Aquatic Toxicology 96 (2010) 234–245

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Hepatic gene expression in flounder chronically exposed to multiply polluted estuarine sediment: Absence of classical exposure 'biomarker' signals and induction of inflammatory, innate immune and apoptotic pathways

Michael J. Leaver[a,*], Amer Diab[a], Evridiki Boukouvala[a,b,c,1], Tim D. Williams[b], J. Kevin Chipman[b], Colin F. Moffat[d], Craig D. Robinson[d], Stephen G. George[a]

[a] Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK
[b] School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK
[c] Fisheries Research Institute, NAGREF, Nea Peramos, Kavala 64007, Greece
[d] Marine Scotland, Marine Laboratory, 375 Victoria Road, Aberdeen AB11 9DB, UK

ARTICLE INFO

Article history:
Received 21 August 2009
Received in revised form 27 October 2009
Accepted 31 October 2009

Keywords:
Pollution
Chronic exposure
Flounder
Microarray
Genomics
Benzo(a)pyrene
Estradiol
Diablo
Smac
Hepatocytes
Cyp1A
Vitellogenin

ABSTRACT

The effects of chronic long-term exposure to multiply polluted environments on fish are not well understood, but environmental surveys suggest that such exposure may cause a variety of pathologies, including cancers. Transcriptomic profiling has recently been used to assess gene expression in European flounder (*Platichthys flesus*) living in several polluted and clean estuaries. However, the gene expression changes detected were not unequivocally elicited by pollution, most likely due to the confounding effects of natural estuarine ecosystem variables. In this study flounder from an uncontaminated estuary were held on clean or polluted sediments in mesocosms, allowing control of variables such as salinity, temperature, and diet. After 7 months flounder were removed from each mesocosm and hepatocytes prepared from fish exposed to clean or polluted sediments. The hepatocytes were treated with benzo(a)pyrene (BAP), estradiol (E2), copper, a mixture of these three, or with the vehicle DMSO. A flounder cDNA microarray was then used to measure hepatocyte transcript abundance after each treatment. The results show that long-term chronic exposure to a multiply polluted sediment causes increases in the expression of mRNAs coding for proteins of the endogenous apoptotic programme, of innate immunity and inflammation. Contrary to expectation, the expression of mRNAs which are commonly used as biomarkers of environmental exposure to particular contaminants were not changed, or were changed contrary to expectation. However, acute treatment of hepatocytes from flounder from both clean and polluted sediments with BAP or E2 caused the expected changes in the expression of these biomarkers. Thus transcriptomic analysis of flounder exposed long-term to chronic pollution causes a different pattern of gene expression than in fish acutely treated with single chemicals, and reveals novel potential biomarkers of environmental contaminant exposure. These novel biomarkers include Diablo, a gene involved in apoptotic pathways and highly differentially regulated by both chronic and acute exposure to multiple pollutants.

1. Introduction

Estuarine sediments are sinks for chemical pollutants present in industrial and domestic effluents, and the health of fish which live in polluted estuaries is of concern. The European flounder lives in close association with estuarine sediments and is a key sentinel species for biological effects monitoring in initiatives such as the OSPAR Joint Assessment and Monitoring Programme (OSPAR, 2003). Such biological effects monitoring programmes have concentrated on the use of biochemical, molecular, histopathological and morphological biomarkers to indicate exposure to harmful pollutants (van der Oost et al., 2003). The development of these biomarkers has most often been based on identifying endpoints which vary after single acute experimental exposure to model pollutants, such as polycyclic aromatic hydrocarbons (PAH), halogenated aromatic hydrocarbons, estrogenic chemicals, and metals. However, changes in these markers are not always observed in field surveys, even in fish exposed to high levels of contaminants (Eggens et al., 1996; Reynolds et al., 2003).

The development of transcriptomic techniques for massively parallel, simultaneous measurement of expression of several thousands of genes may provide a means to assess, not only exposure but

* Corresponding author. Tel.: +44 01786467995; fax: +44 01786472133.
E-mail address: mjl1@stir.ac.uk (M.J. Leaver).
[1] Permanent address: National Agricultural Research Foundation, Fisheries Research Institute, Kavala 64007, Greece.

0166-445X/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2009.10.025

also mechanisms of action and effects of environmental contaminants. Transcriptomic profiling with targeted microarrays has been used to assess the effects of exposure to polluted sediments and in some of these studies it has been possible to obtain a toxicologically relevant signal from amongst the transcripts which were changed. Thus, when comparing a highly PAH-contaminated environment with a 'clean' reference site, flounder transcripts characteristic of acute PAH-response were changed (Williams et al., 2003). Similarly, when comparing hornyhead turbot (*Pleuronichthys verticalis*) from a site receiving an estrogenic effluent with a clean site, a signal characteristic of acute estrogen exposure was observed (Baker et al., 2009). However, in a larger study, involving several European estuaries of differing pollutant status, and using a more comprehensive microarray, a transcriptomic signal of pollutant effect was less clear (Falciani et al., 2008). These results demonstrated that gene expression profiles depended on site, and that the relative expression levels of a subset of 44 genes could predict the source of flounder with high accuracy. However, this subset only contained one currently described biomarker, CYP1A, and furthermore transcriptomic profiling of flounder experimentally exposed to acute doses of model pollutants (Williams et al., 2008) showed large differences to gene expression profiles in fish from polluted environments. These results have general similarities to studies on populations of *Fundulus heteroclitus*, a fish which inhabits severely contaminated estuarine sites in North America. Comparing gene expression profiles of *F. heteroclitus* from several polluted and unpolluted sites indicates that in each of the polluted populations 5–17% of genes are differentially expressed, yet only two genes had a consistent difference in expression among all polluted sites (Fisher and Oleksiak, 2007). Also notable was an apparent under-representation of the type of biomarker genes which might have been expected to be differentially expressed between polluted and unpolluted sites, although in the case of *F. heteroclitus* there appears to be a heritable resistance to the effects of some contaminants, which is believed to prevent CYP1A induction for example (Oleksiak, 2008).

It is perhaps not surprising that comparing gene expression in different populations of fish from different locations is not straightforward. Estuaries in particular are highly variable environments and it is possible that pollutant responses are masked by large changes in expression of other genes that may be responsive to variables such as salinity, temperature, feeding, and, as shown with *F. heteroclitus*, population differences. It is also possible that the long-term response to living on multiply polluted sediments differs from the response to acute, short-term, single-chemical exposures which have been used to identify many of the current commonly used molecular and biochemical biomarkers. The aim of the study reported here was to compare the transcriptomic effects of chronic exposure to a real polluted estuarine sediment with a real 'clean' sediment in a single population of flounder. This was achieved by chronic exposure in a controlled mesocosm study designed to eliminate many of the variables present in different estuaries, followed by microarray and quantitative PCR measurement of gene expression in isolated hepatocytes from exposed and unexposed fish. The use of isolated hepatocytes provided a means to directly compare the transcriptomic effects of acute exposure to prototypical pollutants with long-term chronic exposure to sediments, whilst minimizing the effects of natural genetic and environmental variation in outbred wild populations of fish.

2. Methods

2.1. Experimental animals

Juvenile (0-group) flounder, were collected by beam trawl at low tide in May–June from the Ythan estuary (NE Scotland). Benthic invertebrates (mostly *Tellina* and *Macoma* bivalves, together with some polychaete worms) were collected by wet sieving (4 mm) from clean sediment at the Ythan estuary, Loch Ewe (NW Scotland), and at Balmerino on the Firth of Tay estuary (E Scotland).

2.2. Mesocosm establishment

The mesocosms consisted of four 1.5 m tanks contained 4–5 cm of test sediment over ~3 cm of filter sand, covering ~5 cm of filter gravel, on top of perforated, corrugated plastic sitting on the base of the tank. Four air-riser pipes per tank ensured a circulation of water down through the sediment and up the air-riser in order to prevent anoxia of the sediment, reduce sediment resuspension, and lessen water turbidity. Each mesocosm was supplied with ~100 L/h of carbon-filtered 50% seawater.

Three bulk sediments were collected for use in the mesocosm experiments. Two contaminated sediments were obtained from dredged material from the estuary of the River Tyne, Riverside Quay, Jarrow, and also sediment collected by van Veen grab from the Firth of Forth, adjacent to the Grangemouth petrochemical complex. A clean reference sediment was manually collected from the estuary of the River Ythan, in North East Scotland, adjacent to the site of flounder collection. Each of the sediments was thoroughly mixed, and stored as large (~75 kg) aliquots that were held in plastic packing crates within a commercial frozen storage facility until required.

Prior to introducing flounder to the tanks, the bulk Forth and Tyne sediments were combined (FT). This sediment and the reference Ythan (Y) sediment were each thoroughly mixed (75:25) with filter sand, and aliquoted/stored as described above. The filter sand was added to equalize the grain size of all sediments and to reduce the likelihood of the sediment becoming anoxic during the test. The final test sediments, i.e. mixed and with filter sand added, are hereafter referred to as 'clean' and 'polluted'.

The test sediments were added to duplicate tanks in May and benthic invertebrates were added 30 days later. After a further 30 days 60 0-group flounder (3.1 ± 1.1 g, 56 ± 6 mm) were added to each tank. Benthic *in situ* invertebrate prey species were supplemented daily with frozen mysid shrimp (at a rate of ~1% of body weight/day) and once a week with commercial pellets (Bio-Optimal START 1.5 mm, Biomar, 1% of body weight/week). Water temperature and salinity were recorded weekly. After three months, an additional 1–2 cm of test sediment was added to each mesocosm and the mesocosm-exposed fish were sampled after 7 months of exposure to the sediments.

Five aliquots from each of the 'clean' and 'polluted' sediments were collected for chemical analysis at the point of first addition to the tanks.

Tanks were regularly inspected for mortalities and dead fish removed. After 7 months, surviving fish were counted and taken for biometric, chemical and biological analyses. Salinity over the course of the experiment was $19.8 \pm 1.5\%$ (mean of weekly measurements from each of the 4 tanks) and temperature was $12.3 \pm 2.7\,^{\circ}C$ (mean of weekly measurements from each of the 4 tanks), ranging from a maximum of $16\,^{\circ}C$ to a minimum of $8\,^{\circ}C$, in accordance with the normal environmental variation over the period.

2.3. Sediment chemistry

Unless stated otherwise, chemical analyses were accredited to ISO/IEC 17025:2005 by the UK Accreditation Service.

2.3.1. Determination of sediment characteristics

The particle size distributions of freeze dried test sediments were determined using a laser granulometer, after initial sieving to 2 mm. The concentrations of Total, Inorganic, and Organic Carbon (TC, TIC, TOC) in freeze dried sediments, were determined using