Gene isolation and identification as tools for toxicity evaluation of perfluorooctane sulfonate (PFOS) in the marine environment

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Introduction

Despite the fact that perfluorinad compounds (PFCs) are being produced over 50 years, that they have a broad application spectrum in industry and households and that these chemicals are spread worldwide in wildlife and humans, the toxicological profile of these chemicals is very incomplete and insufficient to assess the impact and the hazard for man and biota. Biochemical effects of perfluorooctane sulfonate (PFOS) exposure are mainly studied in mammalian species such as rats and apes and includes serious chronic toxic effects such as induction of microsomal liver carboxylesterase RL4 and postnatal deaths and developmental problems[1,2]. However, effects of perfluorinated chemicals on fish remain largely unknown[3,4]. This lack of toxicity data point out that there is an urgent need for the mechanistic molecular understanding of the mode of action of these chemicals. Toxicogenomics are potentially well suited to meet this challenge. In the past, most toxicity testing of unknown chemicals used a number of short-term bio-assays. These test are time and money consuming, and generally focus on a single end point. In contrast, the use of DNA micro arrays are a powerful tool for monitoring the expression of thousands of genes simultaneously. Interest in using toxicogenomics to quickly classify pollutants based on characteristic gene expression profiles and to use these to unravel the mechanisms of action has increased significantly. Data are only now becoming available on the effects of PFCs, and more specifically on PFOS, using gene expression profiles as determined by micro array technology[5,6].

Since little precise is known on the effects of PFOS in aquatic species, the goal of this study was to assess the impact of PFOS on the aquatic ecosystem and to characterize the working mechanism of PFOS and related compounds. The species selected in this study was the European sea bass, *Dicentrarchus labrax* (*Moronidae*), a euryhaline marine teleost species which inhabit the rivers and coastal waters in the eastern Atlantic Ocean and the Mediterranean Sea. Sea bass is a commercially important species, which through the food chain can cause a possible treat to human health.

For this purpose, an in-depth study into the molecular mechanisms of PFOS for a marine fish was conducted. A recently established technique for differential gene expression, Suppression Subtractive Hybridization-Polymerase Chain Reaction (SSH-PCR), has been used combined with the technology of cDNA micro array hybridisation. This provided the possibility of a high-through put technique allowing screening of hundreds of individual mRNA products in a single analysis[7].

Materials and Methods

Sexually immature European sea bass (58 ± 3 g) were acclimatised at 12°C for 4 weeks in plastic 25L aquaria filled with fully aerated water with a salinity of 35 ‰. The fish were divided into five groups of six fish and randomly assigned to the experimental tanks. After acclimatisation, fish were exposed for 14 days to PFOS at 0, 0.1, 1, 10 and 100 ppm PFOS through the water. To avoid loss of PFOS, no chemical filtration was effected, but movement and oxygenation of the water was carried out by an oxygen pump. Every two days, the exposure medium was renewed. Fish were not fed during the duration of the experiment. At the end of the experiment, the sea bass were killed by a sharp blow on the head. The livers were removed, frozen in liquid nitrogen and stored at -80°C.

Concentrations of PFOS in liver tissue of all fish were determined using high-performance liquid chromatography (HPLC) combined with electrospray tandem mass spectrometry (LC-MS/MS) as) as described by Hansen et al. with some minor modifications described by Hoff et al.[8,4].

In the present study, Suppression Subtractive Hybridization-Polymerase Chain Reaction (SSH-PCR), has been used

in combination with cDNA microarrays[5]. Briefly, totally RNA was isolated using the Totally RNA™ Kit (Ambion) according to the manufacturer's instructions. For removal of DNA contamination from RNA samples, the DNAse treatment described by Sambrook et al. was performed[9]. SSH was performed with the PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Palo Alto, CA) as described by the manufacturer with modifications according to Moens et al.[5]. This way, cDNA clones of the enriched library were obtained and used to construct a custom made micro array, containing approximately 3200 cDNA spots, representing 737 SSH clones, some housekeeping genes like actin and GAPDH and the Lucidea Universal ScoreCard controls (Amersham Biosciences). Afterwards, all exposure groups were individually hybridised against the control group; this way, four different arrays were hybridised. After post-hybridisation washing, micro array slides were scanned at 532 and 635 nm using a GenePix Personal 410A Scanner (Axon Instruments, Union City, CA, USA). Image analysis was conducted using GenePix Pro 4.1 software (Axon Instruments, Union City, CA, USA) for spot identification and for quantification of the fluorescent signal intensities. Clones with at least an average fold-change of 1.88 were retained for sequencing.

Results

The two week during water exposure experiment, resulted in hepatic mean PFOS concentrations of 12.7 ± 6.4 µg/g for the control group, and 27.3 ± 9.6, 78.7 ± 21.2, 350.1 ± 191.6 and 573.1 ± 422.9 µg/g wet weight (mean ± SD) for the exposure groups of respectively 0.1, 1, 10 and 100 ppm.

A total of 195 cDNAs were differentially expressed in liver tissue of sea bass after exposure to PFOS. For nonmodel species, DNA sequence data are poorly represented in the public data base. By sequence analysis, 28 of the cDNAs could be identified by homology. The genes with known function were classified into various functional groups. Major functional classes of differentially expressed genes included detoxification-related genes, oxidative stress related genes and genes involved in energy or iron/heme metabolism. Some of the genes were classified as "miscellaneous" as they are involved in various cellular processes.

Discussion

The study of PFOS-mediated gene transcription has received only little attention. Information on PFCs-mediated gene induction and repression in marine fish is completely lacking. This study makes a fist attempt to characterize some of the genes influenced by PFOS exposure in the European sea bass.

The selection of the exposure concentrations used, was based on results previously obtained in our lab. It is remarkable that within a rather short space of time (only 14 days) there is already a very high and rapid accumulation, even at the lowest exposure doses. The observation that even fish of the control group contained detectable hepatic PFOS concentrations can be explained by the food of the fish. It consists of artificial sea bass food pellet, containing already 24.2 ng/g PFOS wet weight.

Our toxicogenomic data reveal a broad range of biochemical pathways that may be effected by exposure to PFOS. Several of the differentially expressed genes shared roles in a common physiological process. Interesting in our data is the influence on genes which are known to respond to organic pollutants and are involved in detoxification (28% of the genes). Eight of the identified clones correspond to genes of the cytochrome P450 family (subfamilies 1, 2 and 3). The majority of these genes were up regulated after PFOS exposure. The induction of hepatic cytochrome P450 isomers can lead to DNA-adduct formation and carcinogenesis[10]. In addition with the present study, several cytochrome P450s were induced using the Affymetrix rat genome U34A genechip[5]. It is known that PFOS has potent hepatic peroxisome proliferating capacities in rats, mice and freshwater fish, a phenomenon that has been intimately correlated with hepatocarcinogenesis[11,12].

With regard to the high up regulation of some transcripts even at the lowest exposure dose, genes involved in successive steps of iron/heme catabolism, like ferritin, are of particular interest. The up regulation of ferritin might be an indication of elevated iron concentrations, which can be associated with an increased risk of adverse effects such as liver fibrosis[13]. A previous study has demonstrated that PFOS can cause liver damage[4]. The induction of genes related to iron metabolism might have a link with the reported inflammation-independent leakage of liver cells and disturbance of DNA metabolism homeostasis in liver tissue after exposure to PFOS.

In conclusion, the data obtained in this study identifies several genes whose mRNA is regulated after PFOS exposure

suggesting an array of hypotheses which could be tested to reveal the toxicological mode of action of perfluorinated chemicals. It will know be important to further verify the data, to look for the same patterns in fish collected from field studies and to focus more in detail on the different pathways that have been shown to be affected.

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