Oxidative stress in the marine environment-prognostic tools for toxic injury in fish liver cells
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Sex-specific biotransformation and detoxification after xenobiotic exposure of primary cultured hepatocytes of European flounder

(Platichthys flesus L.)

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AQUATIC TOXICOLOGY
Abstract

Sex-specific effects of sublethal concentrations of known effective pro-oxidants such as 100, 200 and 400 μM benzo[a]pyrene (B[a]p), 50 μM nitrofurantoin (NF) and 100 μM hydrogen peroxide (H₂O₂) on biotransformation pathways were studied in isolated hepatocytes of immature female and male European flounder (Platichthys flesus L.). Cell responses were assessed at the level of (1) stress induction as measured by formation of reactive oxygen species (ROS), mainly superoxide radicals, and induction of cytochrome P450 (CYP450) biotransformation activity, (2) cellular antioxidant defences, both non-enzymatic (reduced glutathione) and enzymatic (DT-diaphorase (DTD)/oxido quinone reductase, EC 1.6.99.2), (3) detoxification (aldehyde dehydrogenase (ALDH), EC 1.2.1.3), and (4) cellular damage as measured by reduced lysosomal membrane stability and cell death. As there is increasing evidence that 17-ß-estradiol interferes with certain pathways of xenobiotic biotransformation, we additionally tested the effects of different concentrations of 17-ß-estradiol (0.2-10 μM) alone and 17-ß-estradiol (1 μM) in combination with 100 μM B[a]p. Parameters were monitored after 1 and 9 d of exposure by quantitative image analysis of chromogenic or fluorogenic reaction products. Our study revealed clearly sex-dependent differences in cellular stress responses. In hepatocytes of female flounder, biotransformation was slower and the capacity of non-enzymatic antioxidant defences and detoxification of toxic aldehydes was lower than in males. Additional administration of 17-ß-estradiol enlarged these differences between the sexes with respect to biotransformation activity and antioxidant defence in xenobiotic-induced injury. These findings may explain the generally higher cancer rates in female flatfish due to higher xenobiotic toxicity.

Keywords: primary flounder hepatocyte culture; biotransformation; CYP450; oxidative stress; antioxidative defence; hydrogen peroxide; benzo[a]pyrene; nitrofurantoin; estrogen; ALDH; DTD
1. Introduction

Sex-dependent differences in late responses to toxicant exposure such as higher incidences of tumors in female fish have been observed by several investigators (Köhler, 1995; Landwüst et al., 1996; Teh and Hinton, 1998). Since the early 90ies it is known that CYP4501A1, the major phase I enzyme of xenobiotic biotransformation, is affected by sexual hormones during vitellogenesis in female flatfish and rainbow trout (Gray et al., 1991; Andersson and Förlin, 1992; Lindström-Seppä and Stegeman, 1995; Anderson et al., 1996; Saborowski, 1996; Lange et al., 1998; Broeg et al., 1999). Yet, induction of CYP 450 biotransforming activity is only one step in the cascade of cellular responses to xenobiotic exposure. Underlying mechanisms of the observed sex differences have not yet been analysed in detail, especially not with respect to the multistep phases of biotransformation and elimination of xenobiotics. The sequence of responses and the triggering factors involved such as specific enzymatic or non-enzymatic defence systems and ROS-induced lipid peroxidation which may finally lead to cellular damage are still not understood in relation to sex. Therefore, a series of analyses of the various metabolic steps is needed to understand cause-effect relationships in xenobiotic-induced adaptive responses to keep up homeostasis and in cell injury in case of their failure (Köhler and Pluta, 1995).

Beneficial aspects of xenobiotic biotransformation, namely facilitation of excretion of hydrophobic compounds, can be overwhelmed by the paradox of bioactivation of parent compounds to more reactive and harmful metabolites. A good example for metabolic activation is B[a]p which itself is relatively unreactive, but -as all high molecular weight polycyclic aromatic hydrocarbons (PAHs)- is bioactivated rapidly to carcinogenic metabolites by CYP1A1 and epoxide hydrolase activity (Varanasi, 1989). In this case, biotransformation activity and the half-life of resulting toxic compounds or metabolites are important determinants of xenobiotic toxicity.

Phenomenons like oxidative stress provide an additional parameter in xenobiotic toxicity. Many chemical compounds such as aromatic diols, quinones which are metabolites of, for example, PAHs such as B[a]p, nitroaromatics such as NF and aromatic amines are known to generate ROS via redox reactions during CYP450-
mediated xenobiotic biotransformation (Mather-Mihaich and DiGiulio, 1986; Kappus, 1987; DiGiulio et al., 1989). Cellular defence systems such as antioxidative enzymes and non-enzymatic scavengers provide only a limited capacity to protect cells against oxidative damage. Thus, xenobiotic challenge may overstress cellular protective mechanisms, and provoke DNA strand breaks, protein oxidation, lipid peroxidation and generation of toxic aldehydes which may consequently result in early aging, cell death and/or carcinogenesis (Esterbauer, 1985; Halliwell and Gutteridge, 1984; Winston and DiGiulio, 1991).

Our earlier studies on gender-specific oxidative stress responses in living flounder hepatocytes revealed xenobiotic-specific differences with respect to cellular damage and total antioxidant scavenging capacity independent of xenobiotic-induced prooxidant challenge (Winzer et al., 2000). Therefore, we anticipated that modulation of xenobiotic biotransformation, antioxidant defence and/or detoxification are likely sex-dependent and are a cause of differences in toxic injury between males and females.

The importance of 17-ß-estradiol in xenobiotic toxicity is increasingly acknowledged. On the one hand, 17-ß-estradiol and other steroid hormones have been found to downregulate xenobiotic biotransformation such as induction of CYP450 (Anderson et al., 1996). On the other hand, estrogenic or anti-estrogenic effects of numerous pollutants on estrogen-regulated reproductive processes and feminisation of males have become evident (Colborne et al., 1993; Peterson et al., 1993; White et al. 1994; ICES, 1996, 1998; Smeets et al., 1999b). Effects of 17-ß-estradiol on metabolic functions such as stress responses and the mechanisms of the effects are still unknown.

We have chosen primary hepatocyte cultures for our studies as they provide a simplified model of the animal and a standardised in vitro system for xenobiotic research without systemic interferences due to the complexity of the intact organism (Moldeus et al., 1978; Moon et al., 1985; Braunbeck, 1993; Segner and Lenz, 1993). European flounder (Platichthys flesus L.) is a marine flatfish that is frequently used in international

The aim of the present study was to analyse the effects of oxidative stress on several molecular and cellular levels in hepatocytes of both immature male and female flounder under defined conditions, to achieve a better understanding of pathways and/or enzymes involved in xenobiotic toxicity. H$_2$O$_2$, B[a]p and NF were selected as stressors, because they have been shown to be effective model inducers of oxidative stress in short-term exposure studies in aquatic organisms (Hartter, 1985; Washburn and DiGiulio, 1988, 1989; Lemaire and Livingstone, 1994; Martinez et al., 1995, Lemaire and Livingstone, 1995, 1997; Buchner et al., 1996; Abele-Oeschger et al., 1997; Abele et al., 1998, Canova et al., 1998; Winzer et al., 2000). H$_2$O$_2$ is of environmental relevance in coastal and surface waters in low concentrations due to UV irradiation, whereas B[a]p is a contaminant of oil spills. NF was chosen as a model compound for redox cycling of nitroaromatics resulting from the production of rubber, pesticides or dyes in chemical industry. In order to estimate the putative role of estrogens on initial stages of oxidative stress responses and xenobiotic biotransformation phase I and II, we used 17-ß-estradiol as a model estrogen.

The cellular responses to toxicant- and 17-ß-estrogen exposure that were measured are superoxide radical (O$_2$•-) formation and CYP450-dependent biotransformation (phase I), phase II biotransformation (DTD and ALDH activity), non-enzymatic antioxidant defences (reduced glutathione) and cellular damage (impaired lysosomal membrane stability and cell death).

2. Materials and Methods

2.1. Chemicals

Ethidium homodimer-1 and calcein AM, compounds of the Live/Dead viability/cytotoxicity kit were purchased from Molecular Probes (Eureka OR, USA).
NAD and NADP were obtained from Roche (Mannheim, Germany), 1-methoxyphenazine methosulfate (PMS) from Serva (Heidelberg, Germany), and collagenase type IV, polyvinyl alcohol (PVA), tetranitro blue tetrazolium salt (TNBT), benzo[a]pyrene (B[a]p), nitrofurantoin (N-(5-nitro-2-furfurylidene)-1-aminohydantoin; NF), Neutral Red (NR) and all other reagents were ordered from Sigma (St Louis MO, USA).

2.2. Animals, primary hepatocyte cultures, cell and tissue preparations

Immature European flounder of both sexes with a body length of 17 - 25 cm were caught from May to September in an area of the German Bight (Tiefe Rinne) near Helgoland (North Sea) that is relatively unpolluted in terms of levels of hydrocarbons and heavy metals in fish and sediment (Landwüst et al., 1996; Broeg et al., 1999). Immature fish were selected in order to eliminate effects of seasonal variations in sex hormone levels and related suppression of biotransformation activity. After catching, fish were adapted for two weeks to standard environmental conditions (30%c salinity/15°C/fed ad libitum). Hepatocytes were isolated by mechanical destruction of the freshly dissected livers as previously described in detail by Winzer et al. (2000) and according to Lowe et al. (1992). Isolated hepatocytes were plated at a density of 2x10^6 cells per well in 24-well plates in (A) vitamin E-containing Medium 199 or (B) Hanks salt solution, each supplemented with 20 mM Hepes, 1% penicillin/streptomycin in the presence or absence of 10% fetal calf serum (FBS) to evaluate effects of the composition of the culture medium. Osmolality of both media was 300 mmol/kg which corresponds to serum levels of flounder (Schlenk et al., 1996). FBS is a serum that is commonly used in primary cultures of fish cells (Braunbeck, 1993; Zahn et al., 1981, 1982). Cells were kept in the dark for up to 10 d at pH 7.5 at 10°C under sterile conditions. Half the volume of the media with or without oxidative stressors was exchanged every second day. Cells were sampled for experiments at 1 d and 9 d after start of exposure and washed in phosphate buffered saline (PBS), pH 7.2, prior to use. For each parameter tested, cells of three different sex-specific pools of at least 5 males and 5 females were used.
In parallel, of each liver sampled for the cell isolation procedure, a 5 mm³ piece was dissected from the central area, immediately frozen in liquid nitrogen and stored at -80 °C for histochemical analysis of neutral lipids in cryostat sections (Köhler and Pluta, 1995).

2.3. Exposure of cells to oxidative stressors
Stock solutions of oxidative stressors were prepared in dimethylsulfoxide (DMSO; final dilution in the media, 1, 2 or 4 %) except for H₂O₂ that was diluted directly in Medium 199 or Hanks salt solution. Final sublethal concentrations of B[a]p (100, 200 or 400 μM), NF (50 μM), H₂O₂ (100 μM) were prepared by adding stock solutions directly to the media. Solvent control was performed by using DMSO only. Cells were exposed also to 0.22 μM (physiologically maximum concentration in rainbow trout according to Anderson et al., 1996; Van Bohemen and Lambert, 1981), to 1 and 2 μM 17-ß-estradiol alone or to 1 μM in addition to 100 μM B[a]p. Exposure was started at 24 h after cell isolation.

2.4. Viability of isolated hepatocytes
Cell viability was determined using the Live/Dead viability/cytotoxicity kit as described by Winzer et al. (2000). Fluorescence was determined with excitation light of 470 nm and emission light of 530 nm in living hepatocytes (green) and of 585 nm in dead hepatocytes (red) using an image analysis system (see section 2.8.). Viability was calculated as percentage living cells of the total number of cells.

2.5. Analysis of enzyme reactions based on tetrazolium salt methods
In the present study, we used chromogenic tetrazolium salt methods for the detection of O₂⁻ and the activity of DTD, NADPH-CYP450 reductase and ALDH on the basis of a histochemical approaches. The histochemical techniques for cryostat sections (Chung and Secombes, 1987; Van Noorden and Frederiks, 1992) were modified in a similar way as was done by Winzer et al. (in preparation) with the method to localise and quantify G6PDH activity in living hepatocytes of flounder. Only 2% PVA (w/v) instead of 18% was dissolved in 5 ml 0.1 M phosphate buffer, pH 7.4, by stirring and heating up to
60°C. After cooling down to 37°C substrates, coenzymes, additional factors and TNBT were added to the media for the respective assays as listed in Table 1. To prevent precipitation of TNBT in the less viscous medium, we selected a final concentration of 1 mM instead of 5 mM, that was dissolved first in equal volumes (100 μl) of ethanol and DMSO. Under these conditions, the capturing of electrons by 1 mM TNBT is just as efficient as by 5 mM (Van Noorden, 1988). Reactions were carried out at room temp in the dark to prevent light-induced formazan formation. Phosphate buffer (pH 5.3) containing 4% formaldehyde was used to stop reactions immediately in the intact cells after 60 min of incubation (Winzer et al., submitted).

Table 1. Components of media for the histochemical assays based on TNBT reduction.

<table>
<thead>
<tr>
<th>TNBT methods</th>
<th>Superoxide radicals</th>
<th>NADPH CYP450 reductase</th>
<th>Biotransformation (phase I)</th>
<th>DTD</th>
<th>ALDH</th>
<th>Antioxidative defence/ detoxification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer [5 ml]</td>
<td>pH 7.4</td>
<td>pH 7.4</td>
<td>pH 7.4</td>
<td>0.1 g</td>
<td>0.1 g</td>
<td>pH 7.0</td>
</tr>
<tr>
<td>PVA</td>
<td>0.1 g</td>
<td>0.1 g</td>
<td>0.5 mM NADPH</td>
<td>0.5 mM NADH/ NADPH</td>
<td>25 mM propion-aldehyde</td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td>0.5 mM NADPH</td>
<td></td>
<td></td>
<td>1.0 mM menadione</td>
<td>25 mM sodium azide</td>
<td></td>
</tr>
<tr>
<td>Coenzyme</td>
<td></td>
<td></td>
<td></td>
<td>0.45 PMS</td>
<td>5 mM sodium azide</td>
<td></td>
</tr>
<tr>
<td>Additional factors</td>
<td></td>
<td></td>
<td></td>
<td>1 mM</td>
<td>5 mM NAD</td>
<td></td>
</tr>
<tr>
<td>TNBT</td>
<td>1 mM</td>
<td>1 mM</td>
<td>1 mM</td>
<td>1 mM</td>
<td>5 mM NAD</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Addition of SOD</td>
<td>(1) no substrate</td>
<td>(2) NADP [5 mM] - inhibition with substrate</td>
<td>no substrate</td>
<td>(1) no substrate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2) sodium pyruvate</td>
<td>1 M] - inhibition with substrate</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.6. Biotransformation via CYP450

CYP450 activity in living cells was analysed using the EROD (ethoxyresorufine-O-deethylase) assay based on formation of fluorescence of resorufln (Burke and Mayer, 1974). The EROD assay described here was a modification of an assay for living rainbow trout hepatocytes in a microplate reader that was developed by Behrens et al. (in press). Auxiliary NADPH is not required in the EROD assay of living cells due to endogenous production. Resorufln reduction by cytoplasmic DTD was inhibited by addition of dicumarol. The assay medium was prepared by adding 5 μl of a stock
solution of ethoxyresorufin (1600 μM in methanol) and 1 μl dicumarol (stock solution, 3 mg/ml DMSO; dilution 1:1000) to 1 ml PBS buffer (pH 7.2). Suspensions of living hepatocytes (50 μl) were placed on Biobond-pretreated glass slides and left to adhere for 15 min at 10°C in a humid chamber. The assay medium (50 μl) was added and fluorescence was read immediately and after 15 min incubation using excitation light of 470 nm and emission light of 530 nm. Grey level images were captured using an image analysis system as described in detail in section 2.8. Grey levels were expressed as percentage of fluorescence.

2.7. Analysis of reduced glutathione in living cells (non-enzymatic defence)
We localised reduced glutathione in living cells using a solution of 5.0 mM Mercury Orange (MO; Sigma) in DMSO after a method we modified from an assay described for cryostat sections by De Jong et al. (submitted). Short incubation times resulted in specific localisation of reduced glutathione due to the formation of a complex of MO with specific SH-groups (Ashgar et al., 1975). Pretreatment of cells as recommended for cryostat sections, such as drying and water steaming (Chieco and Boor, 1983; De Jong et al., submitted) did not improve results. Pretreatment caused morphological deterioration or cell death and was therefore omitted. Optimal results for living hepatocytes attached to Biobond-pretreated slides were obtained by immediate incubation with the MO solution for 45 sec at room temp. The solution was washed off with DMSO and cells were mounted in Euparal. Fluorescence was measured by excitation at 470 nm and emission at 585 nm using an image analysis system (see section 2.9). Data are presented in relation to control cells after substraction of background fluorescence of the mounting medium. Levels of reduced glutathione were measured at 3, 6 and 24 h after start of exposure to any of the stressors. Additionally, effects of different concentrations of 17-β-estradiol (1, 2 and 10 μM) alone and in the presence of 100 μM B[a]p were estimated in both sexes. Controls were not run, because cells depleted of reduced glutathione were not available.
2.8. Estimation of cellular damage

Membrane stability of cellular lysosomes (Neutral Red retention assay)
The NR assay was used to determine effects of culture conditions and treatment on the integrity of the lysosomal membrane system of hepatocytes. NR stains lysosomes of healthy cells as the dye is actively taken up by lysosomes. When their membranes destabilise, the dye is released into the cytoplasm. Suspensions of living hepatocytes (50 µl) were allowed to adhere to Biobond-pretreated slides for 15 min in humid chambers at 10°C and were then incubated in 80 µl NR working solution (5 µl of NR stock in 995 µl PBS; NR stock solution contained 20 mg NR in 1 ml PBS buffer, pH 7.2). Cells were observed under the microscope and the ratio of healthy and damaged cells was determined at time intervals of 10 min until over 50% of the cells were damaged.

The amount of neutral lipids was estimated using a routine histochemical approach for cryostat sections. Cryostat sections of flounder livers (10 µM) were prepared using a cryostat (Microm, Walldorf, Germany) (Köhler et al., 1998). Sections were stained for neutral lipids using the Oil Red O assay (Bancroft, 1967).

2.9. Image analysis of chromogenic and fluorogenic assays
Cytophotometric analysis was performed by quantitative video microscopy using a CCD colour video camera (Sony, ATV Horn, Aalen, Germany) connected to a Zeiss Axioskop light microscope (Zeiss, Oberkochen, Germany) and coupled via a frame grabber (maximum pixel size, 786 x 512) to an image analysis system (Kontron, Eching, Germany) with the KS300 software package. Details of set up, analysis of absorbance in time and the calculation of enzyme activities have been described elsewhere (Chieco et al., 1994; Jonker et al., 1995, 1997; Van Noorden et al., 1997). Absorbance of formazan was measured as grey values in individual hepatocytes using monochromatic light of 580 nm. According to Van Noorden and Jonges (1995), areas of the cytoplasm of cells were measured and concentrations of formazan (c) could be calculated using the Lambert-Beer-law, \( A = \varepsilon \cdot c \cdot d \), with \( A \) = absorbance, \( \varepsilon \) = extinction coefficient of
formazan (19 000; Van Noorden and Frederiks, 1992), and \( d \) = diameter of the hepatocyte (estimated average diameter, 15 \( \mu \)m). An average cell height of 7.5 \( \mu \)m was assumed. Enzyme activity was calculated on the basis of the fact that one molecule formazan is formed by the conversion of two molecules of substrate.

2.10. Statistical analysis
For each parameter a minimum of 30 cells was analysed. All data were analysed with non-parametric statistics (Kruskal-Wallis test and post-hoc Nemenyii test (for uneven sample sizes); Statistica 5.0-StatSoft, Tulsa OK, USA) because the data were not normally distributed. \( P=0.05 \) was taken as level of significance.

3. Results
Early responses such as induction of biotransformation activity, antioxidative defence and detoxification proved to be sensitive to the exposure conditions that were used, whereas cellular stress signals such as \( O_2^- \) formation or cellular damage and cell death were low. Generally, hepatocytes cultured in Hanks salt solution showed to be more susceptible for stress effects caused by exposure than those cultured in Medium 199 (data not shown). This may be caused either by the lack of nutrients or by the supplemented non-enzymatic antioxidant vitamin E in Medium 199 by scavenging ROS and thus protecting hepatocytes against oxidant challenge.

3.1. CYP450 biotransformation activity
Independent of culture conditions and time of incubation, hepatocytes of males and females showed a significant induction of CYP450 as measured as EROD activity due to exposure to different concentrations of B[a]p for 1 d, but induction in males was significantly higher (Fig. 1A, B). Exposure to NF and \( H_2O_2 \) had no effect on CYP450 activity after 1 d. After 9 d of exposure, CYP450 activity showed the same induction profile, independent of culture conditions (data not shown). 17-\( \beta \)-Estradiol alone had no
Fig. 1. Activity of CYP450 (% of total fluorescence) following 1 d of exposure to B[a]p (100, 200, 400 μM), NF (50 μM), hydrogen peroxide (100 μM), 17-β-estradiol (0.2, 1, 2 μM), 17-β-estradiol (1 μM) and B[a]p (100 μM) and solvent (DMSO) control (1 %) in living hepatocytes of male (A), and of female flounder (B). *, significantly different from controls.
effect on CYP450 activity but it inhibited induction by 100 μM B[a]p in both sexes (Fig. 1).

Independent of culture conditions and treatment, NADPH-CYP450 reductase activity was low and not significantly induced in hepatocytes of males and females (data not shown). Hepatocytes also showed almost no formazan production by intracellular O$_2^\cdot$ formation using the TNBT assay (data not shown). In xenobiotic biotransformation O$_2^\cdot$ a direct product of CYP450-mediated redox cycling of various pollutants including B[a]p and NF. We did not observe a significant induction following treatment independently of time of exposure, sex or culture conditions. This indicates that either oxidative stress induction was mainly based on ROS converted from O$_2^\cdot$ via dismutation or metal-catalysed Haber-Weiss-type of reactions (Halliwell and Gutteridge, 1986) and/or the TNBT method was too insensitive for our purpose. All stressors have been shown to be potent prooxidants in short-term exposure of isolated hepatocytes of flounder using the fluorescent assay based on the detection of ROS such as H$_2$O$_2$ and peroxynitrite by dihydrorhodamine 123 (Winzer et al., 2000) and the discussion is based on these previous results.

3.2. Cellular antioxidative defence

3.2.1. Reduced glutathione in living cells

Intracellular concentrations of reduced glutathione varied depending on sex and culture conditions. Reduced glutathione was slightly but significantly decreased in female hepatocytes following 1 d of exposure to B[a]p and H$_2$O$_2$ (Table 2), whereas hepatocytes of males showed a less pronounced decrease if any depending on culture conditions (Table 2). Reduced glutathione showed minimal levels after 6 h of exposure in hepatocytes of males (approx. 20 % reduction) and after 8 h in hepatocytes of females (approx. 40 % reduction) whereas 1 d of exposure had less effect (Table 2). Therefore, we concluded that the capacity of non-enzymatic antioxidant response in females was diminished to a stronger degree after exposure. The smaller effect after 1 d of exposure indicates that reduced glutathione levels can be regenerated (Table 2).
Table 2. Alterations in levels of reduced glutathione in living hepatocytes of flounder after exposure to various concentrations of 17-ß-estradiol, B[a]p and B[a]p plus 17-ß-estradiol, H₂O₂ and NF as compared with levels of reduced glutathione in untreated hepatocytes of each sex. n.e., no effect; -, low but significant effect (20%); --, moderate effect (40%); ---, distinct effect (60%); ---, strong effect (appr. 80% inhibition).

<table>
<thead>
<tr>
<th>exposure</th>
<th>males</th>
<th>females</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-ß-estradiol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µM</td>
<td>n.e.</td>
<td>--</td>
</tr>
<tr>
<td>2 µM</td>
<td>n.e.</td>
<td>--</td>
</tr>
<tr>
<td>10 µM</td>
<td>-</td>
<td>--</td>
</tr>
<tr>
<td>100 µM B[a]p + 17-ß-estradiol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µM estradiol</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>10 µM estradiol</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>xenobiotics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µM B[a]p (6/8 h)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>100 µM B[a]p (1 d)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>100 µM H₂O₂</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>50 µM NF</td>
<td>n.e.</td>
<td>n.e.</td>
</tr>
</tbody>
</table>

Various concentrations of 17-ß-estradiol resulted in sex-specific alterations in non-enzymatic responses (Table 2). 17-ß-Estradiol at all concentrations tested (1, 2 and 10 µM) resulted in significantly decreased levels of reduced glutathione in females, whereas levels only decreased in males following exposure to 10 µM 17-ß-estradiol which is approx. 50 times the maximum physiological concentration. The cumulative effect of 17-ß-estradiol and B[a]p on levels of reduced glutathione was low in females, whereas it was distinct in hepatocytes of males using 10 µM of 17-ß-estradiol (Table 2). This finding supports our idea that effects caused by oxidative stressors are enhanced by 17-ß-estradiol.

3.2.2. DTD activity

DTD can use either NADH or NADPH as substrate, but activity of DTD in hepatocytes of males and females was 50% lower when NADPH was used as substrate instead of NADH. All stressors and 17-ß-estradiol had only a slight sex-independent effect if any on DTD induction with NADPH as substrate.

In contrast, high concentrations of B[a]p (400 µM) were effective in induction of DTD activity with NADH as substrate after 1 d of exposure in both sexes (data not shown).
Sex differences after 1 d of exposure were not observed. Following long-term incubation (9 d) DTD activity was generally 2-fold higher in untreated and exposed cells of males than of females. Hepatocytes of both sexes also showed DTD induction after exposure to lower concentrations of B[a]p (100 μM), but to 50 μM NF only in females (Fig. 2A, B). When the basic levels of DTD activity are taken into consideration, the percentage of B[a]p-induction in males was only 60% as compared to 100% in females, and only females reacted to H2O2.

Various concentrations of 17-β-estradiol (0.2-2 μM) had a slight but significant effect on DTD induction in both sexes after 1 d (data not shown), whereas DTD induction was persistent only in hepatocytes of females after 9 d (Fig. 2A, B). Combined effects of B[a]p and 17-β-estradiol were not cumulative, but resulted in slightly, but significantly increased DTD activity in both sexes (data not shown).
Fig. 2. Activity of DTD (absorbance values) following 9 d of exposure to B[a]p (100 μM), NF (50 μM), hydrogen peroxide (100 μM), 17-β-estradiol (2 μM), and solvent (DMSO) control (1%) in living hepatocytes of male (A), and female flounder (B). *. significantly different from controls.

3.3. Cellular detoxification by ALDH

Induction of ALDH activity was significant after 1 d of exposure to the various concentrations of B[a]p in both sexes. This effect was more pronounced in the absence of FBS (Fig. 3A, B). Yet, only males showed slight induction of ALDH activity after 1 d of exposure to NF, whereas H₂O₂ had no effect on ALDH activity in both sexes independently of the duration of exposure. Induction by B[a]p was slightly higher in hepatocytes of males. ALDH activity was also significantly higher in untreated and exposed male hepatocytes than in female hepatocytes. Following 9 d of exposure, there were no sex differences in ALDH activity in unexposed and exposed hepatocytes, and only exposure to B[a]p induced ALDH activity (data not shown).

Additional 17-β-estradiol in various concentrations (0.2 - 2 μM) resulted in increased ALDH activity only after 9 d of exposure in hepatocytes of both sexes (data not shown) whereas 1 d of exposure did not have an effect (Fig. 3A, B).
Fig. 3. Activity of ALDH (absorbance values) following 1 d of exposure to B[a]p (100, 200 μM), NF (50 μM), hydrogen peroxide (100 μM), and solvent (DMSO) control (1%) in living hepatocytes of male (A), and female flounder (B). * significantly different from controls.
3.4. Cellular injury
Immediately after the isolation procedure, viability of isolated hepatocytes was minimally 95% and decreased gradually with time to approx. 80% at 48 h after isolation. Of the oxidative stressors, only high concentrations of B[alp] and DMSO alone significantly reduced viability of hepatocytes (approx. 15%) after 1 d of exposure (Fig. 4), but differences between exponent and solvent control were not significant. Sex differences or effects of 17-β-estradiol on viability were not observed. At 9 d after isolation, only cells cultured in the presence of FBS were still alive, showing more than 60% viability in unexposed and exposed cells. Thus, FBS treatment did not increase viability of flounder hepatocytes in short-term culture or exposure experiments up to 2 d, but its use is a necessity in long-term experiments. Hepatocytes used for all experiments never showed viability <60%.

Fig. 4. Viability (%) of hepatocytes following 24 h of exposure to B[alp] (100, 200 and 400 μM), B[alp] plus 17-β-estradiol (2 μM), 17-β-estradiol alone (2 μM), NF (50 μM), hydrogen peroxide (100 μM), and solvent (DMSO) control (1, 2 and 4 %). *, significantly different from controls.
Sex, stressors and 17-ß-estradiol did not show significant effects on membrane stability of hepatocellular lysosomes, but FBS treatment of hepatocytes of both sexes resulted in significantly increased membrane stability in control and exposed cells (Fig. 5). This suggests that the stress-reducing effects of FBS such as effective inhibition of intracellular ROS formation and oxidative injury (Winzer et al., 2000 and in preparation) are not caused by direct scavenging of ROS by FBS but rather by improving membrane stability.

Fig. 5. Lysosomal stability (% of damaged cells per time period) following 1 d of exposure to B[a]p (100 µM), NF (50 µM), hydrogen peroxide (100 µM), and solvent (DMSO) control (1%) in hepatocytes of male and female flounder in the presence or absence of FBS.

Sex-specific differences were not detected in the amount of neutral lipids as stained with the Oil Red O assay in primary hepatocyte cultures of immature flounder (data not shown). The two-weeks period of adaptation and standard feeding of fish before sacrifice eliminated possible nutritional effects on lipids in this study.
4. Discussion

In the present study, initial steps of xenobiotic toxicity and oxidative stress responses were investigated in living hepatocytes of flounder. We performed these experiments in order to elucidate xenobiotic-specific and gender-specific phase I and II biotransformation and antioxidant defence in these cells. Our goal was to investigate whether sex-dependent differences in cellular responses could be the basis of the higher vulnerability of female flounder to xenobiotic-induced hepatocellular toxicity and carcinogenesis.

Following exposure to B[a]p, we observed a rapid and sensitive induction of CYP450, DTD and ALDH activity, as well as significant reduction of levels of reduced glutathione. Direct induction of synthesis of phase I and II biotransformation enzymes such as CYP4501A, DTD, ALDH, and glutathione-S-transferase is possible by xenobiotics like polycyclic aromatic hydrocarbons such as B[a]p via direct binding to the aryl hydrocarbon (Ah) receptor (AhR; Nebert et al., 1990; Celander, 1993; Stegeman and Hahn, 1994). Sex differences were apparent in B[a]p-induced enzyme induction and antioxidant response. Males showed a significantly higher induction of CYP450 by B[a]p than females. Thus, their capacity for one-electron reduction and consequently quinone production and generation of ROS through redox cycling was increased, indicating a more rapid one-electron reduction-based biotransformation of B[a]p in male flounder (Stegeman and Chevion, 1980; Edwards et al. 1988; Winzer et al., 2000). Nevertheless, sex differences in ROS production were minor which indicates that factors other than ROS caused sex differences in stress responses (Table 3; Winzer et al., 2000). DTD-related sex differences became apparent only after long-term exposure to low concentrations of B[a]p. Although hepatocytes of females showed a higher induction of DTD activity by B[a]p after 9 d of exposure, hepatocytes of males contained a higher basic activity of DTD. Because DTD catalyses two-electron reduction and therefore prevents redox cycling of xenobiotic diones through inhibition of semi-quinone radical formation (Hajos and Winston, 1991), this enzyme is involved in xenobiotic biotransformation (phase II) of B[a]p without generation of ROS. Consequently, male flounder liver may be more efficient than female flounder liver in
xenobiotic two-electron-based biotransformation and thus, in the elimination of toxicants during long-term exposure. Still, the exact role of DTD in sex-specific xenobiotic toxicity remains to be investigated, because Lemaire et al. (1996) reported a possible prooxidant role of purified hepatic DTD in one-electron biotransformation catalysing NADH-dependent AH-quinone-mediated ROS production but this role has not yet been established in vivo.

ALDHs, the most important aldehyde oxidising and detoxifying enzymes that we know, metabolise not only all endogenous aldehydes that are natural metabolic products (Koivusalo et al., 1982; Vasiliiou, 1993), but also aldehydes that are generated during xenobiotic biotransformation or during xenobiotic-induced lipid peroxidation (Benedetti et al., 1980; Esterbauer, 1982; Esterbauer et al., 1982; Harvey and Lindahl, 1982; Esterbauer, 1985). NAD-dependent ALDH reacts very early to xenobiotic exposure (Winzer and Köhler, 1998). In the present study hepatocytes of males were slightly more effective than hepatocytes of females in NAD-dependent ALDH-mediated detoxification of damaging aldehydes (Esterbauer, 1985, Dianzani, 1985) following short-term exposure to B[a]p. This may well be related to a direct binding of B[a]p to a NAD-dependent ALDH which has been found in mammals (Lesca et al., 1998).

Females showed a higher susceptibility to B[a]p due to the lower levels of reduced glutathione in hepatocytes, a major and effective non-enzymatic low molecular weight protector against lipid peroxidation (Bell et al., 1984). Lower levels of reduced glutathione may result from its antioxidant activity that generates oxidised glutathione. NADPH-dependent activity of glutathione reductase to regenerate reduced glutathione may then explain the re-increase of levels of reduced glutathione that were observed after 1 d of exposure to B[a]p. Depletion of reduced glutathione in oxidative stress can also occur as consequence of increased activity of glutathione peroxidase in removal of H₂O₂ or of glutathione-S-transferase in biotransformation of xenobiotic metabolites or aldehydes produced by lipid peroxidation. Consequently, we conclude that females have a reduced capacity of non-enzymatic antioxidant activity as compared with males following short-term exposure to B[a]p. In conclusion, the disadvantage of increased ROS production by rapid induction of CYP450 in B[a]p metabolism in males may be
ruled out by a more effective defence against oxidative damage and a faster phase I and II biotransformation and thus faster elimination of toxic metabolites.

In addition, enzymatic and non-enzymatic formation of B[a]p-dinones via CYP4501A or cooxidation of B[a]p and products of lipid peroxidation (Teroa et al., 1987) and thus, ROS production during their redox cycling, is relatively low in livers of fish including flounder (Lemaire et al., 1993; Lemaire et al., 1994). However, there are other pathways of B[a]p metabolism which result in DNA or RNA adduct formation as described in mammals that may be of more importance (Lemaire et al., 1994; Smeets et al., 1999a): (1) double monooxygenation of B[a]p also mediated by CYP450 that can generate for example epoxides; (2) one-electron oxidation mediated via CYP450 or peroxidative reactions forming radical cations; and/or (3) bioalkylation that produces benzylic carbenium ions (Sims et al., 1974; Wislocki and Lu, 1988; Varanasi, 1989; Stansbury et al., 1994; Cavalieri and Rogan, 1995). These processes may augment B[a]p-mediated toxicity. To our knowledge gender-specific studies on these different pathways have not yet been carried out in fish.

In contrast to B[a]p, NF did not mediate AhR-related enzyme induction. After NF exposure, we detected only a long-term induction of DTD activity in females, whereas CYP450, ALDH and levels of reduced glutathione were not affected. In general, basic activities of DTD and ALDH were higher in hepatocytes of males than of females providing a higher capacity of phase II biotransformation. Nitroaromatics including NF exert their toxicity in anaerobic and aerobic metabolism via one-electron or two-electron reduction by nitroreductases such as CYP450 reductase, xanthine oxidase or DTD. Direct NAD(P)H-dependent redoxcycling of NF and ROS formation by autoxidation of the nitro anion radical following one-electron reduction (Mason, 1990) was found to be predominantly related to microsomal CYP450 reductase and to cytosolic xanthine oxidase in mussels, cat fish and mammals (Harada and Omura, 1980; Kappus, 1986; Washburn and DiGiulio, 1988; Walker and Livingstone, 1992; Martinez et al., 1995; Mitchelmore et al., 1998). DTD in the cytoplasm limits ROS formation and redox cycling via two-electron reduction of nitroaromatics to nitroso and amino products that may exert toxicity by binding to DNA (Kappus, 1986; Washburn and DiGiulio, 1988;
In accordance to Washburn and DiGiulio (1989), we did not observe a significant induction of NADPH-CYP450 reductase by NF or any of the stressors. Thus, higher susceptibility to NF-mediated oxidative damage in males as detected by increased lipid peroxidation (Table 3; Winzer et al., 2000) may be related to sex-dependent activity of xanthine oxidase or substrate specificity of CYP450 reductase or to the toxicity of two-electron reduction metabolites.

$\text{H}_2\text{O}_2$ exerts its toxicity either directly by oxidation of SH-groups in proteins or indirectly via formation of hydroxyl radicals. Sex differences in $\text{H}_2\text{O}_2$-induced long-term effects may be due to the higher efficiency of antioxidant activity and detoxification of lipid peroxidation products in male livers.

In our study, $17\beta$-estradiol downregulated CYP1A induction by polycyclic aromatic hydrocarbons and reduced the capacity of antioxidant defence systems as demonstrated by decreased levels of reduced glutathione, long-term induction of DTD activity, and delayed induction of ALDH in living hepatocytes. Therefore, $17\beta$-estradiol and B[a]p exerted similar hepatocellular responses at the level of reduced glutathione, DTD, and ALDH which may implicate that these pathways/enzymes are regulated by the same receptor, for example the estrogen receptor (ER) or AhR. Sex differences in response to $17\beta$-estradiol and xeno-estrogens may well depend on sex-specific regulation of the ER (Legler et al., 1996; Janssen et al., 1997). This may explain the higher susceptibility to specific toxic injury and the higher incidence of cancer in livers of female flatfish than in livers of males (Köhler, 1995; Landwüst et al., 1996).

Alterations in the metabolic steps described here clearly precede irreversible cellular damage such as lysosomal membrane stability and reduced viability. Therefore, these parameters have essential function to keep up homoestasis and protect cells from injury. Processes associated with the onset and progression of degenerative toxicopathic liver lesions such as lysosomal destabilisation (Köhler, 1991; Köhler and Pluta, 1995) may not occur in early responses to short-term experimental oxidative stress as long as
Table 3. Xenobiotic-induced alterations in enzyme activities in hepatocytes of male and female flounder following 24 h of exposure. Based on present data and Winzer et al. (submitted). Alterations from levels or activities in untreated hepatocytes of the same sex are indicated by + for induction, - for inhibition for the specific xenobiotic (B[a]p or NF). High numbers of symbols indicate strong effects, n.e., no effects.

<table>
<thead>
<tr>
<th>Biomarkers of exposure</th>
<th>Control (basic)</th>
<th>Responses after B[a]p - exposure</th>
<th>Responses after NF - exposure</th>
<th>Responses after H₂O₂ - exposure</th>
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<tr>
<td></td>
<td>M/F</td>
<td>M</td>
<td>F</td>
<td>M/F</td>
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<tr>
<td>Biotransformation (phase I)</td>
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<tr>
<td>CYP450</td>
<td>M=F</td>
<td>++</td>
<td>+</td>
<td>M&gt;F</td>
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<tr>
<td>CYP450 reductase</td>
<td>M=F</td>
<td>n.e.</td>
<td>n.e.</td>
<td>M=F</td>
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<tr>
<td>ROS formation</td>
<td>M=F</td>
<td>++</td>
<td>+</td>
<td>M=F</td>
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<tr>
<td>Antioxidative defence</td>
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<td>Total scavenging capacity</td>
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<tr>
<td>GSH</td>
<td>M=F</td>
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<td>M&gt;F</td>
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<td>Biotransformation (phase II)/ Detoxification</td>
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<td>DTD</td>
<td>M&gt;F</td>
<td>+</td>
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<td>ALDH</td>
<td>M&gt;F</td>
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<td>+++</td>
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<td>G6PDH</td>
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<td>M&gt;F</td>
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<tr>
<td>damage lipid peroxidation</td>
<td></td>
<td>n.e.</td>
<td>+++</td>
<td>M&lt;F</td>
</tr>
</tbody>
</table>

Protective mechanisms are preventing membrane damage. However, using a sensitive parameter for early responses in short-term exposure, namely lipid peroxidation (Zahn and Braunbeck, 1994), sex-differences in early cellular damage following exposure to oxidative stress were observed previously (Winzer et al., 2000). Induction of lipid
peroxidation following exposure to NF and B[a]p, both xenobiotics that require activation by biotransformation, was different in hepatocytes of male and female flounder (Table 3).

The sex differences described in the present study with respect to biotransformation, antioxidative defense and detoxification may not be dramatic when considered individually, but the synergism of responses indicates that male flounder have a more efficient early defence against xenobiotic toxicity than females (Table 3). In addition, glucose-6-phosphate dehydrogenase, the major NADPH-generating enzyme in cells, was shown to be rapidly inhibited by xenobiotics and again to a far stronger degree in females than in males (Winzer et al., submitted). The lower supply of NADPH in female livers may further decrease efficiency of NADPH-dependent biotransformation (e.g. CYP450) and non-enzymatic defence (e.g. reduced glutathione) in female flounder and thus, higher susceptibility of females to toxicants and carcinogens in marine environments.

In conclusion, our present results indicate that the sex differences in metabolic pathways that are used for xenobiotic biotransformation may be the cause of the sex-dependences of xenobiotic-induced toxicity in flounder liver. Yet, effects like enzyme saturation, concentrations of stressors and their combinations as well as duration of exposure are known to be relevant factors for toxicant assessment as well (Smeets et al., 1999). Therefore, these aspects have to be included as well for our understanding of sex-dependent xenobiotic toxicity.

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CHAPTER VI


Winzer, K., Becker, W., Van Noorden, C. J. F., Köhler, A., in press. Sex-related response to oxidative stress in primary cultured hepatocytes of European flounder (*Platichthys flesus* L.). Aquatic Toxicology


