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The Toxicity of Benzo[a]pyrene on Sole (*Solea Solea*) Hepatocytes: Assessment of Genotoxic and Enzymatic Effects

N. Wessel[1], D. Ménard[1], K. Pichavant-Rafini[2], H. Ollivier[2], J. Le Goff[3], T. Burgeot[1], and F. Akcha1[1]*

[1]IFREMER, Département de Biogéochimie et Ecotoxicologie, Rue de l'Ile d'Yeu, 44311 Nantes Cedex 03, France.
[2]Laboratoire Optimisation des Régulations Physiologiques, Université Européenne de Bretagne, Université de Brest, 6 Avenue Le Gorgeu, CS 93837, 29238 Brest cedex, France.
[3]ADn'tox et Groupe Régional d'Etudes sur le CANcer, EA 1772 Université de Caen Basse Normandie, Centre François Baclesse, Avenue du Général Harris, 14076 Caen cedex 05, France.

The benzo[a]pyrene is a polycyclic aromatic hydrocarbon known to be genotoxic, mutagenic and carcinogenic in higher vertebrates. The aim of this study was to evaluate *in vitro* the enzymatic and genotoxic effects of BaP in a benthic fish species, *Solea solea*. Sole hepatocytes were exposed to BaP in order to measure the modulation of ethoxyresorufin-o-deethylase (EROD) activity and the DNA strand breaks induced by BaP metabolism. Exposures were performed in both culture flasks and microplate wells in order to check for the possible miniaturization of the exposure system. Moreover, sole liver microsomes were exposed to BaP in the presence of standard DNA in order to assess the potential formation of DNA adducts in sole.

The results demonstrated the ability of sole hepatic enzymes to metabolize BaP into reactive species responsible for bulky DNA adducts and DNA strand breakage, whatever the tested exposure concentration and the mode of exposure.

Key Words: Genotoxicity; Sole; BaP; DNA strand breaks; EROD, DNA adducts.

*Corresponding author: Farida Akcha, Ifremer, Department of Biogeochemistry and Ecotoxicology, Rue de l'Ile d'Yeu, BP 21105, 44311 Nantes 03, France. Tel: 33 2 40 37 42 08, Fax: 33 2 40 37 42 41. Mail: fakcha@ifremer.fr

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are ubiquitous environmental pollutants. Their inputs in the marine environment are mainly from pyrolytic origins, with supplementary inputs near the harbors and oil refineries (1). PAHs can reach high concentrations in the particulate phase, and in sediments (2). Due to their toxicity, many of them are listed as priority pollutants by the US Environmental Protection Agency (US EPA), and as dangerous priority substances by the European Water Framework Directive (Directive 2000/60/EC). Due to their hydrophobicity, biotransformation mechanisms are involved in their transformation into more hydrophilic and excretable compounds. Some metabolites produced are responsible for the genotoxic effects associated to the parent compound. The benzo[a]pyrene (BaP) is the PAH the most widely studied. It is a model genotoxicant whose metabolic activation pathways are well known in higher vertebrates (3). In fish, species-related differences were observed in the metabolism of PAHs (4, 5) and PAH genotoxicity was already demonstrated (6). The common sole (*Solea solea*) is a commercial benthic species. Its nursery are located in coastal and estuarine areas, that receive major pollutant inputs. The sole is so particularly exposed to marine pollution.

The aim of this study was to assess *in vitro* (i) the potential modulation of EROD activity in BaP-exposed sole hepatocytes, (ii) the genotoxic effects in terms of DNA strand breaks induced by BaP in sole hepatocytes, and (iii) the potential production of BaP adducts on calf thymus DNA following activation of BaP by sole microsomal fractions. Moreover, hepatocyte exposures were conducted in two different ways (75 cm^2-cell culture flasks and 96-well microplates) in order to check for a possible miniaturization of the exposure system that could allow the forthcoming genotoxicity study of different PAH metabolites.

MATERIAL AND METHODS

Sole Hepatocyte Isolation

Soles used for these experiments came from the Loire estuary in the French West coast. Individuals from 16 to 27 cm were used. The hepatocytes were isolated by a collagenase digestion technique adapted from Ollivier et al. (7). Livers from freshly sacrificed soles were perfused using (i) 5 mL of a standard iso-osmotic solution (IOS) at a flow rate of 0.4 $mL.min^{-1}$, (ii) 5 mL of an IOS enriched with 0.6 mM EDTA at a flow rate of 0.4 $mL.min^{-1}$, and (iii) 5 mL of a collagenase solution (C5138, type IV from Sigma-Aldrich Chemicals, 1 $mg.mL^{-1}$ in IOS) at a flow rate of 0.2 $mL.min^{-1}$. Following perfusion, cells were recovered by filtrations (200, 85 and 48 μm), centrifuged and resuspended

in 5 mL of an IOS enriched with 5 mM glucose and 1.5 mM CaCl2. Cells were seeded in 75 cm^2-tissue culture flasks (24.5×10^6 cells in a final volume of 15 mL) or in 96-well culture plates (0.65×10^6 cells in a final volume of 200 μL) in a medium containing 90% DMEM, 10% fetal bovine serum (FBS), and supplemented with 2% penicillin-streptomycin as antibiotics and 2% fungizone-amphotericin B as antifungics.

BaP Exposure

One night after seeding, cells were exposed to 0.1, 0.5, 1, 5, and 25 μM of BaP (Sigma Aldrich Chemicals) dissolved in DMSO so that the solvent concentration in the medium equaled 0.5%. Exposure were conducted for 24 hrs in the dark at 17°C. A solvent control using DMSO was performed (0.5%). For each tested concentration, at least 2 replicates of flasks and 2 replicates of wells were performed. Following 24 hrs exposure, cells were recovered by using trypsine-EDTA (Gibco, Invitrogen) and were withdrawn in a freezing medium before storage in liquid nitrogen until analyses.

EROD Activity Measurement

For the samples exposed in flasks, the Ethoxyresorufine-O-Deethylase (EROD) activity was determined as an indicator of phase I biotransformation activities. EROD activity was measured directly on the hepatocyte suspensions by a method adapted from Burke and Mayer (8) slightly modified for microplate readers (9).

DNA Strand Breaks Measurement

For genotoxicity assessment, the alkaline comet assay was applied in hepatocytes as previously described for fish erythrocytes by Akcha et al. (10). A trypan blue viability test was performed on each defrosted sample prior to the comet assay. For each sample, two slides were prepared. For each slide, the DNA of 75 nuclei was analyzed by the measurement of the percentage of DNA present in the comet tail (Kinetic Imaging Software 4.0).

Preparation of the Microsomal Fractions

The sole used for the microsomal exposure were 1 year old individuals coming from the Seine estuary (French West coast), which is a high-contaminated area. Sole liver microsomes were extracted by potter homogenization, centrifugation (9 000 g, 15 min, 4°C), and ultracentrifugation of the supernatant (105 000 g, 60 min, 4°C). Supernatants were further discarded and pellets were resuspended into 100 μM phosphate buffer enriched with 1 mM EDTA

and 20% glycerol. Microsomes were then stored into liquid nitrogen until the experiments.

Microsomal Exposure to BaP

Just before use, microsomal proteins were quantified using the Bradford assay. Standard calf thymus DNA (600 μg) and sole liver microsomes (1 mg) were exposed to 20 and 40 μM BaP in a final volume of 1 mL in an incubation buffer containing nicotinamide adenine dinucleotide phosphate (1 mM), glucose-6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (1U/mL). Following 1 hr of incubation, reaction was stopped by plugging the samples into liquid nitrogen, and the production of bulky DNA adducts was measured by applying the ^{32}P post-labeling assay, as described by Le Goff et al. (11).

Statistical Analysis

The data concerning the EROD activity and the DNA strand break levels were respectively analyzed by an ANOVA and a nested design ANOVA following mathematical normalization of the data when necessary.

RESULTS AND DISCUSSION

EROD Activity Following Hepatocyte Exposure

Following 24 hrs of hepatocyte exposure, no cytotoxic effect was observed as measured by the trypan blue assay (data not shown). The EROD activity (Figure 1), normalized by the square root transformation of the activity measured in pmol of resorufin formed per min and per 10^6 cells, followed a standard "bell shape" curve. In fact, the activity was significantly higher than that of the control group following exposure to BaP concentrations of 0.1, 0.5 and 1 μM. For this range of concentrations, increase was not concentration-dependent. Following exposure to the highest tested concentrations of 5 and 25 μM, the activity decreased and reached a level similar to that of the control group. Similar results were obtained following rat hepatocyte exposure to a range of BaP concentrations from 1 nM to 100 μM for 48 hrs: a bell shape curve was obtained with a maximum EROD activity measured at approximately 20 μM, followed by a decrease in activity (12). In fish, the CYP1A response to contaminants is known to be species-specific (13). Nevertheless, BaP is currently known to induced the EROD activity in fish, as in higher vertebrates (14, 15, 16). The bell shape curve observed for EROD activity could be due to a toxic effect. The BaP at the highest concentrations should become cytotoxic (12), and reaches a maximal threshold for inducing a response.

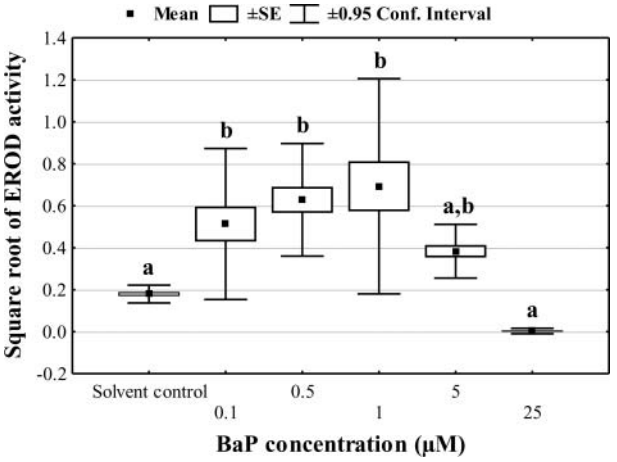

Figure 1: Normalized EROD activity: square root of the EROD activity in pmol of resorufin formed per min and per 10^6 cells. Boxes denote standard errors and vertical bars denote 0.95 confidence intervals. Letters are grouping homogeneous groups (ANOVA, Tukey $p < 0.05$).

DNA Strand Break Level Following Hepatocyte Exposure

The levels of DNA strand breaks measured in hepatocytes exposed in flasks or in wells were not significantly different (ANOVA, $p > 0.05$). Whatever the mode of exposure, in flasks (Figure 2A) or in wells (Figure 2B), the level of DNA strand breaks, expressed as the square root of the percentage of DNA in the comet tail, was significantly increased from the lowest exposure concentration of 0.1 μM. This genotoxic effect was not concentration-dependent: the level of DNA strand breaks was similar whatever the exposure concentration. The genotoxicity of BaP was already demonstrated in trout hepatocytes exposed *in vitro* to similar concentrations of BaP for one hour (17). In these conditions, a concentration-dependent increase in the DNA strand break levels was observed from an exposure concentration of 50 μM of BaP. In the present study, the lack of a concentration-dependent effect indicated that genotoxicity reached its higher level from the lowest exposure concentration. At this level, the antioxidant and DNA repair enzymes might be saturated. No correlation was found between the comet parameter and the EROD activity in hepatocytes exposed in flasks ($R = 0.21$, $N = 12$, $p > 0.5$).

DNA Adduct Production Following Microsomal Exposure

Whatever the exposure concentration (20 or 40 μM), the exposure of calf thymus DNA to BaP in the presence of sole liver microsomes lead to the formation of four bulky DNA adducts following 1 hr of incubation (Figure 3). In the

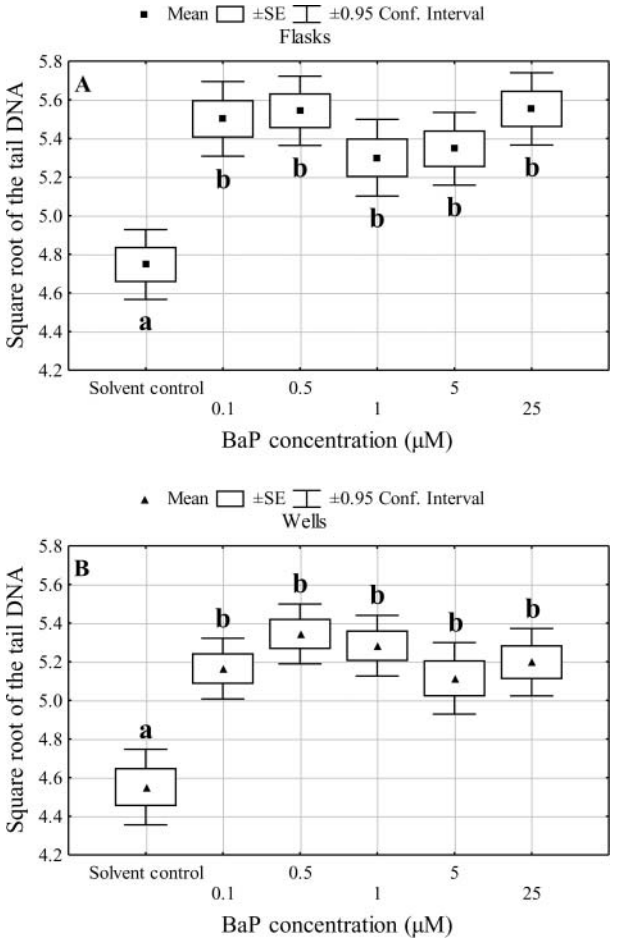

Figure 2: Normalized comet parameter : square root of the percentage of DNA in the tail of the comet. **A**: Exposure in flasks; **B**: Exposure in wells. Boxes denote standard errors (SE) and vertical bars denote 0.95 confidence intervals. Letters are grouping homogeneous groups (Nested design ANOVA, Tukey $p < 0.05$).

English sole, the formation of DNA adducts was already demonstrated *in vivo* following a single exposure to BaP-contaminated food (88 μg BaP.kg^{-1}) (18). In higher vertebrates, the major adduct produced results from the addition of the BaP-diol-epoxide (BPDE) to the dGuo at the N2 position (19, 20). The autoradiograms obtained by exposing calf thymus DNA to BaP activated by sole microsomes showed similarity with the autoradiogram from the positive control obtained following exposure of calf thymus DNA to BaP-diol-epoxide. The comparison with calf thymus DNA containing BAP induced adducts allows to hypothese that one of the four adducts produced in this study could be the BPDE-N2-dGuo adduct. The others may result from the adduction of BPDE

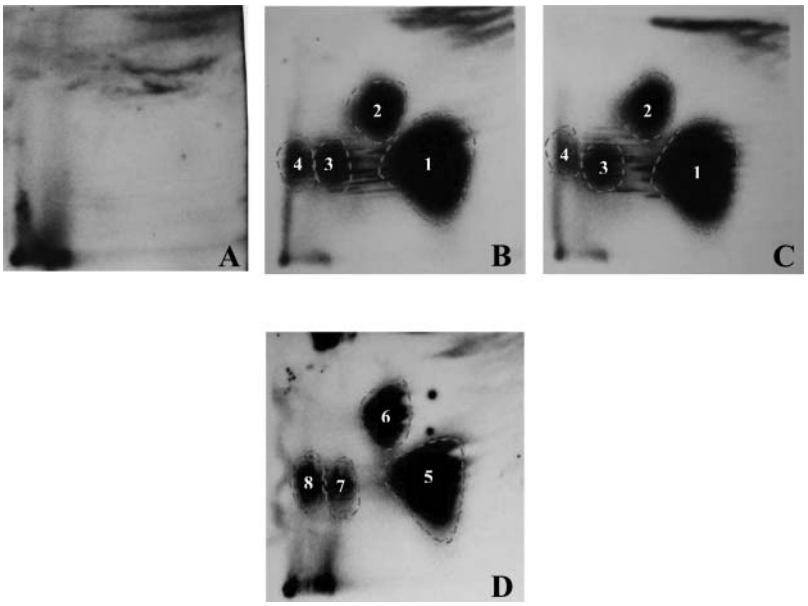

Figure 3: Autoradiograms of ^{32}P-postlabeled DNA from BaP-exposed microsomes. **A**: Solvent control; **B**: Microsomes exposed to 20 μM of BaP; **C**: Microsomes exposed to 40 μM of BaP; **D**: Calf thymus DNA exposed to BaP diol-epoxide used as a positive control.

to the other DNA bases, or could be isomers of the BPDE-dGuo adduct. These hypothesis will be verified by new experiments using mass spectrometry for determining the chemical structure of these adducts.

CONCLUSION

This study demonstrates the ability of sole liver enzymes to metabolize BaP into reactive chemical species leading to DNA damage, such as bulky DNA adducts and DNA strand breaks. The enzymatic modulation and the genotoxicity induced by BaP seem to occur through similar pathways as in higher vertebrates. The DNA adduct production seems to occur at least by the BPDE biotransformation pathway. Further experiments using mass spectrometry will be helpful for the chemical characterization of the produced adducts. The DNA strand breaks measured could result from DNA adduct repair mechanisms or from the production of reactive oxygen species (ROS). The level of DNA strand breaks measured in hepatocytes exposed in flasks or in wells are not significantly different, allowing the validation of a miniaturized exposure system in microplate wells. Because exposure in wells requires a smaller number of hepatocytes and a lower quantity of high value tested compounds, this exposure system will be used for the forthcoming study of the genotoxicity of some PAH metabolites.

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