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Precision-Cut Liver Slices of *Salmo salar* as a tool to investigate the oxidative impact of CYP1A-mediated PCB 126 and 3-methylcholanthrene metabolism

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ABSTRACT

Fish isolated cell systems have long been used to predict *in vivo* toxicity of man-made chemicals. In present study, we tested the suitability of Precision-Cut Liver Slices (PCLS) as an alternative to these models that allows the evaluation of a global tissue response to toxicants, to investigate oxidative stress response to cytochrome P450 1A (CYP1A) induction in fish liver. PCLS of *Salmo salar* were exposed for 21 h to increasing doses of 3-methylcholanthrene (3-MC) and Polychlorobiphenyl 126 (PCB 126). 3-MC (25 μM) strongly induced CYP1A transcription. In dose–response analysis (25–100 μM), EROD activity was strongly increased at intermediate 3-MC concentrations. We found the counter-intuitive decline of EROD at the highest 3-MC doses to result from reversible competition with ethoxyresorufin. No increases of H_2O_2 production, antioxidant enzymes activities or oxidative damage to lipids were found with 3-MC treatments. PCLS subjected to PCB 126 (2–200 nM) showed increased contamination levels and a parallel increased CYP1A mRNA synthesis and EROD activity. H_2O_2 production tended to increase but no oxidative damage to lipids was found. As antioxidant enzymes activities declined at the highest PCB 126 dose, it is suggested that longer incubation periods could be required to generate oxidative stress in PCLS.

1. Introduction

The development of *in vitro* models to study the toxicity of man-made chemicals in fish species emerged from the need to rapidly and consistently predict the deleterious impacts of contaminants at the levels of organisms and populations (Fent, 2001). Primary cultures of hepatocytes (Laville et al., 2004; Tollefsen et al., 2008), hepatoma cell lines (Christianson-Heiska and Isomaa, 2008; Traven et al., 2008) and reporter-gene bioassays (Koh et al., 2001; Fleming et al., 2009) have all proven useful to investigate the toxicity of pollutants in fish liver. However, these isolated cell systems have limited ability to predict *in vivo* toxicity. Indeed, cell lines may respond differently to chemicals through repeated passages (Dehn et al., 2005), primary hepatocytes quickly dedifferentiate during culture (Mitaka, 1998) and reporter-gene bioassays cannot deal with the biokinetics of chemicals (Fent, 2001). These features call for the development of *in vitro* models that could better account for the complexity of fish liver organisation, in a way to improve the predictive power of the observed responses.

In that sense, Precision-Cut Liver Slices (PCLS), which have largely been used in pharmacological and toxicological studies, constitute a valuable tool to overcome the limitations of isolated cell systems, such as accelerated dedifferentiation (reviewed in Groneberg et al., 2002 and Vermeir et al., 2005). Indeed, they maintain tissue integrity (cell-cell and cell-matrix interactions) and viable cell populations for several days in culture (Lerche-Langrand and Toutain, 2000), thus allowing evaluation of a global tissue response (e.g. Van de Bovenkamp et al., 2007). Moreover, cryopreservation protocols have been developed that allow a delayed and more efficient use of liver slices in short-term toxicological investigations (for a review, see De Graaf and Koster, 2003). Importantly, several studies with microarrays confirmed that PCLS more closely predicted *in vivo* toxicity than isolated hepatocytes or established cell lines and that they were valuable tools for toxicologists (Boess et al., 2003; Elferink et al., 2008).

Although the procedure of PCLS preparation has been shown to be easily adapted to different vertebrate species (Lerche-Langrand and Toutain, 2000), this technology has rarely been applied

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to fish (and mostly on the freshwater salmonid *Onchorhynchus mykiss*). To our knowledge, Singh et al. (1996) were the first to demonstrate that fish PCLS retained biotransformation profiles comparable to isolated hepatocytes and that few signs of histological alterations were observed after 24-h culture. Kane et al. (1998), Cravedi et al. (1998) and Ohyama et al. (2004) used this *in vitro* model to investigate the integrated metabolism of model xenobiotics in fish liver (e.g. 7-ethoxycoumarin, methoxychlor, β-naphtoflavone), while Gilroy et al. (1996) demonstrated that the altered disposition of dieldrin in PCLS of pre-treated fish resulted from altered transmembrane kinetics rather than modified metabolism or lipid content. Moreover, several studies have also demonstrate the usefulness of PCLS technology to analyze the estrogenic potential of xenobiotics in fish liver (Shilling and Williams, 2000; Schmieder et al., 2000; Shilling et al., 2001). To our knowledge, PCLS have never been used to investigate xenobiotic-related oxidative stress in fish liver cells, while several studies on rodents and humans have demonstrated the benefits of PCLS technology in the field (Gallagher and Sheehy, 2001; Schmelz et al., 2001).

Oxidative stress constitutes a major toxic pathway for a broad range of chemicals in fish liver, such as the ubiquitous polychlorobiphenyls (PCBs) and pesticides (van der Oost et al., 2003; Slaninova et al., 2009). This pathological state is characterized by increased pro-oxidant forces in cells that lead to irreversible oxidative damages to macromolecules and ultimately cell death (Lesser, 2006). The precise mechanism by which PCBs disrupt the redox balance of fish liver cells is currently unclear, but could depend on the activation of hepatic phase 1 detoxification enzymes, namely cytochromes P450 (CYPs). Indeed, several CYPs, whose activities are increased upon intoxication, generate reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide, as by-products of their NAD(P)H-dependent catalysis (Halliwell and Gutteridge, 1999). In PCB-contaminated fish liver cells, the increased ROS production may impose a drain on antioxidants, notably ROS detoxifying enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX), and lead to oxidative damages and cell death.

PCBs accumulate at high levels in fish liver (e.g. Pastor et al., 1996; Ferreira et al., 2004). Strong increases of hepatic CYP1A activity, measured as ethoxyresorufin-O-deethylase (EROD), have been reported *in vivo* for PCB-contaminated fish (e.g. Kuzyk et al., 2005), as well as increased antioxidant enzymes activities (e.g. Ferreira et al., 2005) and oxidative lesions (e.g. Grinwis et al., 2001). This suggests that PCBs-mediated CYP1A activation, by promoting ROS release, generates oxidative stress in fish liver cells. In favour of this, fish exposed to PCBs in the wild or treated with an IP injection of a PCB mixture showed increased microsomal NAD(P)H-dependent ROS production (Livingstone et al., 2000).

Therefore, the present study was conducted to estimate the usefulness of PCLS technology as an *in vitro* model to study CYP1A-related oxidative impact of PCBs in liver cells of *Salmo salar*. PCB 126 was selected as this dioxin-like congener is a strong CYP1A inducer (Hestermann et al., 2000) that was shown to promote ROS leakage in fish microsomes (Schlezinger et al., 2006). A set of experiments was conducted with 3-methylcholanthrene (3-MC), a model Polycyclic Aromatic Hydrocarbon (PAH) whose rapid metabolism is not reported to increase ROS production in liver cells, as to validate assays and compare possible alterations of pro-oxidant and antioxidant parameters in response to CYP1A induction. In each set of experiments, citrate synthase (CS) activity was used to monitor the rates of aerobic metabolism, which is responsible for basal ROS production and therefore determines basal levels of antioxidant enzymes activities in cells (Janssens et al., 2000; Morales et al., 2004; Lucassen et al., 2006).

2. Material and methods

2.1. Chemicals

Leibovitz's 15 (without phenol red), PenStrep (Penicillin 10,000 U ml^{-1}–Streptomycin 10,000 μg ml^{-1}) and Hank's balanced salt solution (HBSS; with or without Ca^{2+} and Mg^{2+}) were purchased from Gibco (Paisley, UK). Foetal bovine serum (FBS; lot n° 4SB0010) was from Cambrex (Charles City, USA). Polychlorobiphenyls (PCBs) 112 (CAS 74,472-36-9) and 126 (CAS 57465-28-8) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All solvents used in PCB 126 quantification were from Fluka (Buchs, Switzerland) while Mirex (CAS 2385-85-5) was from Ultra Scientific (North Kingston, USA). Florisil Superclean SPE 6 ml cartridges were obtained from Supelco (Bellefonte, USA). 3-methylcholanthrene (3-MC; CAS 56-49-5), α-naphtoflavone (α-NF; CAS 60459-1), tricaine methane sulphonate (MS-222; CAS 886-86-2) and other reagents were purchased from Sigma–Aldrich (Milwaukee, USA). The description of the commercial kits used for the determination of lactate dehydrogenase leakage and adenosine triphosphate content, for total RNA extraction, reverse transcription and sequencing procedure were described in the appropriate sub-sections of Section 2.

2.2. Animals

Atlantic salmon (*Salmo salar* landlocked), weighing 800–1800 g, were purchased as fry from Troutlodge Incorporation (Spring Garden, USA) and cultivated in 3200 l tanks with running freshwater (15 ± 1 °C) under natural solar irradiance. Individuals were fed once daily with extruded Joosen-Luyckx Aquabio BDA 48 pellets (Turnhout, Belgium).

2.3. Slicing procedure

Fishes were anaesthetised with MS-222 (100 mg l^{-1} water). Muscle dissection of a flank, from caudal region to operculum, allowed perfusion of the liver at the portal vein with ice-cold HBSS (HBSS-; lacking Ca^{2+} and Mg^{2+}). Vena cava was cut shortly after liver whitening begun and caused the animal's death (in accordance with the *Code of Ethics of the World Medical Association*). Once perfused, liver was excised and placed in ice-cold HBSS (HBSS+; containing Ca^{2+} and Mg^{2+}). Liver cores (8 mm diameter) were obtained with a coring tool and PCLS were then generated at a thickness of 100 μm with a Krumdieck MD-1100 tissue slicer (Munford, USA), filled with ice-cold HBSS+.

2.4. PCLS culture

Slices were individually incubated for 21 h at 15 °C under moderate horizontal agitation (175 rpm) in 24-well plates with 500-μl Leibovitz's 15 medium containing 10% FBS and 1% PenStrep (L15+ medium). Polychlorobiphenyl 126 (PCB 126) concentrations applied in media ranged from 2 nM to 20 μM and 3-methylcholanthrene (3-MC) concentrations from 25 to 100 μM. α-naphthoflavone (α-NF), a CYP1A inhibitor, was applied at 100 μM. 3-MC, α-NF and PCB 126 were first dissolved in dimethyl sulfoxide (DMSO) prior to addition in media (final solvent concentration of 0.1% for all treatments, including controls).

2.5. PCLS viability

PCLS viability was estimated by lactate dehydrogenase (LDH; EC 1.1.1.27) leakage and adenosine triphosphate (ATP) content measurements. LDH leakage was measured on 50-μl aliquots of media

(removed and replaced), using the Cytotoxicity Detection Kit from Roche (Mannheim, Germany), with a Spectramax 190 spectrophotometer (Molecular Devices, Sunnyvale, USA) set at 490 nm. Slope values, derived from the linear portion of absorbance increase, were recorded. LDH activity remaining in slices was measured on homogenates performed in L15 medium with a Kontes pellet pestle (Daigger, Vernon Hills, USA). Time-specific percentages of LDH leakage were then derived. After 24 h, LDH leakage remained moderate (22 ± 1%; mean ± standard error of the mean; $n = 24$).

ATP content was measured on a luminometer LB96P (Berthold, Bad Wilbad, Germany) with the ATP lite 1 Step Kit (Perkin Elmer, Waltham, USA). PCLS were first sonicated in 1 ml of ice-cold perchloric acid 2%. After a short spin (12,000*g* for 1 min at 4 °C) to pellet proteins, supernatants were neutralised with $KOH/KHCO_3$ (3 mol l^{-1}) and assayed in Tris–HCl 0.04 M buffer (pH 7.8) against a standard curve of ATP (Vanhulle et al., 2001). The ATP content of PCLS increased from time 0 at 24 h culture in L15+ medium (6.05 ± 0.54 nmol mg^{-1} proteins; mean ± standard error of the mean; $n = 29$). At the doses tested, 3-MC, PCB 126 and α-NF did not significantly affect LDH leakage or ATP content after 24 h culture.

2.6. EROD assays

Two methods were used to quantify CYP1A-associated ethoxyresorufin-O-deethylase (EROD) activity. The first method is based on S9 fractions, according to Burke and Mayer (1974). In this assay, individual slices were homogenised with a Kontes pellet pestle (Daigger, Vernon Hills, USA) in 200-µl extracting buffer (phosphate 100 mM buffer at pH 7.8, containing 20% glycerol and 0.2 mM PMSF) and centrifuged at 9000*g* for 20 min at 4 °C. Supernatants were collected in Eppendorf cups and diluted to 1.25 and 2.5 mg proteins ml^{-1} with 100 mM phosphate buffer (pH 7.4), before incubation with ethoxyresorufin (2 µM) in black 96-well plates. NADPH (2.5 mM) started the reaction (25 °C) that was followed on 15-min kinetic basis with a Fluoroskan fluorimeter (Labsystems, Minneapolis, USA) set at 530 nm (excitation) and 590 nm (emission), against a standard curve of resorufin ($\varepsilon = 73.2\ mM^{-1}\ cm^{-1}$; 572 nm). In a second method, EROD assay was performed on living slices incubated in 24-well plates for 3 h at 15 °C and 175 rpm in L15 medium containing ethoxyresorufin (5 µM), as performed on isolated hepatocytes (e.g. Bols et al., 1999). Resorufin was quantified in the culture medium at the end of incubation period as described above. Note that the proportion of resorufin enclosed in slices was low (8.13 ± 0.96% of total; mean ± standard error of the mean; $n = 30$) and considered negligible. As part of the validation process, we analyzed whether PCLS incubated for 21 h with 3-MC (25 µM; CYP1A inducer) and α-NF (100 µM; CYP1A inhibitor) would affect EROD levels as expected. Highly significant effects of 3-MC and α-NF treatments (alone and in combination) on EROD levels were found (data not shown). With 3-MC alone, EROD activity increased to 6.95 ± 0.97 pmol min^{-1} mg^{-1} proteins on S9 fractions and 0.18 ± 0.02 pmol min^{-1} mg^{-1} proteins on living slices (mean ± standard error of the mean; $n = 6$).

2.7. PCB 126 quantification

Pools of ten slices, incubated for 21 h with increasing PCB 126 doses, were lyophilized for 16 h with a Virtis apparatus (Inc Gardiner, New-York, USA). Dry matter was then determined gravimetrically. 50-µl PCB 112 (100 pg $µl^{-1}$ in acetone) were applied as surrogate. Lipid extraction was performed at 80 °C and 1500 psi with a mixture of *n*-hexane, dichloromethane and methanol (5:2:1, v:v:v) on ASE 2000 (Dionex, Sunnyvale, USA). Samples were then evaporated at 40 °C under nitrogen flow, with a TurboVap (Zymark, Hopkinton, USA). Gravimetric determination of the fat content was performed afterward. Acid clean-up with 95% concentrated sulphuric acid preceded Florisil clean-up, as described in Debier et al. (2003). Final elution was performed with a mixture of *n*-hexane/diethylether (1:1). 50-µl Mirex (100 pg $µl^{-1}$ in acetone), used as an internal standard for Gas Chromatography/Electron Capture Detection (GC/ECD) procedure, were then added to samples. GC/ECD was performed on AS 2000 (Thermo Quest, Milan, Italy) at a pressure of 130 kPa, with 3.8 ml min^{-1} hydrogen flow as vehicle. The make-up gas was argon/methane (95:5) at 30 ml min^{-1} flow rate. The capillary column was a 30 m × 0.25 mm (0.25 µm film thickness) Rxi-5 ms (Restek, Bellefonte, USA) and the detector was ^{63}Ni. The temperature gradually increased from 60 to 140 °C (20 °C min^{-1}), stayed for 3 min at 140 °C, then gradually increased to 270 °C (2.5 °C min^{-1}) and stayed for 12 min at 270 °C. Chromatograms were analyzed with the ChromCard 2.2 software (Fisons Instrument, East Grinstead, UK). Quality control was performed by regular analyses of *n*-hexane blanks, PCBs enriched milk cream and BCR RM 349 (cod liver). Samples with a recovery between 70% and 130% only were used. The limit of detection was 2 ng g^{-1} wet weight. The quality control procedure is regularly checked through interlaboratory comparisons with the IAEA-MEL Marine Environment Laboratory (Monaco, France).

2.8. Semi-quantitative RT-PCR

Total RNA from needle-disrupted slices was extracted with the Nucleospin RNA II Kit (Macherey–Nagel, Düren, Germany), according to manufacturer's guideline. Total RNA was quantified and the purity checked with $OD_{260/280}$ on a spectrophotometer Ultrospec 3000 (Pharmacia Biotech, Québec, Canada). RNA integrity was checked by resolving samples on 1.2% agarose gels. Reverse transcription was performed on 1-µg total RNA using $oligo(dT)_{20}$ with the Superscript III First Strand Synthesis Kit (Invitrogen, Carlsbad, USA). Primer pairs, designed from mRNA sequences available on GenBank®, were selected with Primer Express 2.0 software (Foster City, USA) and further analysed *in silico* with Amplify 3.1.4. software (Wisconsin, USA). Prior to semi-quantitative experiments, titration reactions were operated to screen for optimal primer pairs concentrations (Table 1). Real-time reverse transcriptase polymerase chain reaction (RT-PCR) assays were performed at a final volume of 20-µl per well on ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, USA), with SYBR green ROX mix (Thermo Scientific, Epsom, UK). 2 µl of ten-fold diluted cDNA samples were used and a dissociation stage always performed. To confirm the specific amplification, randomly selected amplicons of each preparation were sequenced on ABI 3100 Genetic Analyzer with Big Dye Terminator 1.1 Kit (Applied Biosystems, Foster City, USA), after ligation/amplification of sequences in pCRII-TOPO vector using the TOPO TA Cloning Kit and TOP 10F′ competent cells (Invitrogen, Carlsbad, USA) and plasmid isolation with the Plasmid DNA Purification Kit (Macherey–Nagel, Düren, Germany). Caution was put on the selection of reference gene, as classical housekeeping genes were affected by organochlorines and physiological state in liver of *Salmo salar* (Olsvik et al., 2005; Arukwe, 2006). Results proved the stability of elongation factor 1Aα as reference gene in our experiments (Ct = 18.16 ± 0.14; mean ± standard error of the mean; $n = 54$).

2.9. Antioxidant enzymes and citrate synthase activities

PCLS were homogenised with a Kontes pellet pestle (Daigger, Vernon Hills, USA) at 4 °C in phosphate buffered saline (PBS) containing 1% Triton X-100. Homogenates were centrifuged at 15,000*g* for 10 min (4 °C) and assays performed on supernatants (25 °C). Catalase (CAT; EC 1.11.1.6) was quantified on a microplate luminometer LB96P (Berthold, Bad Wilbad, Germany) against a

Table 1
Characteristics of amplicons for reference (EF1Aα) and CYP1A mRNA sequences as well as associated GenBank® accession number and primer concentrations selected for RT-PCR experiments on PCLS of *Salmo salar*.

Sequence	GenBank®	Forward primer	Reverse primer	Amplicon size (bp)	Primers concentrations (nM)
EF1Aα	AF321836	5′- TTG GTC GTT TTG CTG TGC GT -3′	5′- AGA CTT TGT GAC CTT GCC GCT T -3′	101	300
CYP1A	AF361643	5′- CCC CGT CTC TCA GAC AAA ACC -3′	5′- GGT GAA CGG CAG GAA GGA A -3′	87	200

standard of bovine catalase, according to Janssens et al. (2000). Spectrophotometric quantifications of superoxide dismutase (SOD; EC 1.15.1.1), glutathione peroxidase (GPX; EC 1.11.1.9) and citrate synthase (CS; EC 2.3.3.1) activities were performed with a Spectramax 190 spectrophotometer (Molecular Devices, Sunnyvale, USA). SOD assay, based on the inhibition of cytochrome c reduction by superoxide anions, was adapted from the method of Flohé and Ötting (1984). GPX assay was based on the method of Paglia and Valentine (1967), using hydrogen peroxide (GPX_H) and *tert*-butyl hydroperoxide (GPX_T) as substrates. CS assay was based on the method of Bailey et al. (2005), using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as a sensor of free-thiol groups of de-acetylated coenzyme A.

2.10. H_2O_2 production

Hydrogen peroxide (H_2O_2) production was quantified with a chloromethylated dichlorodihydrofluorescein diacetate probe (CM-DCFHDA). Slices were incubated for 3 h in 24-well plates at 15 °C under moderate agitation (175 rpm) in 500-µl HBSS+ containing CM-DCFHDA (10 µM). The kinetics of dichlorofluorescein (DCF) production in slices was followed at 20-min intervals (25 °C) with a Fluoroskan fluorimeter (Labsystems, Minneapolis, USA). Excitation and emission wavelengths were 485 nm and 520 nm, respectively. Slope values were used to estimate the relative rates of H_2O_2 production.

2.11. TBARS assay

Oxidative damage to lipids were evaluated as Thiobarbituric Acid Reactive Substances (TBARS), with a method adapted from Wey et al. (1993). In black 96-well plates, 30-µl slices homogenates (performed in HBSS-with a Kontes pellet pestle) were mixed with 48-µl thiobarbituric acid (0.67%) and 24-µl trichloroacetic acid (15%). Plates were sealed, incubated at 95 °C for 30 min and 100-µl butanol was then added at 4 °C. Plates were centrifuged for 5 min at 1500 rpm and TBARS were quantified against a standard curve of malondialdehyde, with a Fluoroskan fluorimeter (Labsystems, Minneapolis, USA) set at 515 nm and 555 nm excitation and emission wavelengths, respectively.

2.12. PCLS protein content

Protein contents were determined with the DC Protein Assay Kit (Bio-Rad, Richmond, USA) on slices homogenates diluted in 5% sodium dodecyl sulfate (SDS). When proteins were assayed in parallel to ATP, pellets were first sonicated in 200-µl 5% SDS prior to dilution. Absorbance (750 nm) was measured on a Spectramax 190 spectrophotometer (Molecular Devices, Sunnyvale, USA) against a standard curve of bovine serum albumin.

2.13. Statistics

Linear regressions, ANOVA I (with post hoc Tukey's test) and ANOVA II (with post hoc Fisher's test) were calculated with Statistica 7.1 software (Statsoft, Tulsa, USA). Graphs were obtained with Sigma Plot 8.0 software (Systat, San Jose, USA). Results were expressed as mean ± standard error of the mean (S.E.M.) and statistical data with a p-value $\leqslant 0.05$ were considered significant.

3. Results

3.1. Dose–response effects of 3-MC

3.1.1. CYP1A transcription and EROD activity

3-MC (25 µM) strongly induced CYP1A transcription, with mRNA levels increased to 58 ± 2 folds that of controls ($p = 0.004$). As to further investigate the impact of 3-MC treatment on CYP1A activation, PCLS were incubated with increasing 3-MC concentrations. EROD activity was then assayed on living slices (Fig. 1). Strong increases of EROD activity were observed, with maximal EROD values at 50 µM 3-MC (52 ± 11 folds the basal level) and significant EROD activation up to 75 µM 3-MC ($p \leqslant 0.014$). The counter-intuitive decrease of EROD activation at doses exceeding 50 µM 3-MC prompted us to analyse whether 3-MC could compete with EROD's substrate (ethoxyresorufin) during the assay.

In a first experiment, EROD was measured on living slices ($n = 6$) that had been first incubated for 21 h with 25 µM 3-MC (induction phase) and then assayed in the presence of 3-MC (25 or 100 µM) or in its absence (controls). A 50% lower EROD activity was found with both 3-MC doses ($p = 0.002$). In a second experiment, PCLS ($n = 6$) that had been incubated for 21 h with 3-MC (25 or 100 µM; induction phase) were then incubated in contaminant-free medium (L15) for 2 to 6 h prior to EROD assay. In this case, time-dependent gains of activity were observed with both 3-MC doses ($p < 0.0001$), the recovery reaching 165% and 290% the level of control slices (assayed directly after the induction phase). Note that 3-MC concentration by itself did not significantly affect EROD levels, but the interaction of time and dose was significant ($p = 0.001$). Altogether, these results suggest that a reversible competition for CYP1A occurred in slices between 3-MC and ethoxyresorufin.

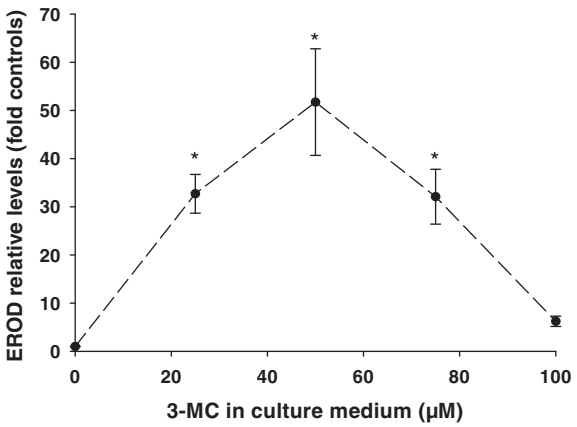

Fig. 1. EROD activity (pmol min^{-1} mg^{-1} proteins, reported here as fold increase from controls) measured in PCLS of *Salmo salar* after 21 h culture in L15+ medium containing increasing doses of 3-methylcholanthrene (3-MC). Data are presented as mean ± S.E.M. ($n = 4$). Results with a p-value $\leqslant 0.05$ were considered significant (*).

3.2. Pro-oxidant and antioxidant responses to 3-MC

As 3-MC treatments increased CYP1A mRNA and EROD levels in slices, we analyzed whether these increases were associated to alterations in the balance between pro-oxidant and antioxidant processes in liver cells. We therefore measured antioxidant enzymes activities and the possible oxidative damage to lipids in response to 3-MC treatments (Table 2). CAT, SOD and GPX_T activities were not significantly affected, even though GPX_T activity tended to be higher with 3-MC doses up to 75 μM. GPX_H activity was significantly lowered at 75 μM and 100 μM 3-MC doses ($p \leqslant 0.013$). CS activity, a proxy for oxidative metabolism, tended to increase with 3-MC dose. This increase became significant at 75 μM 3-MC ($p = 0.0009$). TBARS levels did not differ from controls at any dose tested. H_2O_2 production, which was only evaluated at 25 μM 3-MC, tended to be lower than in controls (76 ± 8%; $n = 8$; not shown).

3.3. Dose–response effects of PCB 126

Results obtained with the model CYP1A inducer 3-MC indicate that PCLS allow the assay of CYP1A activation and antioxidant/pro-oxidant status on single PCLS. We then applied this model to the effects of PCB 126.

3.3.1. PCB 126 contamination levels

PCLS contamination levels increased linearly with the PCB 126 dose present in the culture medium (Fig. 2). The scale of contamination ranged from 9 ± 2 ng g^{-1} wet weight (w.w.) to $39{,}200 \pm 4200$ ng g^{-1} w.w. Since the two highest PCB 126 treatments yielded contamination levels well beyond those found in liver from wild fish, we restricted our subsequent investigations to slices incubated with doses up to 200 nM. For this environmentally relevant range of PCB 126 contamination levels, the equation was: [PCB 126 (ng g^{-1} w.w.) = 1.2791 * PCB 126 (nM) + 4.7476; $R^2 = 0.99$; $p < 0.0001$].

3.3.2. CYP1A transcription and EROD activity

CYP1A transcription was determined in PCLS incubated with increasing PCB 126 doses (Fig. 2). CYP1A mRNA levels were affected by treatments ($p < 0.0001$), with significant increase from controls detected at 200 nM PCB 126 ($p < 0.001$). A linear relationship was found between PCB 126 content in slices and CYP1A mRNA levels: [CYP1A mRNA level = 0.2408 * PCB 126 (ng g^{-1} w.w.) + 2.1646; $R^2 = 0.99$; $p = 0.003$].

EROD activity was also determined through that dose range (Fig. 3) but the observed pattern of activation diverged from that of CYP1A transcription. Indeed, although PCB 126 significantly affected EROD levels ($p = 0.0002$), only a modest increase of EROD from controls was found in 200 nM PCB 126 treated PCLS ($p = 0.0006$).

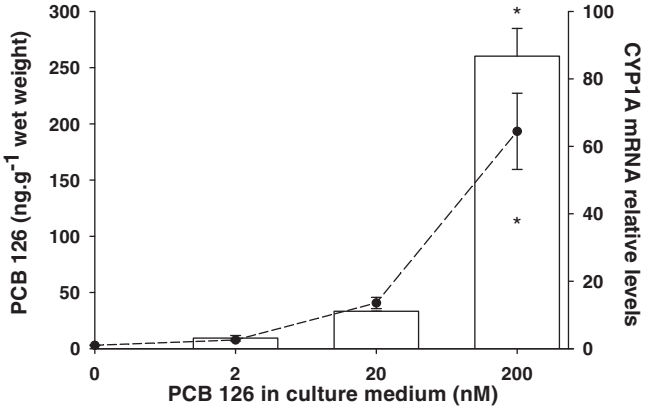

Fig. 2. CYP1A mRNA levels (relative to controls) measured in PCLS of *Salmo salar* after 21 h culture in L15+ medium containing increasing PCB 126 doses. Vertical bars represent PCB 126 contamination levels in PCLS (ng g^{-1} wet weight). Data are presented as mean ± S.E.M. ($n = 4$). Results with a p-value $\leqslant 0.05$ were considered significant (*).

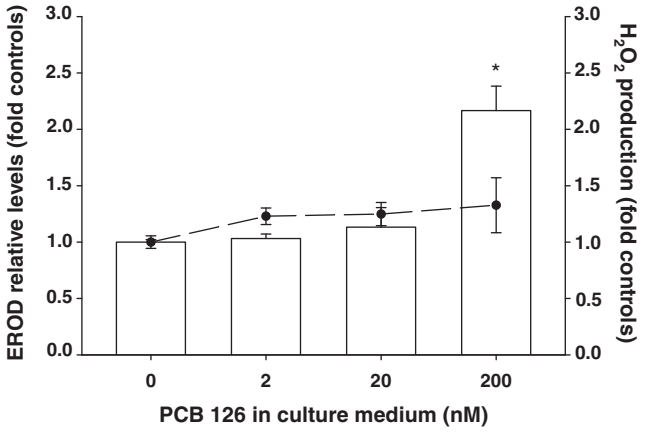

Fig. 3. H_2O_2 production and EROD activity in PCLS of *Salmo salar* cultivated for 21 h in L15+ medium with increasing PCB 126 doses. Vertical bars represent relative EROD activity (pmol min^{-1} mg^{-1} proteins, reported here as fold increase from controls). Data are presented as mean ± S.E.M. ($n = 4$ for EROD; $n = 8$ for H_2O_2 production). Results with a p-value $\leqslant 0.05$ were considered significant (*).

3.4. Pro-oxidant and antioxidant responses to PCB 126

As CYP1A transcription and EROD activity increased in contaminated slices, we analyzed the pro-oxidant and antioxidant status of PCLS exposed to PCB 126 (Table 3). Amongst antioxidant enzymes, CAT activity was significantly affected ($p = 0.012$). However, this was not the result of significant differences with controls, but rather consisted in a moderate decrease of activity in slices treated with 200 nM compared to 20 nM PCB 126 ($p = 0.007$). A similar

Table 2
Effects of increasing 3-MC doses on the relative levels of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX_H and GPX_T) as well as citrate synthase (CS) activities (U mg^{-1} proteins) and TBARS levels (nmol mg^{-1} proteins) in PCLS of *Salmo salar*. Data are presented as mean ± S.E.M. ($n = 8$ for antioxidant enzymes; $n = 4$ for CS; $n = 9$ for TBARS) and results with a p-value $\leqslant 0.05$ were considered significant. Asterisks and triangles indicate significant differences from controls and between treatments, respectively.

3-MC (μM)	CAT	SOD	GPX_H	GPX_T	CS	TBARS
0	1.00 ± 0.06	1.00 ± 0.10	1.00 ± 0.07	1.00 ± 0.13	1.00 ± 0.18	1.00 ± 0.06
25	0.96 ± 0.15	1.03 ± 0.21	0.73 ± 0.04	1.70 ± 0.19	2.13 ± 0.16	1.04 ± 0.09
50	0.92 ± 0.17	1.34 ± 0.33	0.80 ± 0.12	1.82 ± 0.34	1.80 ± 0.26▲	0.93 ± 0.09
75	0.92 ± 0.09	1.15 ± 0.25	0.62 ± 0.07*	1.52 ± 0.30	3.20 ± 0.54*,▲	0.92 ± 0.05
100	0.88 ± 0.11	0.75 ± 0.16	0.56 ± 0.07*	1.39 ± 0.24	1.99 ± 0.15	0.82 ± 0.08
ANOVA	N.S.	N.S.	0.003	N.S.	0.002	N.S.

Table 3
Effects of increasing PCB 126 doses on the relative levels of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX_H and GPX_T) as well as citrate synthase (CS) activities (U mg^{-1} proteins) and TBARS levels (nmol mg^{-1} proteins) in PCLS of *Salmo salar*. Data are presented as mean ± S.E.M. ($n = 8$ for antioxidant enzymes; $n = 4$ for CS; $n = 9$ for TBARS) and results with a p-value $\leqslant 0.05$ were considered significant. Asterisks and triangles indicate significant differences from controls and between treatments, respectively.

PCB 126 (nM)	CAT	SOD	GPX_H	GPX_T	CS	TBARS
0	1.00 ± 0.05	1.00 ± 0.12	1.00 ± 0.16	1.00 ± 0.13	1.00 ± 0.18	1.00 ± 0.06
2	0.96 ± 0.07	1.17 ± 0.19	1.26 ± 0.12	1.55 ± 0.17	1.19 ± 0.05	1.06 ± 0.12
20	1.29 ± 0.16▲	1.34 ± 0.29	1.56 ± 0.24▲	2.16 ± 0.40*	1.11 ± 0.09	0.88 ± 0.11
200	0.79 ± 0.04▲	1.02 ± 0.37	0.68 ± 0.16▲	1.18 ± 0.28	1.08 ± 0.08	0.79 ± 0.11
ANOVA	0.012	N.S.	0.015	0.025	N.S.	N.S.

pattern was detected for GPX_H ($p = 0.015$) whose activity was significantly reduced from 20 to 200 nM ($p = 0.011$). On the other hand, GPX_T activity was significantly increased from controls at 20 nM PCB 126 ($p = 0.025$) while the decrease observed between 20 and 200 nM was nearly significant ($p = 0.07$). SOD, CS and TBARS levels were not significantly affected by PCB 126 treatments. Nevertheless, a slight and non significant increased H_2O_2 production was observed with doses up to 200 nM (Fig. 3).

4. Discussion

The present study was set up to estimate the usefulness of PCLS technology for addressing the impact of PCB 126 and 3-MC contaminations on CYP1A induction, H_2O_2 production, antioxidant enzymes activities and oxidative damage to lipids in fish liver cells. The results indicate that PCLS technology allows the measurement of a great variety of parameters in relation to CYP1A induction and oxidative stress and suggest that the model is reliable. Indeed, strong positive correlations were observed between the dose of PCB 126 applied in media, the resulting PCB 126 contamination level in slices and the associated CYP1A mRNA production.

While both 3-MC and PCB 126 treatments led to strong increases of CYP1A transcription levels at 21-h culture, 3-MC was most potent for activating EROD. However, the low EROD values measured at the highest 3-MC doses tested were suggestive of a competition with ethoxyresorufin during the assay. The subsequent EROD assays performed in the presence/absence of 3-MC confirmed the reversible competition between 3-MC and ethoxyresorufin for CYP1A. Such competition has already been demonstrated in isolated rat hepatocytes (Petrulis and Bunce, 1999).

Amongst antioxidant enzymes, no significant variations with 3-MC treatments were observed, except for GPX_H which was lowered at the highest 3-MC doses. However, GPX_T activity tended to increase at intermediate 3-MC doses. Interestingly, 3-MC treated slices had an increased rate of oxidative metabolism, as measured by the higher CS activity. Nevertheless, no signs of increased H_2O_2 production or oxidative damage to lipids were detected, suggesting that exposure to 3-MC did not overwhelm basal antioxidant capacity of liver cells.

PCB 126 was far less potent than 3-MC to activate EROD at 21-h culture. It is noteworthy that bell-shaped patterns of EROD were previously observed in primary hepatocytes of four different fish species exposed to doses up to 30 nM PCB 126 in medium for two days (Smeets et al., 2002), further supporting that a competitive inhibition occurs in fish liver cells upon PCB 126 intoxication, as already demonstrated on fish microsomes (Schlezinger et al., 2006). In favour of this, we found that PCLS that were first incubated with 3-MC (as to increase CYP1A level in slices) and that were then co-incubated with ethoxyresorufin and 2 μM PCB 126 during EROD assay had a 25% decreased activity (data not shown).

There was a trend to an increased H_2O_2 production (roughly 20%) with the highest PCB 126 doses. CAT and GPX_H tended to increase and GPX_T increased significantly at intermediate PCB 126 doses. However, decreases of those antioxidant activities were obvious at the highest PCB 126 dose. As CS activity was fairly stable through the dose range tested, the observed patterns of altered pro-oxidant and antioxidant status were not attributed to increased rates of aerobic metabolism in slices. Such modifications further suggest that the measured EROD activity did not fully reflect the existing pool of CYP1A enzyme responsible for ROS production. It should be kept in mind that, as we did not investigate the time course of activity of the various parameters assayed in present study, we also cannot rule out a possible depletion of CYP1A levels with time in cultivated slices. Nevertheless, it is suggested that the observed concomitant increased H_2O_2 production and the decreased antioxidant capacity at 21-h culture with 200 nM PCB 126 could favour the emergence of oxidative stress. However, we did not observe increased TBARS levels at any PCB 126 dose.

Many workers, though, firmly established that PCB 126 was pro-oxidant *in vivo*. For example, during a 30 weeks survey that followed a single IP injection of PCB 126, Palace et al. (1996) found that *Salvelinus namaycush* juveniles had increased EROD activity concomitantly to increased TBARS levels. Antioxidant enzymatic activities were not affected in this case. Brown et al. (2002) found depleted antioxidant stores in liver of *Oncorhynchus mykiss* juveniles fed 30 days with PCB 126 and then fed 160 days with uncontaminated food. Arzuaga et al. (2006) found increased ROS production in *Fundulus heteroclitus* developing embryos exposed to PCB 126. In mammals, Hori et al. (1997) found that a single IP injection of PCB 126 to mice decreased hepatic selenium-dependent GPX activity while increasing non-selenium-dependent activity. In rats, Lai et al., 2010 found that PCB 126 decreased selenium-dependent GPX activity and Robertson et al. (2007) also found decreased CAT activity. To our knowledge, no data are available on the oxidative stress response of isolated hepatocytes to PCB 126. However, Lin and Lin (2006), using human T47D and MDA-MB-231 breast cancer cell lines observed increased ROS production after 24 h exposure to micromolar doses of PCB 126 and found the cytotoxicity to be CYP1A-dependent.

In present study, trends to an increased pro-oxidant and a decreased antioxidant status were noticed at the highest PCB 126 dose after 21-h culture. However, such alterations were not sufficient to generate oxidative stress in PCLS of *Salmo salar*. Our results suggest that increasing the dose of PCB 126 would not be environmentally relevant and not helpful in promoting the onset of oxidative stress after 21-h culture. Rather, it is likely that an exposure to moderate PCB 126 doses but for longer periods would yield oxidative damage in slices as antioxidant defences appear to be reduced.

5. Conclusion

Results demonstrate that PCLS were viable and functional for 21 h and allowed the evaluation of CYP1A induction and EROD activation on a broad range of contamination levels, concomitantly to the determination of antioxidant enzymes activity and oxidative

damage to lipids. Nevertheless, as pro-oxidant and antioxidant status were not strongly altered in PCLS after 21 h culture, it is suggested that longer incubation periods would be required to generate oxidative stress.

Conflict of interest statement

None declared.

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