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

Citation for published version (APA):
Short-time induction of oxidative stress in hepatocytes of the European flounder (*Platichthys flesus* L.)

Katja Winzer,
Wilhelm Becker, Cornelis J.F. Van Noorden, Angela Köhler
Abstract

Oxidative stress induced by xenobiotic compounds has been studied using primary hepatocytes of juvenile European flounder (Platichthys flesus L.) caught in a low polluted area of the German Bight, Tiefe Rinne (Landwüst et al., 1996). Cells were exposed to known oxidative stressors such as hydrogen peroxide and benzo[a]pyrene (B[a]p) in various concentrations (50 and 100 μM) up to 6 days. Cell mortality was determined using fluorescent ethidium homodimer-1 and calcein AM. Oxidative stress response was measured by image analysis using dihydrorhodamine 123, which is converted to fluorescent rhodamine 123 in the presence of intracellular reactive oxygen species (ROS), mainly hydrogen peroxide and peroxynitrite. ROS formation was initiated already after two hours of exposure to low concentrations of B[a]p and hydrogen peroxide. Probably due to a membrane stabilising effect of the serum factors the addition of fetal bovine serum (FBS) to the culture medium had a protecting influence on the hepatocytes and resulted in (a) an increased cell viability and (b) reduced formation of intracellular ROS during exposure. In conclusion, the assay is a sensitive tool for testing the potential of various xenobiotics to induce oxidative stress in living hepatocytes.

Key words: oxidative stress; primary flounder hepatocyte culture; dihydrorhodamine 123; benzo[a]pyrene; hydrogen peroxide

1. Introduction

Many compounds are known to induce the formation of reactive oxygen species (ROS) in organisms during biotransformation via the redox-cycle (DiGiulio et al., 1989; Kappus, 1987; Mather-Mihaich and DiGiulio, 1986). Cellular defence systems like antioxidative enzymes (e.g. catalase, DT-diaphorase) and non-enzymatic scavengers (e.g. vitamin A and E, glutathione) protect the organisms against ROS-induced damage such as DNA strand breaks, protein oxidation and the induction of lipid peroxidation.
(Winston and DiGiulio 1991). Overstressing the potential of the cellular defence, the loss of lipid structures of membranes and the production of toxic aldehydes provoke early aging and cell death or carcinogenesis (Esterbauer, 1985; Halliwell and Gutteridge, 1984). Primary cultures of hepatocytes, cells of the main biotransforming organ, of freshwater fish are frequently used as a standard model to observe cellular responses to xenobiotic exposure (Braunbeck, 1993), because they allow the parallel investigation of control and exposed cells. Our aim was to develop a test system to study ROS formation in hepatocytes isolated from the European flounder (Platichthys flesus L.), because this marine flatfish species is frequently used in monitoring potential biological effects of xenobiotics in the North Sea (ICES, 1984; Landwüst et al., 1996).

2. Methods

Immature European flounder (body length, 17-25 cm) were caught with a bottom trawl (30 min fishing periods) in the Tiefe Rinne close to Helgoland, an area of the German Bight that is relatively low polluted concerning levels of hydrocarbons and heavy metals in sediments and fish (Landwüst et al., 1996). To allow stress recovery and adaption, fish were kept in tanks at 15 °C with permanent water flow-through and aeration for two weeks before use and fed ad libitum with artificial food (Trouvit, Milkivit-Werke, Burgheim, Germany). Animals were killed by cervical transection, and the freshly dissected liver was cut into 1 mm³ slices which then were incubated in Hanks salt solution with additional collagenase (20 mg per liver) similar to the method described by Lowe et al. (1992). Cells were kept at 10 °C (which corresponds to the mean temperature at the sample site) up to 6 days in Medium 199 (Sigma, St. Louis, MO, USA) supplemented with 20 mM Hepes, 1% streptomycin and penicillin in the presence or absence of 10% fetal bovine serum (FBS; Sigma). Stock solution of B[a]p was prepared in DMSO (with a final concentration in media of only 1% DMSO (solvent control) or DMSO and 100 μM B[a]p), whereas hydrogen peroxide was diluted directly in Medium 199 (final concentration 50 or 100 μM). Exposure started at 24 h after cell isolation. Culture and exposure medium were changed every second day. Cell viability
was determined by using fluorescent ethidium homodimer-1 and calcein AM (Molecular Probes, Eureka, OR, USA). Induction of oxidative stress in the cells was observed after incubation of hepatocytes for 15 min in 7μM dihydrorhodamine 123 (Molecular Probes; cf. Moore 1992). The reaction was stopped by adding 25 μl of a solution of 1 mM N-t-butyl-α-phenylnitrone, and 10 μl of 10% formaldehyde in 0.5 ml phosphate buffer (0.1 M, pH 7.2). Relative fluorescence was measured by excitation with light of 450-490nm and emission at >520nm and an image analysis system (Zeiss Axioskop, Kontron KS300 software). Liver cryostat sections were stained with H&E and incubated to demonstrate glucose-6-phosphate-dehydrogenase (G6PDH) activity (Köhler and Van Noorden, 1998). Data were analysed with non-parametric statistics (Mann-Whitney-U-test, p<0.05; Statistica 5.0-StatSoft, Tulsa OK, USA).

3. Results and Discussion

Histology of flounder livers varied with respect to degenerative toxipathic lesions despite the fact that fish was caught in a relatively low polluted area concerning sediment and tissue data (Landwüst et al., 1996). Healthy livers consisted of two cell strings around sinusoids with high G6PDH activity whereas injured livers showed focal up to extensive necrosis combined with reduced activity of G6PDH (Winzer and Köhler, 1998). Correspondingly, variation in viability and oxidative stress response were observed in isolated hepatocytes from individual fish.

Viability of freshly isolated hepatocytes was up to 95% and declined moderately with duration of culture. Up to day 4 of culture, exposed and control hepatocytes of a liver diagnosed as healthy (referred to as “healthy hepatocytes” in the following) always showed more than 70% viability (Fig. 1). Additional FBS had no effect on the viability of these hepatocytes. However, viability of isolated hepatocytes of livers, which showed pathological alterations and decrease in enzyme activity (referred to as “injured hepatocytes” in the following), declined faster, down to less than 50% on day 3 of
culture. Here, FBS obviously reduced death rates of control and exposed hepatocytes and viability declined to less than 50% only on day 4 of culture.

*Endogeneous concentrations of ROS* varied considerably in the isolated hepatocytes but were related with diagnosed histopathology and altered G6PDH activity of the liver of each animal. ROS concentrations were significantly higher in hepatocytes obtained from

### Healthy

![Graph](image1)

### Injured

![Graph](image2)

**Fig. 1.** Viability (%) of primary cultured hepatocytes obtained of individual healthy (A, B) and injured (C, D) flounder livers, in the absence (A, C) or presence (B, D) of 10% FBS. Exposure to 1% dimethylsulfoxide (DMSO), 50 and 100 µM benzo[a]pyrene (b) and hydrogen peroxide (h) started at day one of culture. Viability was measured every day up to day 6 of culture using the fluorescent probes ethidium homodimer-1 and calcein AM.
injured livers as compared with healthy livers. FBS-treatment of hepatocytes generally resulted in decreased intracellular ROS concentrations as compared with those in non-FBS-treated cells (Fig. 2).

Fig. 2. Endogenous formation of intracellular ROS shown as % relative fluorescence of rhodamine 123 in unexposed hepatocytes of healthy and injured livers of individual fish (median of 15 cells each) (A) in the absence and (B) presence of 10% FBS in the culture medium, measured at 2 h, 1 d and 3 d after the start of the culture (day one). Significant differences with hepatocytes of healthy livers are marked by *.
Significant induction of oxidative stress in healthy hepatocytes was observed already after 2h of exposure to 50 and 100 μM hydrogen peroxide and B[a]p (Fig. 3A, 4A). The level of ROS concentration returned to almost control levels within 24 h, probably due to an increased cellular antioxidative activity, but again significant induction was observed after renewing the exposure medium and consequently the oxidative stressor on day 2 after isolation. As in unexposed hepatocytes, FBS-treatment had protecting and buffering effects on oxidative stress during exposure experiments. However, hydrogen peroxide at 100 μM concentration showed an immediate and persistent induction of intracellular oxidative stress already after 2 h in healthy hepatocytes, whereas B[a]p induced only a minor transient increase after 1 d of exposure (Fig 3B, 4B).

**INDUCTION OF ROS FORMATION by hydrogen peroxide**

![Graphs showing ROS formation](image)

Fig. 3. Induction of ROS formation by 50 and 100 μM hydrogen peroxide as compared with control cells shown as % relative fluorescence of rhodamine 123 in hepatocytes of healthy (A, B) and injured (C, D) liver tissue of individual fish (median of 15 cells each) (A, C) in the absence and (B, D) presence of 10% FBS in the culture medium, measured at 2 h, 1 d and 3 d after the start of exposure. Significant differences with control hepatocytes are marked by *.
When cells from primarily injured livers, both in the presence and absence of FBS, were exposed experimentally to additional oxidative stressors, a significant decrease in the potential to form ROS was observed (Fig. 3C, 4C, D) that corresponded with the xenobiotic induction of cell death. Only in injured hepatocytes cultured in stabilising FBS-containing medium cell death was delayed, so that a spontaneous high induction of ROS by the more effective stressor hydrogen peroxide was measured after 2 h of exposure. This obvious induction of oxidative stress in the presence of FBS contradicts its general protective activity in our experiments and excludes an ROS scavenging influence of FBS itself. A stabilising effect on intact cell membranes could explain the

**INDUCTION OF ROS FORMATION**

by benzo[a]pyrene

![Graphs showing induction of ROS formation by benzo[a]pyrene](image)

**Fig. 4.** Induction of ROS formation by 100 μM benzo[a]pyrene as compared with control cells [1% dimethyl-sulfoxide (DMSO)] shown as % relative fluorescence of rhodamine 123 in hepatocytes of healthy (A, B) and injured (C, D) liver tissue of individual fish (median of 15 cells each) (A, C) in the absence and (B, D) presence of 10% FBS in the culture medium, measured at 2 h, 1 d and 3 d after the start of exposure. Significant differences with control hepatocytes are marked by *. 
delayed induction of ROS formation whereas the influence of FBS on injured hepatocytes with already destabilised membranes might be too low. Studies on the effect of FBS on the membrane stability of flounder hepatocytes using the neutral red assay among others are in progress in our laboratory.

The present study demonstrates not only the applicability of hepatocytes in primary cultures of marine flatfish for xenobiotic research, but the extreme sensitivity of dihydorhodamine 123 to detect oxidative stress in living cells. The induction of ROS formation by both stressors, B[a]P and H₂O₂ (Abele et al., 1998; Lemaire et al., 1994), was obvious even within only 2 h of exposure, as well as variations of exposure were detectable during prolonged experimentation by specific cell signals. Whether the reversibility of stress induction is caused by the lag-phase of beginning induction of antioxidative defence or a decay of oxidant has to be further investigated. The described approach appears not only to be useful for monitoring in feral populations, but also provides a sensitive tool to identify sequential steps of xenobiotic metabolism, cellular defence and breakdown of homoeostasis in living cells.

Literature cited


