro inhibition studies with pooled liver microsomes from Atlantic cod confirmed that the alkylphenols mixture efficiently inhibited the CYP1A activity ($IC_{50} = 10 \mu M$), although the inhibitory effect of each individual alkylphenol varied. The IC_{50} values for each individual alkylphenol on the CYP1A activity were, in a descending order of magnitude: $[C_7 > C_6 > C_5 \gg C_4]$, ranging from 12 to 300 μM with decreased length of the 4-alkyl chain. The effect of alkylphenols on the CYP3A activity in vitro in liver microsomes also was investigated, using the fluorescent 7-benzyloxy-4-[trifluoromethyl]-coumarin (BFC) as a diagnostic CYP3A substrate. The alkylphenol mixture inhibited CYP3A activity with IC_{50} value at 100 μM. The IC_{50} values for each individual alkylphenol on CYP3A activity were, in a descending order of magnitude: $[C_5 > C_6 > C_7 > C_4]$, ranging between 60 and 250 μM. Taken together, our results show that the alkylphenol mixture and 17β-estradiol resulted in elevated hepatic CYP1A and CYP3A expression in male Atlantic cod. The alkylphenol mixture strongly inhibited CYP1A activities, whereas it weakly inhibited CYP3A activity in Atlantic cod liver microsomes in vitro. In addition, 17β-estradiol was a weak inhibitor of CYP3A activity ($IC_{50} = 75 \mu M$) and did not notably inhibit the CYP1A activity in vitro.
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1. Introduction

Alkylphenols are released into the ocean as a result of offshore oil production. In year 2000, approximately 44 tons alkylphenols were released on the Norwegian continental shelf in connection with discharge of produced water from oil platforms. Alkylphenols with a 4-alkylchain length, ranging from C_4 to C_7, have been reported at concentrations from 2 ppb up to 237 ppb in produced water from different oil platforms outside the Norwegian coast (Brendehaug et al., 1992). Furthermore, release of municipal wastewater is another major source of alkylphenols and alkylphenol ethoxylates in marine waters (reviewed in Ying et al., 2002). Nevertheless, relatively little is known about fate and the long-term effects of alkylphenols in the marine environment (Røe, 1998). The biodegradation rate of alkylphenols is markedly decreased with increasing length of alkyl chain. The bioconcentration factors for alkylphenols C_4 to C_7 range from 118 to 578 in fish (McLeese et al., 1981; Freitag et al., 1985; Tollefsen et al., 1998).

The estrogenic activity of alkylphenols in fish is well described (Nimrod and Benson, 1996; Christiansen et al., 1998; Arukwe et al., 2000, 2001). Induction of vitellogenesis in male or juvenile fish, mediated through the estrogen receptor (ER), is an established biomarker used to assess estrogenic contamination in the aquatic environment (Sumpter and Jobling, 1995). Induction of CYP1A expression in fish, mediated through the aryl hydrocarbon receptor (AhR), is a biomarker used to estimate exposure to polyaromatic hydrocarbons and planar halogenated aromatic hydrocarbons in the aquatic environment (Stegeman and Hahn, 1994). Several AhR agonists have been shown to exhibit anti-estrogenic activities and possible cross-talk between the AhR and ER has been suggested in mammals (e.g. Safe et al., 1991; Klinge et al., 2000; Safe, 2001), and possible also in fish (Elskus et al., 1992; Anderson et al., 1996; Arukwe et al., 1997; Solé et al., 2000; Navas and Segner, 2001).

Estrogens (i.e. 17β-estradiol and estrone) and some xenoestrogens (e.g. octylphenol, nonylphenol, 17α-ethynylestradiol and bisphenol A) have been shown to interact with CYP enzymes, including members of the CYP1A and CYP3A subfamilies in mammals (e.g. Lee et al., 1996a,b; Yamazaki et al., 1998; Hanioka et al., 1999, 2000; Cheng et al., 2001). The CYP1A subfamily normally is expressed in low levels in unexposed fish, but can be highly induced by AhR agonists. The major sites for CYP1A induction are the digestive and respiratory tract and the vascular endothelium (reviewed in: Stegeman and Hahn, 1994). The CYP3A subfamily is the dominant CYP form expressed in the digestive and respiratory tract in fish (Husøy et al., 1994; Cok et al., 1998; Lee et al., 1998, 2001; Hegelund and Celander, 2003). In human, CYP3A4 is the major steroid hydroxylation catalyst. However, due to the wide variety of chemically diverse substrates the physiological significance of CYP3A4 has been questioned (reviewed by Guengerich, 2003). The responsiveness of CYP1A and CYP3A genes to xenobiotics and the strategic locations of expression, suggest that CYP1A and CYP3A enzymes may act as first line defence to xenoestrogens exposure, including alkylphenols.

The objective of this investigation was to study effects of alkylphenols on hepatic CYP1A and CYP3A in first spawning Atlantic cod exposed to high and environmental relevant doses of an alkylphenol mixture (C_4:C_5:C_6:C_7 ratio 1:1:1:1). The level of uptake of alkylphenols by pelagic fish species has been estimated to <10 ppb, based on a computer simulation of the discharge of alkylphenols in produced water from the Halten Bank area outside the Norwegian West coast (Rye et al., 1996). The lowest exposure dose used in the present study (0.02 ppm) was calculated based on alkylphenol concentrations in produced water, uptake in fish and the bioconcentration factor 600 for the alkylphenol mixture. In addition to investigating the effects on CYP1A and CYP3A expression of alkylphenol exposure in vivo, we also characterized the effects of the alkylphenol mixture and each individual alkylphenol in vitro on hepatic microsomal CYP1A and CYP3A catalytic activities in Atlantic cod.

2. Material and methods

2.1. Chemicals

The 4-*tert*-butylphenol (C_4) and 4*n*-hexylphenol (C_6) were purchased from Sigma–Aldrich Norway AS (Oslo, Norway). The 4*n*-pentylphenol (C_5) was obtained from Acros (Gell, Belgium) and 4*n*-heptylphe-

nol (C_7) from Avocado Research Chemicals Ltd. (Lancashire, England). The BCA Coomassie® Plus Assay Reagent was purchased from Pierce (Rockford, IL, USA). The BCA[TM] protein assay kit from Pierce was purchased from Boule Nordic AB (Huddinge, Sweden). Ready gels 12% continuous acrylamide in Tris–HCl and supported nitrocellulose membrane (0.45 μm) were purchased from BioRad (Sundbyberg, Sweden). The 1-12-3 p6 mouse monoclonal antibody (MAb) against scup (*Stenotomus chrysops*) CYP1A1 was kindly provided by Dr. John J. Stegeman, Woods Hole Oceanographic Institution, MA, USA. Horseradish peroxidase conjugated donkey-anti-rabbit IgG and ECL[TM] Western blotting detection reagents were from Amersham Pharmacia Biotech (Uppsala, Sweden). Horseradish peroxidase conjugated goat-anti-mouse IgG, β-naphthoflavone (BNF), 7-ethoxyre-sorufin, resorufin, ketoconazole, dimethylsulphoxide (DMSO) were obtained from Sigma–Aldrich (Stockholm, Sweden). The 7-benzyloxy-4-[trifluoromethyl]-coumarin (BFC), 7-hydroxy-4-[trifluoromethyl] coumarin and the CYP3A4 inhibition kit were from Gentest[TM] (Woburn, MA, USA). Reduced nicotin-amide-adenine-dinucleotide-phosphate (NADPH) was purchased from Roche Diagnostics (Bromma, Sweden). Kodak X-OMA X-ray film was from VWR International (Stockholm, Sweden). All other chemicals used were of analytical grade available in Sweden and Norway from Sigma–Aldrich, Fluka Chemie AG or VWR international.

2.2. Animals, treatment and sampling

First time spawning Atlantic cod (*Gadus morhua*) of both sexes with body weights ranging between 600 and 800 g were obtained from Austevoll Aquaculture Research Station, Bergen, Norway and transported to the Institute of Marine Research in Bergen. The fish were divided into seven groups, each consisting of 40 fish, placed in $10\,m^3$ indoor tanks, provided with continuously flowing seawater at a temperature of $9\pm1\,°C$ with a salinity of 34.5% and subjected to a natural photoperiod (November–December, Bergen, 60°N). During the experimental period, the fish were force-fed, once a week, with 0.02, 2, 20, 40 and 80 ppm alkylphenol mixture. The alkylphenol mixture consisted of $C_4:C_5:C_6:C_7$ ratio 1:1:1:1. Control fish received vehicle (1,2-propandiol) and positive estrogenic control

fish received 5 ppm 17β-estradiol. The alkylphenols and 17β-estradiol were dissolved in 1,2-propandiol and mixed into a paste consisting of dry fish pellets, water and fish oil. After 1 and 4 weeks of exposure, respectively, fish were anaesthetized with benzocaine (20 mg/l seawater). Blood samples were collected from the *Sinus caudalis* for plasma steroid hormones and vitellogenin analyses (Meier et al., 2002). After blood sampling, fish was killed by a blow to the head and the liver was quickly dissected out, snap frozen and stored in liquid nitrogen. Each liver was next homogenized in 0.1 M sodium phosphate buffer pH 7.4, 0.15 M KCl, 1 mM EDTA, 1 mM dithiothreitol and 10% (v/v) glycerol (4 ml homogenization buffer/g liver) on ice using a Potter-Elvehjem glass-teflon homogenizer. Homogenates were centrifuged ($12,000\times g$ for 20 min at 4 °C) and the postmitochondrial supernatants, S9-fractions, were stored at −80 °C.

For in vitro inhibition studies, adult Atlantic cod of both sexes were caught off the west coast of Sweden and placed in concrete basins provided with recirculating aerated seawater at $10\pm2\,°C$. Eight fish were injected intra peritoneally with 50 ppm BNF dissolved in peanut oil (5 mg BNF/ml). After injection the fish were placed in 100 l glass-aquaria with aerated seawater at $10\pm2\,°C$ and 30% of the water volume was replaced each day. Black plastic was used to cover the sides of the aquarium to eliminate visual stress and the fish were subjected to a 12 h light:12 h dark photoperiod. After 3 days exposure, fish was sacrificed by a blow to the head. Livers were quickly dissected out and the S9-fractions from pooled untreated and BNF-treated Atlantic cod were isolated as described above. The microsomal fractions were isolated ($100,000 \times g$ for 1 h at 4 °C), homogenized in ice-cold homogenization buffer (1 ml/g liver) containing 20% (v/v) glycerol and stored at −80 °C.

2.3. Assays

Total protein content in S9-fractions were analyzed in Norway using the Coomassie® Plus assay reagent from Pierce with bovine gamma globulin as standard. Total protein content in microsomes were analyzed in Sweden using the BCA[TM] protein assay kit and bovine serum albumin as standard at A_{540} in a FLUOstar plate reader from Labvision (Stockholm, Sweden). Protein blot analyses of liver S9-fractions

using the MAb 1-12-3 (diluted 1:500) against scup CYP1A1 (Park et al., 1986) and polyclonal antibodies (PAb) against rainbow trout CYP3A and detected using enhanced cheminoluminescence as described in Celander et al. (1996). These PAb recognize one hepatic CYP3A protein (previously denoted P450b)in Atlantic cod (Celander et al., 1996). The protein band intensities on the scanned fluorograms were analyzed using densitometry and the Scion Image software from Scion Corporation (Frederick, MD, USA).

The CYP1A activity was determined as EROD activity using the protocol provided in Nilsen et al. (2000). The CYP3A catalytic activity was measured using BFC as a diagnostic fluorescent substrate based on the protocol described in Miller et al. (2000), and optimized for rainbow trout liver microsomes (Hegelund et al., unpublished data). The reaction mixture consisted of $24 \mu g$ microsomal protein, $200 \mu M$ 7-BFC, $1.6 \, mg/ml$ bovine serum albumin and $2 \mu M$ NADPH in $200 \, mM$ potassium phosphate buffer pH 7.4. The metabolite, 7-hydroxy-4-[trifluoromethyl] coumarin, was used as standard. Both CYP1A and CYP3A activities were analyzed in a VICTOR[TM] 1420 Multilabel Counter from Wallac Sverige AB (Upplands Väsby, Sweden).

2.4. In vitro inhibition studies

The median inhibition concentrations (IC_{50} values) on CYP1A and CYP3A activities for 17β-estradiol, the alkylphenol mixture (C_4:C_5:C_6:C_7 ratio 1:1:1:1), each individual alkylphenol (C_4 to C_7) and ketoconazole were determined in liver microsomes as described for ketoconazole in rainbow trout liver microsomes (Hegelund et al., in press). All substances were dissolved in DMSO and diluted in ethanol and the final concentrations of solvent never exceed 0.001% (v/v) DMSO and 0.01% (v/v) ethanol. For determination of IC_{50} values for CYP3A activity, pooled liver microsomes from untreated Atlantic cod were used with a total protein concentration of $24 \mu g/\mu l$. For determination of IC_{50} values for CYP1A activity, pooled liver microsomes from BNF-treated Atlantic cod were used diluted $50\times$ in homogenization buffer containing 20% glycerol to a final protein concentration of $0.4 \mu g/\mu l$.

For comparison, the IC_{50} values for 17β-estradiol, the alkylphenol mixture, each individual alkylphenols and ketoconazole on CYP3A activity were determined

in cDNA expressed human CYP3A4 baculovirus supersomes, using the CYP3A4 inhibition kit from BD Gentest[TM].The IC_{50} values in Atlantic cod liver microsomes are presented as means from two to five separate assays and in cDNA expressed human CYP3A4 baculovirus supersomes as means from two separate assays.

2.5. Statistical analyses

Data were analyzed using Kruskal–Wallis followed by a two-tailed Mann–Whitney U-tests. Data are presented as the mean of 5–15 individuals $\pm$ standard error (S.E.). Significance levels are $(*)$ $P \leq 0.05$ and $(**)$ $P \leq 0.01$. Statistical analyses were performed using SPSS 11.0 software from SPSS Sweden AB (Sundbyberg, Sweden).

3. Results

3.1. CYP1A and CYP3A protein expression in vivo

Female Atlantic cod displayed slightly higher CYP1A protein expression than males (Fig. 1A). Four weeks exposure to 17β-estradiol and the alkylphenol mixture resulted in elevated CYP1A protein levels
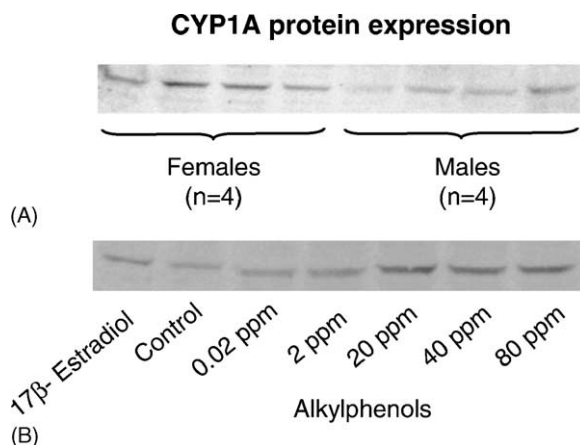

Fig. 1. Hepatic CYP1A protein expression in S9-fractions from adult Atlantic cod in protein blots using MAb 1-12-3 against scup CYP1A1. (A) Lanes 1–4: females and lanes 5–8: males. (B) Representative samples from male Atlantic cod treated with 17β-estradiol, control and 4 weeks exposure to various doses of 4-alkylphenols (C_4:C_5:C_6:C_7 ratio 1:1:1:1).

CYP3A protein expression

(A)

Females
(n=4)

Males
(n=4)

(B)

Male Controls
(n=5)

Males 17β-Estradiol
(n=5)

(C)

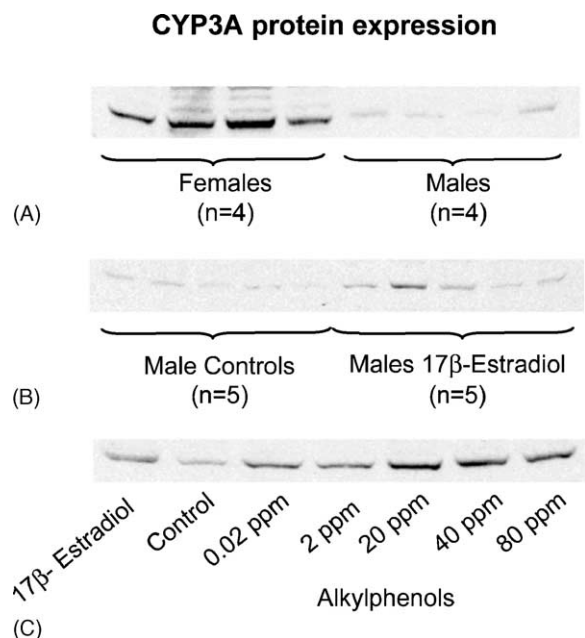17β- Estradiol   Control   0.02 ppm   2 ppm   20 ppm   40 ppm   80 ppm

Alkylphenols

Fig. 2. Hepatic CYP3A protein expression in S9-fractions from adult Atlantic cod in protein blots using PAb against rainbow trout CYP3A. (A) Lanes 1–4: females and lanes 5–8: males. (B) Lanes 1–5: control male and lanes 6–10: 17β-estradiol treated males. (C) Representative samples from male Atlantic cod treated with 17β-estradiol, control and 4 weeks exposure to various doses of 4-alkylphenols ($C_4:C_5:C_6:C_7$ ratio 1:1:1:1).

CYP1A activities in males

Fig. 3. Hepatic CYP1A (EROD) activities in S9-fractions from male Atlantic cod after 4 weeks exposure to various doses of 4-alkylphenols ($C_4:C_5:C_6:C_7$ ratio 1:1:1:1). Each bar represents the mean of 10–15 fish $\pm$ S.E. (*) Statistically different from controls, (***) $P \leq 0.001$.

sure to 17β-estradiol resulted in a significant decrease in CYP1A activity in males (Fig. 3), whereas CYP1A protein expression was increased in these fish (Fig. 1B). In contrast, 4 weeks exposure to various doses of alkylphenols had no effect on CYP1A activities in males (Fig. 3). Nevertheless, CYP1A protein levels were increased in these fish (Fig. 1B).

In female Atlantic cod, treatment with 17β-estradiol had no effect on either CYP1A protein expression (not shown) or CYP1A activity in vivo after either 1 or 4 weeks exposure (Fig. 4). However, exposure to 20 and 40 ppm alkylphenols resulted in increased CYP1A activities in females after 1 week (Fig. 4). After 4

in male Atlantic cod (Fig. 1B), but not in females (not shown). There was a dose-dependent relationship between doses of alkylphenols and CYP1A protein levels in male fish after 4 weeks exposure (Fig. 1B), whereas this was not seen in females (not shown). A similar pattern was observed for CYP3A. Thus, female Atlantic cod displayed seven-fold higher CYP3A protein levels than males (Fig. 2A). Exposure to alkylphenols resulted in increased expression of CYP3A proteins (Fig. 2C). In addition, exposure of male fish to 17β-estradiol resulted in slightly elevated CYP1A protein levels (Fig. 2B).

3.2. CYP1A enzyme activities in vivo

In control male Atlantic cod, CYP1A activity was almost three-fold higher than that in females (Figs. 3 and 4). However, CYP1A protein expression was significantly lower in control males compared to control females (Fig. 1A). Four weeks expo-
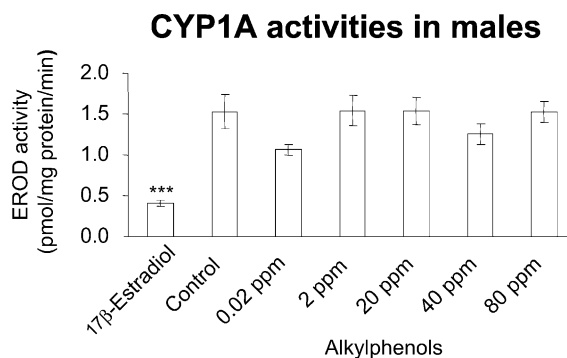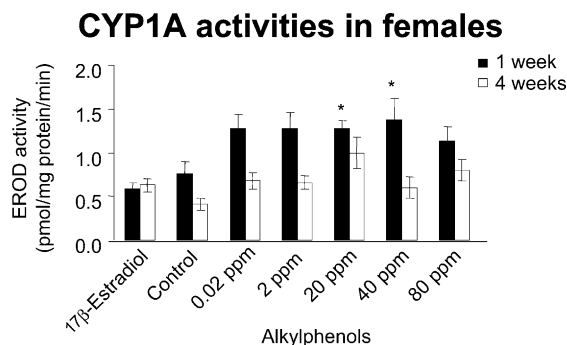
CYP1A activities in females

Fig. 4. Hepatic CYP1A (EROD) activities in S9-fractions from female Atlantic cod after 1 and 4 weeks exposure to various doses of 4-alkylphenols ($C_4:C_5:C_6:C_7$ ratio 1:1:1:1). Filled bars represent the mean of 6–11 females $\pm$ S.E. after 1 week exposure, (*) statistically different from controls, $P \leq 0.05$. Open bars represent the mean of 5–10 females $\pm$ S.E. after 4 weeks exposure.

weeks exposure to alkylphenols, the CYP1A activities were not significantly different from control female fish (Fig. 4). In addition, the CYP1A protein expression in females treated with the 80 ppm alkylphenols for 4 weeks were not significantly different from controls (not shown).

3.3. Inhibition studies of CYP1A and CYP3A activities in vitro in liver microsomes

The possible inhibitory effect of 17β-estradiol and the alkylphenols were determined as IC_{50} values. The CYP3A activity was not detectable in hepatic S9-fractions in Atlantic cod from the study above using BFC as substrate (not shown), whereas this activity was readily detected in Atlantic cod liver microsomes. The IC_{50} values for 17β-estradiol, the alkylphenol mixture and each individual alkylphenol on CYP1A and CYP3A activities were determined in pooled liver microsomes from BNF treated and untreated Atlantic cod, respectively. In addition, the IC_{50} values for these substances were determined in CYP3A4 cDNA expressed baculovirus supersomes, using BFC as substrate. For comparison, the prototypical mammalian CYP3A inhibitor ketoconazole was used, which in addition to inhibiting human CYP3A4 (Boxenbaum, 1999), also inhibits CYP1A and CYP3A activities in fish (Hegelund et al., in press). The IC_{50} values are summarized in Table 1.

The inhibitory effect of 17β-estradiol on CYP1A activity was insignificant ($IC_{50} = 500\,\mu M$). However, the alkylphenol mixture efficiently inhibited CYP1A activity ($IC_{50} = 10\,\mu M$). The inhibitory effects of each individual alkylphenols also were determined. The IC_{50} values for C_4, C_5, C_6 and C_7 were 300, 30, 15 and 12 μM, respectively. Thus, the potency for inhibition of CYP1A activity increases with increasing length of the 4-alkylchain. For comparison, the IC_{50} value for ketoconazole on CYP1A activity was 0.6 μM (Table 1).

Estradiol was a weak inhibitor of CYP3A activity ($IC_{50} = 75\,\mu M$). Furthermore, the alkylphenol mixture only weakly inhibited CYP3A activity ($IC_{50} = 100\,\mu M$). In addition, the inhibitory effects of each individual alkylphenol on CYP3A activity were weak or insignificant. The IC_{50} values ranged between 60 and 250 μM and the rank order were [$C_5 > C_6 > C_7 > C_4$]. For comparison, the IC_{50} value for ketoconazole on CYP3A activity was 0.3 μM. A similar pattern was observed in cDNA expressed human CYP3A4 supersomes (Table 1).

4. Discussion

4.1. Effects of alkylphenols in Atlantic cod

Alkylphenols are continuously released into the aquatic environment as a result of offshore oil

Table 1
The IC_{50} values for 17β-estradiol and 4-alkylphenols compared to ketoconazole on CYP1A and CYP3A catalytic activities in Atlantic cod liver microsomes and on cDNA expressed human CYP3A4 activity in baculovirus supersomes

Compound(s)	IC_{50} (μM)		
	Hepatic microsomal CYP1A activity[a]	Hepatic microsomal CYP3A activity[b]	cDNA expressed human CYP3A4 activity[b]
17β-Estradiol	500	75	Not analyzed
4-*tert*-Butylphenol (C_4)	300	250	800
4*n*-Pentylphenol (C_5)	30	60	60
4*n*-Hexylphenol (C_6)	15	150	80
4*n*-Heptylphenol (C_7)	12	200	300
Alkylphenol mixture (C_4:C_5:C_6:C_7 ratio 1:1:1:1)	10	100	60
Ketoconazole	0.6	0.3	0.4

The IC_{50} values in Atlantic cod liver microsomes are presented as means from two to five separate assays and in cDNA expressed human CYP3A4 baculovirus supersomes as means from two separate assays.

[a] Substrate: 7-ethoxyresorufin.

[b] Substrate: 7-benzyloxy-4-[trifluoromethyl]-coumarin.

production and the extensive use of nonionic surfactants in industrial processing and in various cleaning products. Disruption of endocrine systems has been described in animals in the wildlife, such as induction of the ER mediated vitellogenesis in fish. An alkylphenol mixture (C_4 to C_7) and 17β-estradiol were shown to induce vitellogenesis in adult male Atlantic cod (Meier et al., 2002). In addition, exposure to alkylphenols resulted in elevated hepatic glutathione content and glutathione reductase activity in these fish (Hasselberg et al., 2001). Thus, the alkylphenol mixture not only was estrogenic but also resulted in oxidative stress in first-time spawning Atlantic cod. In the present study, we further investigated effects of alkylphenols on hepatic CYP1A and CYP3A protein expression in these animals.

4.2. Effects of alkylphenols on CYP1A

Treatment of male Atlantic cod with alkylphenols resulted in induction of CYP1A protein expression. However, this increase in CYP1A protein levels was not reflected on CYP1A catalytic activities in vivo, possible due to enzyme inhibition. Although, in females 1 week exposure to 20 and 40 ppm alkylphenols resulted in elevated CYP1A activities. In vitro inhibition study showed that the alkylphenol mixture efficiently inhibited the CYP1A (EROD) activity. The alkylphenols C_6 and C_7 seem to be primarily responsible for this inhibitory effect. In Atlantic salmon (*Salmo salar*), treatment with nonylphenol (125 ppm) resulted in reduced CYP1A protein expression and EROD activity (Arukwe et al., 1997). Nonylphenol also was a potent inhibitor of hepatic microsomal EROD activity ($IC_{50} = 5 \mu M$) in vitro in Atlantic cod (Hasselberg et al., 2003). In rat liver, nonylphenol competitively inhibited EROD activity and down-regulated CYP1A protein expression (Lee et al., 1996a). Although, in mice Hepa-1c1c7 cells, nonylphenol treatment had no effect on EROD activity (Jeong et al., 2001). In cultured rainbow trout hepatocytes, exposure to 4-*tert*-octylphenol and 17β-estradiol both induced vitellogenesis, but only the 17β-estradiol treatment resulted in markedly reduced basal EROD activities (Navas and Segner, 2000, 2001). Hence, effects on CYP1A catalytic activity vary between different estrogenic compounds and between different studies. Variables such as species, sex, reproductive stage, compound, route of exposure, time of exposure, mixed exposure and/or dose may influence the effects.

4.3. Sexually dimorphic expression of CYP1A

Male fish often have higher CYP1A levels than females (e.g. Stegeman and Woodin, 1984; Pajor et al., 1990; Arukwe and Goksøyr, 1997). However, in first spawning Atlantic cod females displayed higher CYP1A protein levels than males. Furthermore, treatment of male Atlantic cod with 17β-estradiol resulted in elevated CYP1A protein levels, whereas this treatment had no effect in females. The sex difference in CYP1A protein expression was not reflected on the CYP1A activity. Hence, untreated males had two-fold higher CYP1A activities than females. Furthermore, the sex difference in CYP1A activity was abolished by treatment with 17β-estradiol, which resulted in pronounced suppression of CYP1A activity in vivo. This treatment with 17β-estradiol had no effect on CYP1A activity in females. In vitro inhibition studies show that the inhibitory effect of 17β-estradiol on CYP1A activity was insignificant and thus does not explain the discrepancy between CYP1A protein levels and CYP1A activities in Atlantic cod.

Previously, an inverse relationship between ER agonists and the AhR mediated induction of CYP1A genes was reported in fish (e.g. Elskus et al., 1992; Arukwe et al., 1997; Solé et al., 2000; Navas and Segner, 2001). A cross-talk between ER and AhR has been described in mammals. However, the molecular mechanisms involved in the AhR and ER cross-talk are not yet fully understood. Recently, a ligand-activated AhR mediated recruitment of proteosomes, resulting in degradation of AhR and ER, has been shown in human breast cancer cells (Wormke et al., 2003). In the present study, alkylphenol exposure resulted in decreased plasma levels of 17β-estradiol in female Atlantic cod, and decreased testosterone and 11-keto-testosterone plasma levels in males (Meier et al., 2002). Future studies will address the possibility that feed-back mechanisms within the hypothalamic-pituitary axis are involved in this down-regulation of sex steroid plasma levels upon exposure to alkylphenols.

4.4. Effects of alkylphenols on CYP3A

Alkylphenols have been show to interact with CYP3A enzymes in rat (Lee et al., 1996b; Hanioka et al., 1999, 2000), implying that these enzymes are involved in alkylphenol clearance. In vitro inhibition studies revealed that alkylphenols are poor inhibitors CYP3A activity BFC metabolism. This is in contrast to efficient inhibition the CYP1A activity by these compounds.

In male Atlantic cod, exposure to alkylphenols resulted in a dose-dependent elevation of CYP3A protein levels, whereas this treatment had no effect on CYP3A protein levels in females. Expression of CYP3A genes in mammals is mediated by the nuclear pregnane X receptor (PXR), which can be activated by steroids and xenobiotics (reviewed in: Kliewer et al., 2002). The existence of a piscine PXR ortholog recently was suggested by cloning of the ligand-binding domain of a zebra fish (*Danio rerio*) PXR gene (Moore et al., 2002). Nonylphenol was shown to activate the PXR mediated expression of CYP3A1 mRNA in rat and to block the proteasome dependent PXR degradation in mouse mammary cancer cells, resulting in elevated CYP3A expression (Masuyama et al., 2000, 2002). Thus, it is possible that similar mechanisms are responsible for the observed elevation of CYP3A expression in Atlantic cod.

4.5. Sexually dimorphic expression of CYP3A

There was a pronounced sexually dimorphic expression of CYP3A proteins in adult Atlantic cod, with seven-fold higher protein levels in females compared to males. Sexually dimorphic expression of CYP3A genes also has been reported in other fish species. In rainbow trout and winter flounder (*Pseudopleuronectes americanus*), females displayed higher CYP3A expression or CYP3A activities than males (Stegeman and Woodin, 1984; Cok et al., 1998; Lee et al., 1998). However, in rainbow trout during spawning and in killifish (*Fundulus heteroclitus*), males displayed higher CYP3A mRNA and protein expression (Celander et al., 1989; Hegelund and Celander, 2003). The mechanism for the sexual dimorphic CYP3A expression is not fully understood, though it is possible that sex steroids are involved. Treatment with 17β-estradiol resulted in down-regulation of CYP3A

protein expression in immature brook trout (*Salvenius fontinalis*) and testosterone 6β-hydroxylase activity (presumably CYP3A) in late post-spawning winter flounder (Pajor et al., 1990; Gray et al., 1991). In addition, treatment with 17β-estradiol resulted in induced vitellogenesis and decreased CYP3A mRNA and protein levels in juvenile rainbow trout (Buhler et al., 2000; Hasselberg et al., unpublished data). An inverse relationship between PXR and ER expression was shown in human breast tumors (Dotzlaw et al., 1999) and possible cross-regulatory mechanism(s) between PXR and ER cannot be ruled out. However, in the present study in first spawning Atlantic cod, 17β-estradiol treatment resulted in elevated vitellogenesis and CYP3A protein expression in males. Thus, this sex steroid appears to be involved in regulation of CYP3A expression in fish, although species differences are apparent. It is not yet clear if 17β-estradiol mediates a direct effect on CYP3A expression or indirect effect(s) through other hormones, feed-back mechanisms or possible receptor cross-talks in fish.

5. Conclusions

This study shows that treatment with 17β-estradiol and alkylphenols induce CYP1A and CYP3A protein expression in male Atlantic cod, but not in females. Treatment with 17β-estradiol resulted in increased CYP1A protein expression and decreased CYP1A activities in males, whereas this treatment had no effect in females. In contrast, treatment with alkylphenols had no effect on CYP1A activities in vivo in males and slightly induced CYP1A activities in females. Thus, 17β-estradiol and alkylphenols both are estrogenic but have different effects on CYP1A activities in vivo. In vitro inhibition studies show that the alkylphenol mixture (but not 17β-estradiol) efficiently inhibited CYP1A activity, suggesting that these two classes of estrogenic compounds have diverse effects on CYP1A activities in Atlantic cod.

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