Oxidative stress in the marine environment—prognostic tools for toxic injury in fish liver cells
Winzer, K.

Citation for published version (APA):
Sex-related responses to oxidative stress in primary cultured hepatocytes of European flounder (*Platichthys flesus* L.)

Katja Winzer,
Gary W. Winston, Wilhelm Becker,
Cornelis J.F. Van Noorden, Angela Köhler
CHAPTER III

Abstract

Effects of oxidative stress induced by xenobiotic compounds were studied in primary cultures of isolated hepatocytes of immature European flounder (Platichthys flesus L.) of both sexes caught in a relatively unpolluted area of the German Bight (North Sea). Cells were exposed to oxidative stressors such as 100 μM hydrogen peroxide (H₂O₂), 100 μM benzo[a]pyrene (B[a]p) and 50 μM nitrofurantoin (N-(5-nitro-2-furfurylidene)-1-aminohydantoin; NF) for 2 and 24 h. Cell mortality was determined with the use of the fluorescent ethidium homodimer-1 and calcein-AM. Oxidative stress response was assessed by quantitative analysis of (1) intracellular reactive oxygen species (ROS) formation with dihydrorhodamine 123, (2) lipid peroxidation on the basis of concentrations of lipid hydroperoxides and the lipid peroxidation products malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) and (3) cellular total oxidant-scavenging capacity (TOSC) using the TOSC assay (Winston et al., 1998). An increase in ROS formation was detected as early as 2 h after exposure to H₂O₂, B[a]p and NF. After 24 h, stress responses were lower, except following exposure to NF. The pattern of responses differed with the different oxidative stressors. Lipid peroxidation and the capacity to scavenge ROS were increased significantly in both sexes only after exposure to H₂O₂, whereas B[a]p and NF provoked sex-dependent responses. B[a]p-induced lipid peroxidation and increase in scavenging capacity were observed only in hepatocytes of females, whereas NF initiated these responses only in cells of males. Sex differences in oxidative stress response only after exposure to pro-oxidants that require enzymatic activation infer the importance of biotransformation pathways in stress responses. Because of their sensitivity to oxidative stress, flounder hepatocytes provide a useful model for early risk assessment of xenobiotics.

Keywords: ROS; total oxidant scavenging capacity; primary flounder hepatocyte culture; hydrogen peroxide; benzo[a]pyrene; nitrofurantoin; Platichthys flesus L.
CHAPTER III

1. Introduction

Reactive oxygen species (ROS) are formed in various metabolic steps in aerobic organisms, such as reduction of molecular oxygen to water during mitochondrial electron transport, microsomal electron transport, activity of various enzymes such as xanthine oxidase (EC 1.1.3.22) and autoxidation of small molecules (Freeman and Crapo, 1982; Winston and Cederbaum, 1983; Cadenas et al., 1984). These cellular processes mainly form superoxide anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (HO$^-$) and peroxy radicals (ROO$^-$). The reactivity of the different ROS and thus their damaging effect on biological macromolecules varies depending on the radical species (Halliwell and Aruoma, 1991). Especially, HO$^-$ is a highly potent oxidant. Cellular defence systems such as antioxidant enzymes (e.g. superoxide dismutase, EC 1.15.1.1, catalase, EC 1.11.1.6, NAD(P)H dehydrogenase/DT-diaphorase, EC 1.6.99.2) and non-enzymatic scavengers (e.g. vitamins A and E, reduced glutathione) protect cells against DNA strand breaks, protein oxidation and lipid peroxidation which may result in loss of integrity of lipid membranes and generation of toxic aldehydes (Winston and Di Giulio, 1991).

Many pollutants are known to induce formation of ROS during biotransformation via redox reactions (Mather-Mihaich and DiGiulio, 1986; Kappus, 1987; DiGiulio et al., 1989). Compounds such as quinones, nitroaromatics and aromatic amines undergo univalent reduction that is catalysed by NAD(P)H-dependent flavoprotein reductases (Kappus, 1986). The resulting xenobiotic anion radicals react with molecular oxygen to produce O$_2^-$ and concomitant regeneration of the parent compound. Other ROS, such as H$_2$O$_2$ and HO$, are formed through dismutation or via metal-catalysed Haber-Weiss-type of reactions (Halliwell and Gutteridge, 1986). Increased production of intracellular ROS may lead to imbalance between antioxidant defence and pro-oxidant challenge. Overstressing the capacity of cellular protective mechanisms provokes processes like early aging, cell death and/or carcinogenesis (Halliwell and Gutteridge, 1984; Esterbauer, 1985).
Primary cell cultures are frequently used as standardised tools for metabolic research (Braunbeck, 1993; Anderson et al., 1996) although isolated cells may not reflect the functional integrity of the entire organ. Yet, the fact that primary cell cultures allow parallel investigations of control and exposed living cells in large quantities from the same animal is a major advantage for the interpretation of results because inter-individual variations is ruled out. As the liver is the main organ for biotransformation and vitellogenesis, hepatocytes of vertebrates have been used routinely in xenobiotic research (Moldeus et al., 1978; Moon et al., 1985; Braunbeck, 1993; Segner and Lenz, 1993). The present study is one of the first attempts to apply primary hepatocyte cultures of a marine fish species to monitor early stages of ROS damage (Moore, 1992; Fessard and Livingstone, 1998). The aim of our investigation was to develop a test system in order to analyse quantitatively the effects and early stages of oxidative stress in viable hepatocytes of a marine flatfish, the European flounder (*Platichthys flesus* L.). Marine flatfish are frequently used as sentinel species in international monitoring programmes of biological effects of contaminants in coastal waters and estuaries (Malins et al., 1984, 1985; Myers et al., 1990; Vethaak and Jol, 1995; ICES, 1996; AMAP 1998; ICES, 1999). Field studies in highly contaminated estuaries of the North Sea have revealed frequencies of liver cancer up to 40% in adult female European flounder although this tendency is decreasing (personal communication, Köhler and Vethaak; Köhler and Van Noorden, 1998; Winzer and Köhler, 1998). A sensitive test system for early signs of toxicity of various pollutants would be of significant benefit for the understanding of the cascade of oxidative stress responses and the putative sex-dependent initiation of carcinogenesis.

In the present study, we selected stressors that have been shown to be effective model inducers of oxidative stress in short-time exposure studies in aquatic organisms such as \( \text{H}_2\text{O}_2 \) (Buchner et al., 1996; Abele-Oeschger et al., 1997; Abele et al., 1998), \( \text{B}[\text{a}]\text{p} \) (Lemaire and Livingstone, 1994, 1995, 1997; Canova et al., 1998; Winzer et al., in press) and NF (Hartter, 1985; Washburn and DiGiulio, 1988, 1989; Lemaire and Livingstone, 1994; Martinez et al., 1995). Cellular responses to stress exposure were
measured at the levels of initiation of ROS formation, capacity of antioxidative defences and cellular damage in terms of lipid peroxidation.

2. Materials and Methods

2.1. Chemicals
Ethidium homodimer-1, calcein-AM, compounds of the Live/Dead viability/cytotoxicity kit and dihydrorhodamine 123 were purchased from Molecular Probes (Eureka OR, USA). Lipid peroxidation kits, Bioxytech ® LPO-560TM and LPO-586TM, were obtained from Oxis Health Products (Portland OR, USA). Collagenase Type IV and all other reagents were obtained from Sigma (St. Louis MO, USA).

2.2. Animals
Immature European flounder of both sexes with a body length of 17 - 25 cm were caught 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). Fish were caught with a bottom trawl (opening, 1.5 m; mesh width at the cod end, 40 mm). Fishing periods were limited to 30 min to minimise sampling stress. To allow stress recovery and adaptation, fish were kept in tanks at 15°C with permanent water flow-through and aeration and were fed ad libitum with artificial food (Trouvit, Milkivit-Werke, Burgheim, Germany) for at least 2 weeks before sacrifice.

2.3. Primary hepatocyte cultures
Animals were killed by cervical transection with a knife followed by severing the spines, and livers were removed immediately. Hepatocytes were isolated by mechanical destruction of the freshly dissected organs as previously described by Winzer et al. (in press) according to Lowe et al. (1992). After washing in Hanks salt solution, liver pieces were incubated for 40 min under gentle shaking conditions in Hanks salt solution to
which 20 mg collagenase per liver was added. Subsequently, cell suspensions were passed through 250 μm and 50 μm gauzes to complete cell isolation. After washing 3 times in Hanks salt solution, hepatocytes were plated at a concentration of 2x10^6 cells per well in 24-well plates in vitamin E-containing cell culture medium, Medium 199 (Sigma), supplemented with 20 mM Hepes, 1% penicillin/streptomycin in the presence or absence of 10% fetal bovine serum (FBS). Cell isolation and cell culture were performed at 10°C under sterile conditions. Cells were kept in the dark for up to 48 h at pH 7.5.

2.4. Exposure of cells to oxidative stressors

Stock solutions of oxidative stressors were prepared in DMSO (final dilution in the media, 1%) except for H_2O_2 that was diluted in Medium 199. Final concentrations of H_2O_2 (100 μM), NF (50 μM), B[a]p (100 μM) and DMSO alone were obtained by adding the necessary amount of the stock solutions directly to the media. Exposure was started at 24 h after cell isolation. For each chemical and time of exposure tested, cells of 10 different immature fish, five males and five females each, were tested individually. Only data for lipid peroxidation and TOSC were obtained from samples of the same fish.

2.5. Cell viability

Cell viability was determined by using the Live/Dead viability/cytotoxicity kit. Aliquots (50 μl) of hepatocytes in 0.1 M phosphate-buffered saline (PBS; pH 7.2) were incubated for 5 min in 100 μl of a solution of 2 μM calcein-bisacetyloxymethylester (calcein-AM) and 4 μM ethidium homodimer-1 diluted in 0.1 M PBS (pH 7.2). Calcein-AM penetrates into living cells through the intact cell membrane and is hydrolysed by intracellular esterases to green fluorescent calcein, whereas ethidium homodimer-1 does not penetrate and therefore does not stain viable cells. It can only penetrate cells with damaged membranes such as dying or dead cells and then, stain their nucleus red. Fluorescence was determined with excitation light of 470 nm and emission light of 530 nm in living green hepatocytes and 585 nm in dead red hepatocytes. Cells were counted.
in a haemocytometer and viability was calculated as percentage of the total number of cells.

2.6. Analysis of intracellular ROS formation

Dihydrorhodamine 123 appeared to be a sensitive marker of ROS, mainly hydrogen peroxide formation in hepatocytes. Induction of oxidative stress in living cells was observed after exposure for 2 and 24 h and after incubation of the hepatocytes for 15 min in 7 μM dihydrorhodamine 123 in phosphate buffer (0.1 M; pH 7.2) (modified after Moore 1992). The reaction was stopped to prevent photo-oxidation by adding 25 μl of a solution of 1 mM N-t-butyl-α-phenylnitrone (Moore 1992) and 10 μl of 10% formaldehyde in 0.5 ml phosphate buffer (0.1 M; pH 7.2). Fluorescence was measured by excitation at 450-490 nm and emission at >520 nm using an image analysis system (Sony 3-chip CCD colour video camera, Zeiss Axioskop, Kontron KS300 software package). Data are shown as percentages of total fluorescence.

2.7. Cell preparation for TOSCA and lipid peroxidation

Cells were sampled after 2 or 24 h of exposure, washed 3 times with 0.1 M PBS (pH 7.2), frozen in liquid nitrogen and stored at −80°C. Hepatocytes were homogenised and sonicated (a) in 0.15% NaCl solution for the TOSC assay, (b) in 10 mM PBS, pH 7.4, containing 4 mM butylated-hydroxytoluene (BHT) to limit sample oxidation, for the determination of lipid hydroperoxides, and (c) in 20 mM Tris buffer, pH 7.4, containing 5 mM BHT for the determination of concentrations of MDA and 4-HNE. After centrifugation for 10 min at 3000 g, parts of the supernatants were immediately used for the assays and for protein determination.

2.8. Analysis of cellular lipid hydroperoxide production

Concentrations of lipid hydroperoxides were measured using the Biotech kit LPO-560TM. This colorimetric assay is based on the detection of lipid hydroperoxides on the basis of their potential to oxidise ferrous ions to ferric ions under acidic conditions. To eliminate interference of H₂O₂, the samples were pretreated with catalase. The change in absorbance at 560 nm was measured in the presence and absence of Tris(2-
carboxyethyl)phosphine (TCEP) which inactivates lipid hydroperoxides by their reduction to the corresponding organic alcohols.

Thawed supernatants were used within 24 h. Samples (90 μl) were incubated for 2 min with 10 μl catalase (3800 u/ml) at room temp. TCEP or deionised water (10 μl) were added to each sample and the samples were incubated for an additional 30 min at room temp. Addition of 900 μl of a solution of Xylenol orange started the colour reaction and samples were measured after 1 h of incubation and centrifugation (10 min, 3000 g) at 560 nm using a spectrophotometer (Lambda 6; Perkin Elmer, Norwalk CT, USA). Data are expressed as μg hydroperoxides per g protein.

2.9. Analysis of cellular MDA and 4-HNE production

Peroxidation of polyunsaturated fatty acids generates MDA and 4-hydroxyalkenals such as 4-HNE. These lipid peroxidation products were measured with a chromogenic reaction using N-methyl-2-phenylindole at 45°C (Bioxytech ® LPO-586TM). One molecule of MDA in hydrochloric acid and one molecule of MDA or 4-HNE in methanesulfonic acid react with two molecules of indole to yield a stable chromophore with maximal absorbance at 586 nm.

Supernatants of cells (200 μl) or standards (10 mM 1,1,3,3-tetramethoxypropane in 20 mM Tris-HCl, pH 7.4, as MDA standard and 10 mM 4-HNE diethylacetal in acetonitrile as 4-HNE standard) were mixed with 650 μl of the chromogenic reagent and then incubated for (a) 60 min at 45°C after addition of 150 μl of 12 N HCl for determination of MDA levels alone or (b) 45 min at 45°C after addition of 150 μl of 15.4 M methane sulfonic acid to determine levels of both MDA and 4-HNE. The colour reaction was measured after centrifugation (10 min, 3000 g) at 586 nm using a spectrophotometer (Lambda 6; Perkin Elmer). Data are expressed as μg MDA or 4-HNE per g protein.

2.10. Cellular TOSC assay

TOSC in supernatants of cell homogenates was measured as described previously (Winston et al., 1998; Regoli and Winston, 1999). ROS were generated by thermal decomposition of either 20 mM azo-bis-amidinopropane to produce peroxyl radicals or
1.8 μM ferric chloride and 180 μM sodium ascorbate to produce hydroxyl radicals at 35°C in 100 mM potassium phosphate buffer, pH 7.4. ROS oxidise 0.2 mM α-keto-γ-methiolbutyric acid to ethylene. Reactions were carried out in 10 ml vials sealed with air-tight Mininert™ valves (Supelco, Bellefonte PA, USA). Aliquots (200 μl) of headspaces of the reaction vials were analysed in a Hach Carle series 100 AGC gas chromatograph (Hach Carle, Loveland CO, USA) equipped with a stainless steel 80/100 mesh Poropak Q column (6 ft x 0.18 cm I.D.) and a flame ionization detector. Oven, injection and detector temperatures were 35, 160 and 220°C, respectively. Helium was the carrier gas with a flow rate of 1 ml/min. Ethylene formation was quantified as the integrated area under the kinetic curve.

TOSC values were quantified from the equation $TOSC = 100 - \frac{SA}{CA} \times 100$, where $SA$ and $CA$ are the integrated areas under the best fitting curve of the experimental points during the reaction time course for sample (SA) and control reactions (CA) (Winston et al., 1998). A TOSC value of 0 corresponded to a sample with no ROS scavenging capacity and thus, no inhibition of ethylene formation. Complete inhibition of ethylene formation during the assay indicated a maximal TOSC value of 100. Specific TOSC values were determined for all samples per g protein. When samples containing 30 to 50 μg protein were analysed, the peroxyl and hydroxyl radical scavenging activity was linear during at least 60 min. Therefore, assays were simplified by taking only 3 measurements after 12, 24 and 36 min, respectively.

Because catalase may be present in cell homogenates due to lysis of peroxisomes during homogenisation and freeze-thaw procedures, measurements of HO·-scavenging capacity of hepatocytes were also performed in media containing 1 mM sodium azide to inhibit catalase (Regoli and Winston, 1999). Under conditions of the TOSC assay, H$_2$O$_2$ is the obligatory precursor of HO· and thus, catalase may interfere in HO· production by removing its precursor. Catalase activity then results in higher TOSC measurements due to decreased levels of HO· present in the assay medium.

2.11. Determination of protein concentrations

Protein concentrations in homogenates were determined using the fluorescamine protein assay (Böhlen et al., 1973). Samples were incubated against a series of bovine serum
albumin standard dilutions for 5 min with 6 mg fluorescamine/20 ml dioxane and then read in a microplate reader (CytoFluor 2350; Millipore, Framingham MS, USA) with excitation at 360 nm and emission at 460 nm.

2.12. Statistical analysis

All data were analysed with non-parametric statistics (Kruskal-Wallis test and post-hoc Dunn’s test for small 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. Statistical significances of the differences between males and females were tested for each chemical with the Kruskal-Wallis test and post-hoc Dunn’s test.

3. Results

3.1. Viability

Viability of freshly isolated hepatocytes was minimally 95% immediately after the isolation procedure and decreased gradually with time to approximately 70% at 48 h after isolation. Of the oxidative stressors, only H$_2$O$_2$ significantly reduced viability of hepatocytes after one day of exposure (Table 1). Hepatocytes never showed less than 50% viability (Table 1) and sex differences in viability were not observed (data not shown).

Table 1: Viability of isolated hepatocytes in primary culture after exposure to no oxidative stressor (control and solvent-control (DMSO)) or 100 μM hydrogen peroxide (H$_2$O$_2$), 100 μM benzo[a]pyrene (B[a]p) or 50 μM nitrofurantoin (NF) in the presence (+) or absence (-) of fetal bovine serum (FBS). Significant differences (P=0.05) from control values (median ± standard error SE) are indicated with *.

<table>
<thead>
<tr>
<th>exposure</th>
<th>Viability - FBS (%) ± SE</th>
<th>Viability + FBS (%) ± SE</th>
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</thead>
<tbody>
<tr>
<td>2h Control and exposed</td>
<td>86 ± 8</td>
<td>86 ± 7</td>
</tr>
<tr>
<td>1d Control</td>
<td>70 ± 16</td>
<td>76 ± 8</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>55* ± 16</td>
<td>66* ± 9</td>
</tr>
<tr>
<td>NF</td>
<td>60 ± 25</td>
<td>72 ± 9</td>
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<tr>
<td>DMSO</td>
<td>66 ± 17</td>
<td>72 ± 8</td>
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<tr>
<td>B[a]p</td>
<td>55 ± 18</td>
<td>66 ± 12</td>
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</table>
3.2. Effects of fetal bovine serum (FBS)

In order to optimise culture conditions for future large scale screening tests of hepatotoxic and carcinogenic xenobiotics, effects of FBS on oxidative stress responses in culture were monitored. FBS is commonly used in primary cultures of cells including fish cells (Zahn et al., 1981, 1982; Braunbeck, 1993). In the presence of FBS, viability of control and exposed hepatocytes was slightly but not significantly higher. However, FBS-treatment had a strong inhibitory effect on ROS formation in isolated hepatocytes. In the presence of FBS, neither significant induction of intracellular oxidant production, nor of lipid peroxidation and of total antioxidative defence was observed in unexposed or exposed cells, irrespective of the type of stressor or exposure time. Therefore, FBS was omitted in all further experiments.

Recent data on membrane stability (Winzer, K., in preparation) suggest that the stress-inhibiting character of FBS is not caused by direct scavenging of ROS by FBS, but rather by enhancing membrane stability. FBS-treatment does not increase viability of flounder hepatocytes in short-term culture or exposure experiments. Thus, its use is not a necessity.

3.3. Formation of intracellular ROS

Significant induction of intracellular ROS formation was found in hepatocytes of male and female flounder after 2h of exposure to H$_2$O$_2$ and NF. B[a]p-mediated induction of ROS production was significantly increased only in hepatocytes of male flounder (Fig. 1). Levels in females were almost as high as in males (no significant sex difference as determined with the Kruskal-Wallis test). In general, stress responses were lower after 24 h than after 2 h of exposure.
Fig. 1. Formation of ROS (% relative fluorescence) in 2 and 24 h-exposed hepatocytes of male and female flounder in the absence of FBS. * mark significant differences from the specific control (control or DMSO-control at 2 or 24 h).

3.4. Formation of lipid peroxidation products

A significant increase in lipid hydroperoxide levels was found in cells of both sexes exposed for 2 h to H$_2$O$_2$ (Fig. 2). Because of their high lipid hydroperoxide concentrations after 2 h of exposure, H$_2$O$_2$-exposed hepatocytes were selected for determination of lipid peroxidation products. Increased lipid hydroperoxide concentrations were concomitant with increased concentrations of MDA and 4-HNE in the hepatocytes (Fig. 3). Sex differences may be relevant in B[a]p- and NF-mediated lipid peroxidation (Fig. 2). A strong increase in levels of lipid hydroperoxides was found in B[a]p-treated hepatocytes of females after 2 h, whereas no significant induction in lipid peroxidation was observed in hepatocytes of males after B[a]p exposure. In contrast, NF seemed to have an effect on hepatocytes of male flounder only after 24 h of exposure resulting in significantly increased levels of lipid hydroperoxides (Fig. 2).
Fig. 2. Lipid peroxidation (μg lipid hydroperoxides/g protein) in 2 and 24 h-exposed hepatocytes in the absence of FBS. * marks significant differences from the specific control (control or DMSO-control at 2 and 24 h); nd, not detected.

Fig. 3. Lipid peroxidation (μg MDA or 4-HNE/g protein) in 2 h-hydrogen peroxide-exposed hepatocytes of flounder (both sexes are pooled) in the absence of FBS. * mark significant differences from control.
Fig. 4. Specific TOSC (TOSC/g protein) for 2 and 24 h-exposed hepatocytes of male and female flounder in the absence of FBS. * mark significant differences from the specific control (control or DMSO-control at 2 or 24 h).

3.5. Total antioxidative capacity

Flounder hepatocytes displayed a significant increase in their ability to scavenge peroxyl radicals in both sexes after 2 h of exposure to H$_2$O$_2$ only (Fig. 4). A slight sex-dependent induction of the scavenging capacity was observed in hepatocytes of males after 24 h of exposure to NF and hepatocytes of females after 2 h of exposure to B[a]p as was determined with the Kruskal-Wallis-test, corresponding with increased lipid peroxidation as described above. Sex differences in TOSC were only significant after induction with NF.

When the scavenging capacity of flounder hepatocytes was measured as their ability to scavenge hydroxyl radicals, TOSC values were approximately 10% of the TOSC
values that were obtained with the peroxyl radical-generating system (data not shown). In contrast with the specific TOSC with respect to peroxyl radicals, there were no detectable differences in the HO'-scavenging capacity of hepatocytes exposed to H₂O₂, or non-exposed controls (data not shown).

Addition of the catalase inhibitor NaN₃ eliminated the contribution of catalase scavenging activity to TOSC and resulted in a residual HO'-scavenging capacity of the hepatocytes of approximately 10% in controls and 26% in cells that were exposed to H₂O₂ for 2 h (data not shown).

4. Discussion

The present study describes the potentials of primary cultured hepatocytes of marine flatfish for the study of cellular responses to oxidative stress. The cells provide a valuable tool for large scale screening of biological effects of specific compounds and identification of sequential steps involved in xenobiotic biotransformation, cellular defence and cell death. Our aim was to assess the potentials and limitations of hepatocellular antioxidative defence against oxidant challenges in the flounder hepatocyte culture system. It appeared that hepatocytes variably responded to oxidative stress by increased lipid peroxidation and antioxidant activity as indicated by elevated TOSC, which was dependent upon the period of exposure, the specific toxic challenge and sex of the animals. Xenobiotic-specific responses of hepatocytes were higher after a 2 h-exposure period than after 24 h of exposure, except for responses to NF in males. The reversible nature of the early response is probably due to a delay in antioxidant defence as described by Winzer et al. (in press) or to a decay of oxidant. It is likely that this phenomenon is specific for hepatocellular responses to direct and acute high-dose exposures in short-term cell culture, as compared with a more continuous lowe-dose exposure to xenobiotics of fish in a polluted environment. Nevertheless, our results indicate a strong potential of the cells to regenerate from a limited oxidative challenge.
In the present study, \( \text{H}_2\text{O}_2 \) appeared to be a strong oxidant as was demonstrated by the increased formation of intracellular ROS. There was no indication for the triggering factors and the sequence of the onset of lipid peroxidation and antioxidative defence because we observed significant induction in both. We also observed an elevated scavenging capacity following \( \text{H}_2\text{O}_2 \)-exposure which was independent of catalase. Although TOSC values do not distinguish between enzymatic or non-enzymatic defences, this elevated TOSC levels may reflect, in part, an increase in levels of low molecular weight and non-enzymatic antioxidants such as glutathione (GSH) and/or uric acid (Regoli and Winston, 1998). GSH is an effective protectant against lipid peroxidation (Bell et al., 1984) and GSH-depletion or oxidation may lead to irreversible cell damage (Baker et al., 1990). A shift to enhanced non-enzymatic antioxidative defence is supported by our preliminary data which showed increased levels of total GSH in hepatocytes (K. Winzer, unpublished data).

NF appeared to be a very potent and persistent inducer of ROS formation in both sexes too, but in contrast with \( \text{H}_2\text{O}_2 \), sex differences were apparent in stress responses. Only in hepatocytes of males, a significant induction of lipid peroxidation and peroxyl radical scavenging capacity was noted after 24 h of exposure. NF is redox cycled under aerobic conditions via nitroreductases and NADPH-cytochrome (c) P450 (CYP450) reductase or by xanthine oxidase. This pathway produces \( \text{O}_2^- \) (Hartter, 1985; Washburn and DiGiulio, 1988, 1989; Walker and Livingstone, 1992; Martinez et al., 1995) which in turn promotes propagation of other ROS (Kappus, 1986). Sex differences in the formation of intracellular ROS were not found by Washburn and Di Giulio (1988) on the basis of nitroreductases activity in freshwater teleost fish which is in accordance to our results. Yet, sex-related variation in cellular responses may be caused by for example, differences in ROS quenching or formation of toxic compounds as a result of anaerobic NF-metabolism. Nevertheless, our results in vitro indicate that male flounder are more susceptible to damage caused by NF exposure.

Our results on the effects of B[a]p in intact cells show a lower potential of B[a]p to generate ROS than NF. This is also reported for the microsomal fraction of flounder.
liver by Lemaire et al. (1994). Additionally, Nishimoto et al. (1992) observed no differences in B[a]p-biotransformation pathways between intact livers of female English sole in vivo and isolated hepatocytes. Generally, the pro-oxidant capacity of B[a]p is known to be lower in fish than in mammals or invertebrates, which indicates that other (additional) biotransformation pathways are more important in fish (Lemaire and Livingstone, 1995, 1997; Canova et al., 1998). We are not aware of any reports on sex differences in ROS production due to B[a]p in fish or any other species, as gender was never specified in these studies. Yet, we observed a lower pro-oxidant activity of B[a]p in hepatocytes of female flounder. We assume that in both sexes B[a]p is metabolised to B[a]p-diones by the CYP450-dependent monooxygenase system, particularly by CYP450 1A (Lemaire et al., 1994, Lemaire and Livingstone, 1995) or non-enzymatically by co-oxidation of B[a]p by products of lipid peroxidation (Teroa et al., 1987). B[a]p-diones readily undergo redox cycling that is catalysed in flounder by liver microsomes via one-electron reduction resulting in an enhanced ROS production (Lemaire et al., 1994). DT-Diaphorase prevents this redox cycling of the diones by inhibiting semi-quinone radical formation through two-electron reduction as shown in rat liver (Hajos and Winston, 1991). Thus, sex differences in ROS production in hepatocytes may well reflect differences in the activity of one- and two-electron pathways (K. Winzer, in preparation). In addition, recent investigations indicated a stronger induction of CYP450 activity by B[a]p and NF in cells of immature male flounder than in those of females (K. Winzer, in preparation). This enhances the potential for redox-cycling and ROS formation in males. Higher turn-over rates of B[a]p in male rainbow trout (Stegeman and Chevion, 1980) and a generally increased B[a]p hydroxylase activity in male winter flounder (Edwards et al., 1988) support our findings of a more rapid and mainly one-electron-reduction-based B[a]p biotransformation in male flounder.

We selected immature fish in our study to eliminate effects of seasonal variations in sex hormone levels and related suppression of CYP1A activity and diminished induction by polycyclic aromatic hydrocarbons as observed during the estradiol-induced onset of vitellogenesis in female flatfish and rainbow trout (Gray et al., 1991; Andersson and
Förlin, 1992; Lindström-Seppä and Stegeman, 1995; Anderson et al., 1996; Broeg et al., 1999; A. Köhler, unpublished). Nevertheless, endogenous levels of estradiol, other sex hormones and/or estrogenic compounds may reduce the inducibility of CYP1A activity by B[a]p and consequently ROS formation due to xenobiotics in immature female flounder. To our knowledge there is no information on basal levels of estradiol in immature flounder. Yet, the fact that it partly inhibits relevant detoxification pathways and functions as a tumor promotor in medaka (Teh and Hinton, 1998; Cooke and Hinton, 1999) may explain the significantly higher incidence of tumors in livers of mature female flatfish than in livers of males (Köhler, 1995; Landwüst et al., 1996).

Recent investigations on the content of neutral lipids in flounder liver indicate no gender-specific differences in immature flounders (K. Winzer, in preparation). This excludes sex differences in quenching of ROS due to lipids present in cells as an explanation for both, the lack of responses in lipid peroxidation and antioxidative defence to B[a]p in hepatocytes of males as compared with the strong stress responses in hepatocytes of females and the sex differences in responses to NF-exposure. Moreover, the lack of sex differences in responses to the most-likely direct acting pro-oxidant H₂O₂ in contrast to NF and B[a]p, which both require activation by biotransformation enzymes supports the idea of sex differences in activity of enzymes or the preference for different pathways for biotransformation of these two compounds. Therefore, we conclude that sex-related modulation of biotransformation plays a key role in gender-specific stress responses.

Variations in time and extent of responses to elevated levels of ROS ask for additional signals other than ROS for the onset of lipid peroxidation and antioxidative response. In our study it became clear that induction of TOSC matches induction of lipid peroxidation. This suggests that lipid peroxidation or its metabolites are involved as triggering factors in induction of cell defences. Therefore, the validation of the toxicity of a specific xenobiotic should not only depend on its potential to induce ROS formation but also on the various pathways of xenobiotic biotransformation and their potential to induce cellular damage. The methods described here can provide
information on temporal and causal relationships between increased ROS formation, onset of antioxidative responses and the capacity of living cells to protect themselves against oxidative damage. Thus, they are valuable tools for a more comprehensive understanding of the initial stages and long-term effects of oxidative stress.

Acknowledgements

This investigation was part of the PhD study of Katja Winzer at the Faculty of Biology, University of Hamburg, Germany. We like to thank Captain C. Lührs and his crew of the m/v "Uthörn" for sampling flounder. This study was supported by the Biologische Anstalt Helgoland in the Alfred-Wegener-Institute for Polar and Marine Research, Bremerhaven, Germany and by the Deutsche Akademische Austausch Dienst, Bonn, Germany.

Literature cited


CHAPTER III


CHAPTER III


