Oxidative stress in the marine environment-prognostic tools for toxic injury in fish liver cells
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Glucose-6-phosphate-dehydrogenase: the key to sex-related xenobiotic toxicity in hepatocytes of European flounder (*Platichthys flesus* L.)?

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Abstract

The role of glucose-6-phosphate dehydrogenase (G6PDH) in oxidative stress responses was investigated in isolated living hepatocytes of immature female and male European flounder (Platichthys flesus L.). Cells were exposed to sublethal concentrations of effective prooxidants such as 100 μM hydrogen peroxide (H₂O₂), 100, 200 and 400 μM benzo[a]pyrene (B[a]p) and 50 μM nitrofurantoin (NF). Because there is evidence that 17-β-estradiol inhibits certain pathways of xenobiotic biotransformation, we tested the effects of different concentrations of 17-β-estradiol (0.2, 1 and 2 μM) alone and 17-β-estradiol (1 μM) in combination with 100 μM B[a]p on G6PDH activity. After short-term (1 d) and long-term (9 d) exposure, G6PDH activity was quantified in living hepatocytes by a tetrazolium salt method using tetranitro blue tetrazolium salt (TNBT). Cells obtained from male fish generally showed a higher G6PDH activity. We observed significant inhibition by all oxidative stressors and 17-β-estradiol in both sexes of fish independently of culture conditions, but inhibition was stronger in cells of females than of males. A cumulative effect of the steroid and B[a]p was not found. Our results indicate a sex-dependent inhibitory effect of all stressors and 17-β-estradiol on G6PDH activity in flounder liver independent of prooxidant activity of the specific compound. Consequently, NADPH supply for xenobiotic detoxification and other cellular antioxidative defence mechanisms may be different in livers of female and male fish. The strongly decreased supply of NADPH in females may explain the reduced and/or delayed NADPH-dependent activity of xenobiotic biotransformation systems such as cytochrome P450 (CYP450) activity and a lower capacity of non-enzymatic defence systems such as reduced glutathione that is particularly observed in female flounder. Moreover, the strong inhibition of G6PDH in livers of female flounder may cause higher susceptibility for xenobiotic toxicity and therefore, higher incidences of cancer in contaminated environments.

Keywords: G6PDH; tetrazolium salt method; primary hepatocyte culture; antioxidative response; biotransformation; hydrogen peroxide; benzo[a]pyrene; nitrofurantoin; estrogen; image analysis; Platichthys flesus L.
1. Introduction

The major function of G6PDH, the key enzyme of the pentose phosphate pathway, was always considered to be the supply of riboses for DNA and RNA synthesis, particularly in proliferating cells (Stryer, 1988). However, a putatively more important function of G6PDH is the concomitant production of NADPH, the major cytoplasmic reducing compound, as is increasingly acknowledged (Spolarics, 1998; Biagiotti et al., 2000). These authors have provided evidence that G6PDH is the regulatory enzyme in NADPH-dependent xenobiotic biotransformation and defences against oxidative stress.

In aerobic metabolism various reactive oxygen species (ROS) are formed (Winston and Cederbaum 1983; Cadenas et al. 1984) that may cause DNA strand breaks, protein oxidation and lipid peroxidation resulting in loss of integrity of membranes and generation of toxic aldehydes (Winston and DiGiulio, 1991). Cellular defence systems such as antioxidative enzymes (e.g. superoxide dismutase, EC 1.15.1.1, catalase, EC 1.11.1.6, NAD(P)H dehydrogenase/DT-diaphorase/oxidoquinone reductase (DTD), EC 1.6.99.2) and non-enzymatic scavengers (e.g. vitamins A and E, reduced glutathione) protect cells against oxidative damage. Most of these pathways use directly or indirectly NADPH to regenerate their antioxidant activity (Spolarics, 1998). An additional prooxidant challenge can be caused by NADPH-dependent biotransformation of various xenobiotics and concomitant generation of ROS via redox reactions catalysed by CYP450 (Mather-Mihaich and DiGiulio, 1986; Kappus, 1986, 1987; DiGiulio et al., 1989). These processes may overstress the physiological capacity of protective mechanisms in cells under certain conditions, such as a polluted environment, and thus, provoke early aging, cell death and/or carcinogenesis (Esterbauer, 1985; Halliwell and Gutteridge, 1984, 1986; Halliwell and Aruoma, 1991).

17-ß-estradiol and other steroids inhibit xenobiotic biotransformation by downregulation of CYP450 induction (Anderson et al., 1996, Winzer et al., submitted). Yet, these effects of steroids are hardly acknowledged, despite the fact that involvement of steroids in xenobiotic toxicity is of increasing importance because many toxic
compounds are released into the aquatic environment and exert estrogenic or anti-
estrogenic effects on reproductive organs of marine organisms (Colborne et al., 1993;

G6PDH is very sensitive to inactivation by chronic exposure in highly contaminated
marine habitats (Köhler, 1995; Köhler and Van Noorden, 1998; Van Noorden et al.,
1997). Moreover, focal alterations in its activity provide a sensitive histochemical
parameter to detect early stages of xenobiotic-induced hepatocellular carcinogenesis in
humans and rodents before morphological changes appear (Pitot, 1989; Bannasch et al.,
1989; Bannasch, 1990; Harada et al., 1989; Stumpf and Bannasch, 1994). Therefore,
G6PDH is a relevant experimental marker for carcinogenesis in mammals (Van Driel et
al., 1997), but also has been identified as a sensitive indicator of early steps of pollution-
induced carcinogenesis in fish (Couch, 1993; Hinton, 1994; Köhler and Van Noorden,

Livers of vertebrates are the main organs of biotransformation, and therefore they are
the organs of choice for biomonitoring effects of chemical pollution of the sea
species such as the European flounder (*Platichthys flesus* L.) are often strongly affected
by liver cancer because of their close contact to sediments loaded with hepatotoxic
compounds (Malins et al., 1984, 1985; Myers et al., 1990; Vethaak and Jol, 1995;
Köhler, 1995). Living hepatocytes of flounder have proven to be very sensitive for low
dose toxic stress (Moore, 1992; Fessard and Livingstone, 1998; Winzer et al., in press).
The use of primary cultures of fish hepatocytes as *in vitro* system in xenobiotic research
eliminates inter-individual differences because many assays can be performed with
hepatocytes of one animal (Braunbeck 1993; Segner and Lenz, 1993; Anderson et al.,
1996). Therefore, primary cultures provide a standardised model that allows large scale
screening of toxicity of chemical compounds under defined conditions, despite the fact
that isolated cells may not completely reflect the functional integrity and
biotransforming activity of intact livers.
In the present study, a recently developed simple and fast cytochemical method is used for quantitative detection of G6PDH activity in individual living hepatocytes of flounder (Winzer et al., submitted) following exposure to compounds that are effective model inducers of oxidative stress during short-time exposure such as $\text{H}_2\text{O}_2$, B[a]p and NF (Hartter, 1985; Washburn and DiGiulio, 1988, 1989; Lemaire and Livingstone, 1994, 1995, 1997; Martinez et al., 1995; Buchner et al., 1996; Abele-Oeschger et al., 1997; Abele et al., 1998, Canova et al., 1998; Winzer et al., in press). Our aim was to detect early xenobiotic-induced alterations of G6PDH activity in single flounder hepatocytes under clearly defined conditions in order to establish the role of G6PDH activity in sex-dependent oxidative stress responses \textit{in vivo}.

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). G6P 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) and all other reagents were ordered from Sigma (St Louis MO, USA).

2.2. Animals and primary hepatocyte cultures

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, Broeg et al., 1999). Fish were caught, adapted for two weeks, then sacrificed and hepatocytes were isolated by mechanical destruction of the freshly dissected liver as previously described in detail by Winzer et al. (in press) and
according to Lowe et al. (1992). Isolated hepatocytes were plated at a density of $2 \times 10^6$ 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 the effects of the composition of the culture medium and to optimise culture conditions. FBS is commonly used in primary cultures of fish cells (Zahn et al., 1981, 1982; Braunbeck, 1993) because it exerts membrane stabilising effects. It also effectively inhibits intracellular ROS formation due to xenobiotic exposure (Winzer et al., in press). 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 and/or 17-ß-estradiol were exchanged every second day.

2.3. Exposure of cells to oxidative stressors and 17-ß-estradiol

Stock solutions of oxidative stressors were prepared in DMSO (maximum final dilution in the media, 1%); only H$_2$O$_2$ was diluted in Medium 199. Final sublethal concentrations of H$_2$O$_2$ (100 µM), NF (50 µM), B[a]p (100, 200 or 400 µM) were prepared by adding stock solutions directly to the media. Solvent control was performed using DMSO only. Cells were also exposed to 0.22 µM (physiological maximum in rainbow trout according to van Bohemen and Lambert, 1981; Anderson et al., 1996), 1 and 2 µM 17-ß-estradiol only or to 1 µM 17-ß-estradiol in addition to 100 µM B[a]p. Exposure was started at 24 h after cell isolation. Cells were sampled for experiments at 1 d and 9 d after the start of exposure and washed in phosphate buffered saline (PBS), pH 7.2, prior to use.

For each parameter tested, 30 cells of 3 different pools of at least 5 male or female fish were used. Cell viability was quantified using a fluorescent Live/Dead viability/cytotoxicity kit as described before (Winzer et al., in press). Fluorescence was determined with excitation light of 470 nm and emission light of 530 nm in living hepatocytes (green) and 585 nm in dead hepatocytes (red) and viability was calculated as percentage of the total number of cells.
2.4. TNBT staining procedure for the endpoint detection of G6PDH activity

We used a cytochemical assay for the demonstration of G6PDH activity in living hepatocytes of flounder as described by Winzer et al. (submitted) which was slightly modified after a tetrazolium salt method used for cryostat tissue sections (Van Noorden and Frederiks, 1992). Untreated hepatocytes were incubated in Eppendorf tubes for 15 min at room temp in 5 ml 0.1 M phosphate buffer (pH 7.4) containing 2% PVA, 6 mM G6P, 0.8 mM NADP, 0.45 mM PMS, 4 mM MgCl\(_2\), 5 mM sodium azide and 1 mM TNBT that was dissolved first in equal volumes (100 µl) of ethanol and DMSO. Reactions were carried out in the dark to prevent light-induced formazan formation (Van Noorden and Frederiks, 1992). Reactions in intact cells were stopped after 15 min using 4% formaldehyde in phosphate buffer (pH 5.3). This stopping buffer immediately terminated the cytochemical reaction. The reaction product was stable for at least 12 d when samples were kept at 4°C in the dark (Winzer et al., submitted). Controls were run in the absence of substrate or substrate and coenzyme (Van Noorden and Frederiks, 1992).

2.5. Quantification of formazan production by image analysis

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 (maximal 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 µm). An average cell height of 7.5 µm was
assumed. Enzyme activity was calculated on the basis of the fact that one molecule formazan is formed by the conversion of two molecules G6P.

2.6. Statistical analysis

As data were not normally distributed, non-parametric statistics (Kruskal-Wallis test and post-hoc Nemenyi test; Statistica 5.0-StatSoft, Tulsa OK, USA) were used for statistical analysis. P≤0.05 was taken as level of significance. Statistical differences between male and female fish were tested for each chemical compound.

3. Results

3.1. Activity of G6PDH in untreated living hepatocytes and effects of culture conditions

Cell suspensions after isolation and culturing did not contain hepatocytes only, but also nonparenchymal cells such as erythrocytes. However, morphological discrimination of these cell populations was easy using image analysis. All suspensions of hepatocytes tested showed more than 60% viability immediately before experimental use (data not shown). Again, dead cells were excluded from measurements. They were easy to distinguish on the basis of disruption of the cell membrane.

In the present study, gender-specific differences were observed. Significantly higher G6PDH activity (30-50%) was found in cells of males than of females (Fig. 1). Hepatocytes of both sexes showed a relatively high variability in activity as was also found previously (Winzer et al.; submitted). G6PDH activity was higher in cells cultured in M199 medium than in cells cultured in Hanks salt solution lacking nutrients (Fig. 1), both in the presence and absence of FBS. Therefore, we used M199 as culture medium in all further experiments. Addition of FBS to the media did not affect viability of flounder hepatocytes in short-term cultures or exposure experiments up to 2 d (data not shown). Generally, G6PDH activity was slightly but not significantly higher in cells incubated in the presence of FBS as compared with hepatocytes cultured in its absence,
and xenobiotic inhibition and sex-differences were less pronounced, but still present (data not shown).

At 9 d after isolation, G6PDH activity in hepatocytes showed a similar profile as after 2 d, but only cells cultured in the presence of FBS survived this longer culture period, showing still 60% viability in unexposed and exposed cells. Thus, the use of FBS was a necessity in long-term, but not in short-term experiments.

![Fig. 1. Activity of G6PDH (expressed as μM G6P converted per min per ml cytoplasm; median +/- quartile range) against 6 mM G6P after 15 min incubation at room temp in isolated living hepatocytes of male and female flounder (Platichthys flesus L.) cultured for 48 h in medium M199 or Hanks salt solution in the absence of FBS.](image)

**3.2. Activity of G6PDH in hepatocytes following exposure to oxidative stress**

In the present study, oxidative stressors were used in sublethal concentrations that did not influence viability of hepatocytes after short- and long-term exposure. Yet, a significant xenobiotic-induced decrease of G6PDH activity was observed independently of culture conditions and duration of cell culture in both sexes. After 1 d of exposure, hepatocytes of male flounder showed significantly higher activities than hepatocytes of
Fig. 2. Activity of G6PDH (expressed as µM G6P converted per min per ml cytoplasm; median +/- quartile range) against 6 mM G6P after 15 min incubation at room temp in isolated living hepatocytes of (A) male and (B) female flounder (Platichthys flesus L.) following 24 h of exposure to 1% DMSO (solvent control), 50 µM nitrofurantoin, 100µM hydrogen peroxide, 100, 200 or 400 µM benzo[a]pyrene, 100 µM benzo[a]pyrene combined with 1 µM 17-β-estradiol and 1 µM 17-β-estradiol alone.
females following exposure to all stressors in the absence of FBS (Fig. 2A, B). In hepatocytes of males, G6PDH activity decreased by 25-75% due to 1 d of exposure to any of the xenobiotics, whereas in female hepatocytes the decrease was 65-90%. Variation of the concentration of B[a]p (100-400 µM) did not have an effect on G6PDH activity in both sexes.

In the absence of FBS, differences in the effects of the xenobiotic compounds H2O2, NF and B[a]p on G6PDH activity were not observed, although their fate in cells is different due to their biotransformation (Winzer et al., in press).

We also observed less but significant inhibition of G6PDH activity by xenobiotic compounds in the presence of FBS (data not shown) despite the fact that FBS was found to be an effective inhibitor of xenobiotic-induced formation of ROS and thus of oxidative stress (Winzer et al., in press). Significant inhibition of enzyme activity of 15-60% in cells of males and 45-80% in females was observed in the presence of FBS following exposure to various concentrations of B[a]p and to NF, whereas H2O2 did not affect G6PDH activity.
Fig. 3. Activity of G6PDH (expressed as μM G6P converted per min per ml cytoplasm; median +/- quartile range) against 6 mM G6P after 15 min incubation at room temp in isolated living hepatocytes of (A) male and (B) female flounder (*Platichthys flesus* L.) following 9 d of exposure to 1% DMSO (solvent control), 100 μM benzo[a]pyrene, 50 μM nitrofurantoin, 100 μM hydrogen peroxide or 1 μM 17-β-estradiol in the presence of FBS.

After 9 d of exposure to 100 μM B[a]p and 50 μM NF, hepatocytes showed a similar enzyme inhibition profile as after 1 d of exposure, whereas significant inhibition by H2O2 was observed only in cells of males (Fig. 3 A, B). Thus, exposure time and FBS treatment had no further effects on G6PDH activity following exposure to compounds that undergo biotransformation in hepatocytes. Sex differences in G6PDH activity were again present in untreated and exposed hepatocytes after 9 d of culture in a similar way as after an exposure period of 24 h.

3.3. Activity of G6PDH in hepatocytes following exposure to 17-β-estradiol

17-β-estradiol had no effect on the viability of hepatocytes in either sex (data not shown). Yet, 17-β-estradiol inhibited G6PDH activity independently of its concentration (0.22, 1 and 2 μM), exposure time and culture conditions in both sexes. However, the
steroid effect was strongest in females (males: 30% inhibition, females 80% inhibition; Figs. 2 and 3). Thus, estrogen exerted similar sex-specific inhibitory effects on G6PDH activity as oxidative stressors.

Combination of oxidative stressor and estrogen was tested only during short-time exposure to 100 μM B[a]p and 17-ß-estradiol. Combined exposure to B[a]p and 17-ß-estradiol did not show an cumulative effect after this short period of exposure in females (Fig. 2B). Yet, we observed a protecting effect of 17-ß-estradiol in cells of males that compensated significant inhibition of G6PDH activity by B[a]p only or 17-ß-estradiol only (Fig. 2A). This effect was not affected by FBS.

4. Discussion

In our study, G6PDH activity in living hepatocytes appeared to be sensitive in a sex-specific manner to xenobiotic exposure. We measured rapid xenobiotic-induced inactivation of the enzyme which seemed to be independent of the prooxidant capacity and the cellular fate of the specific xenobiotic stressors. A possible mechanism of enzyme inhibition may be related to oxidation of the SH-groups in the active site of G6PDH which would explain the less pronounced inhibition by xenobiotics in the presence of FBS. This serum effect was found to completely inhibit ROS formation (Winzer et al., in press). Thus, other mechanisms including biotransforming activity or metabolites such as products of lipid peroxidation may also be involved. Rees et al. (1996) concluded that 3 mechanisms are possible for the regulation of pentose shunt activity in vivo: (1) G6PDH redistribution as relatively inactive form bound to „cellular structures“ and a more active soluble form that has been observed in sea urchin eggs after fertilisation (Swezey and Epel, 1986) or in cultured mammalian renal cells following stimulation by growth factors (Stanton et al., 1991), (2) regulation of G6PDH by the NADP/NADPH ratio (Baquer et al., 1988; Zimmer, 1992) or (3) an alteration in G6PDH isozymes (Barber et al., 1982). The first 2 mechanisms are rapid and posttranslational whereas the 3rd mechanism is transcriptional and more slowly. As the
"cellular structures" mentioned under the first option, likely involve endoplasmatic reticulum and/or ribosomes (Ishibashi et al., 1999; Köhler, unpublished results), toxically induced damage of the endoplasmatic reticulum and dispersion of ribosomes that have been observed at electron microscopic level in highly contaminated flounder livers (Köhler, 1989) may additionally be related to inhibition of G6PDH. We also cannot exclude the participation of the aryl hydrocarbon receptor in G6PDH inhibition by B[a]p. This receptor - known to be responsive to various xenobiotics including polychlorinated biphenyls PCBs and polycyclic aromatic hydrocarbons (PAHs) like B[a]p - was found to be involved in the induction of G6PDH following exposure to PCBs in mammals (Hori et al., 1997).

Reduction or inhibition of G6PDH activity may result in decreased production of riboses for DNA and RNA synthesis and NADPH. The flow of electrons is supposed to be channelled by NADPH in the endoplasmatic reticulum towards biosynthetic reactions or biotransformation activity according to the need (Butcher, 1982). Consequently, the cellular capacity to generate reducing power for growth, proliferation and biotransformation can become a limiting factor in challenged hepatocytes. Direct oxidation of NAD(P)H as a consequence of oxidative stress in xenobiotic biotransformation can add to the limitation of reducing agents (Halliwell and Gutteridge, 1999). In xenobiotic metabolism, reduced NADPH supply may not only limit NADPH-dependent biotransformation but also NADPH-dependent antioxidative defence systems such as the activity of glutathione reductase and SOD (Spolarics, 1998).

We excluded an additional supply of reducing power by the two other major NADPH-generating enzymes, malate dehydrogenase (MDH) and isocitrate dehydrogenase (IDH), to compensate for reduced G6PDH activity (Winzer et al., submitted). As G6PDH catalyses the rate-limiting NADPH-producing step in the pentose phosphate pathway, the activity of phosphogluconate dehydrogenase (PGDH), the second NADPH-producing enzyme of the pentose shunt, can also be neglected (Jonges and Van Noorden, 1989). Alternative NADH-dependent mechanisms in antioxidant defence based on ascorbate or the detoxification of toxic aldehydes by the aldehyde dehydrogenases may compensate a lack of NADPH. Yet, NADH in fish is responsible
for an increased microsomal formation of ROS than NADPH (Winston and Di Giulio, 1991). Therefore, a shift to NADH-dependent processes may elevate the risk of oxidative damage. Another compensatory mechanism to counteract pollution-induced decreases in G6PDH activity is posttranslational lowering of its $k_m$, thereby increasing NADP production at low G6PDH activity (Van Noorden et al. 1997) by mechanisms as described in option (1). Whether short-time exposure also has effects on the $k_m$ of G6PDH in living hepatocytes still remains to be studied.

In the present investigation, G6PDH activity in cells of female fish but not of male fish was almost completely inhibited by all oxidative stressors. Therefore, the supply of NADPH for NADPH-dependent biotransformation or antioxidant defence is likely to be reduced to a stronger degree in females. These sex-dependent differences in G6PDH activity may be the main cause for xenobiotic- and gender-specific variances of xenobiotic biotransformation and antioxidant response which may result in different susceptibility to the initiation of cancer (Köhler, 1995; Winzer et al., in press). Sex-related modulations of biotransformation and specific enzymatic or non-enzymatic defence systems were shown to play a role in stress responses and toxic injury in flounder hepatocytes (Winzer et al., submitted).

We summarised the sex-dependent responses to oxidant exposure in Table 1 (Winzer et al., submitted). The stronger inhibition of G6PDH activity in females by xenobiotic exposure may lead to the selection of other pathways than NADPH-dependent CYP450 biotransformation such as two-electron reduction by DTD and thus biotransformation without ROS generation (Winzer et al., submitted). In contrast, males show a higher capacity of quinone production and consequently generation of ROS through redox cycling following xenobiotic-induction by a more rapid, one-electron reduction-based NADPH-dependent biotransformation (Stegeman and Chevion, 1980; Edwards et al., 1988; Winzer et al., submitted). Furthermore, the non-enzymatic antioxidative defence capacity based on reduced glutathione is significantly lower in females than in males after oxidative exposure (Winzer et al., submitted). As the reduction of glutathione to its antioxidant agent is NADPH-dependent, again inhibition of G6PDH activity may be related to this specific stress response. For instance, Beutler (1994) showed that
G6PDH-deficient human erythrocytes were unable to induce adaptation to oxidative stress. This phenomenon was also observed in cells of yeast (Izawa et al., 1998) independent of the antioxidant function of reduced glutathione (Babiak et al., 1998) which is another indication that G6PDH is essentially involved in adaptive stress responses. Still, whether a lack of NADPH in females causes a higher susceptibility to xenobiotic toxicity or is the result from a larger direct sensitivity of G6PDH molecules to toxicants in females remains uncertain. Yet, we can clearly exclude a regulation of G6PDH activity in xenobiotic exposure by the NADH/NADPH ratio.

Table 1. 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).

<table>
<thead>
<tr>
<th>Biomarkers of exposure</th>
<th>Control (basic)</th>
<th>Responses after B[al]p-exposure</th>
<th>Responses after NF-exposure</th>
<th>Responses after H₂O₂-exposure</th>
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In the present study, we demonstrated that 17-β-estradiol inhibits G6PDH activity in living hepatocytes. We selected immature fish for our study in order to eliminate effects of seasonal variations in sex hormone concentrations and related reduction in stress responses (Gray et al., 1991; Andersson and Förlin, 1992; Lindström-Seppä and Stegeman, 1995; Anderson et al., 1996; Broeg et al., 1999; Winzer et al.; in preparation; Köhler A., unpublished). We found young females to be more susceptible than males to 17-β-estradiol-induced reduction of G6PDH activity. 17-β-Estradiol is known to induce decreased gluconeogenesis and increased glycolysis as a slow, long-term transcriptional effect after long-term exposure of mature flounder (Sand et al., 1980). This suggests that physiological levels of estrogen may already cause lower G6PDH activity in untreated immature females under natural conditions. Although mechanisms of enzyme inhibition by 17-β-estradiol are not clearly identified yet (Lindström-Seppä and Stegeman, 1995), the stronger effects of 17-β-estradiol treatment in female than male flounder (Janssen et al., 1997) are likely due to sex-dependent regulation of the estrogen receptor which is present in both sexes (Legler et al., 1996). 17-β-estradiol and stressors selected in the present study caused a similar but non-additive hepatocellular response, and therefore, a regulation by the same receptor e.g. the estrogen receptor is probable. This hypothesis is supported by the fact that many anthropogenic compounds such as various PAHs and PCBs, dichlorodiphenyltrichloro-ethylene and chlorodecone are known to exert estrogenic or anti-estrogenic effects on reproductive activity such as imposex and intersex phenomena like follicles in testis and premature vitellogenesis in juvenile females, and to a stronger degree in females (Colborne et al., 1993; Peterson et al., 1993; White et al. 1994; Harris et al., 1995; Bettin et al., 1996; ICES, 1996; Janssen et al., 1997; ICES, 1998; Kuballa et al., 1998).

Other studies showed varying results on effects of organic pollutants on G6PDH activity. Following long-term exposure to PAHs in male and female rainbow trout, Tjärnlund et al. (1996) observed no alterations in G6PDH activity of the liver. Instead, induction was found in nile tilapia after long-term exposure to PCBs (Bainy et al., 1996) and in seabream to oil (Bagnasco et al., 1991). Only, Bucher et al. (1993) observed a reduction in G6PDH activity by paper mill effluents in the liver of bullheads. Yet,
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gender was not specified in all these studies. Induction of G6PDH at the transcriptional level by increased mRNA levels as observed also in human hepatomas (Stumpf and Bannasch, 1984) or at a posttranscriptional level by e.g. nutritional regulation (Hodge and Salati, 1997), may well be related to carcinogenesis following long-term exposure. G6PDH activity becomes elevated in mammalian epithelia in early adenoma stage (Van Driel et al., 1997).

In conclusion, our data on G6PDH activity as short-term responses to toxic insults suggest posttranslational inactivation of G6PDH causing enhanced susceptibility to xenobiotic toxicity. This is related to NADPH deficiency in both sexes, but to a stronger degree in females. The additional downregulation of G6PDH activity by estrogens such as 17-β-estradiol during reproduction adds to distinctly lower NADPH supply in females which may well be related to their greater vulnerability by toxic insults observed in field studies. Livers of female fish are the main organ not only for biotransformation but also for production of yolk precursor proteins (vitellogenin). The production of fertile oocytes needs a much higher energetic supply than the corresponding reproductive activities in males. Thus, enhanced vulnerability in the livers of females may indicate a functional priority of those metabolic processes supporting reproductive success. We suggest that G6PDH inhibition reflects early cellular imbalances after xenobiotic stress and thus, is a very sensitive prognostic marker to predict manifestations of toxipathic lesions and caused by pollution.

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