ANALYSIS OF POLYCHLORINATED BIPHENYL (PCB)-INDUCED MODIFICATION OF PROTEIN EXPRESSION IN PRIMARY HEPATOCYTES FROM HARBOUR SEALS

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Introduction

Marine mammals like harbour seals are considered as sensitive indicators for environmental changes in the marine ecosystem due to their position as top predators in the food web, their long life spans, relatively low reproduction rates and their propensity to bioaccumulate pollutants. The Trilateral Monitoring and Assessment Program of the Wadden Sea ecosystem, for instance, uses common seals (*Phoca vitulina*) as one of its biological parameters, regularly monitoring population size and reproduction rates. Post-mortem examinations of stranded animals as well as blood sampling of free-ranging seals are also frequently used[1]. To identify protein patterns as additional sensitive and reliable biochemical markers for the early diagnosis of disorders caused by anthropogenic pollutants, an *in vitro* approach using primary seal hepatocytes has been established.

There is an ongoing need for biomarkers and cause-effect studies because top predators still show high burdens of pollutants. In addition, the distribution, bioavailability and transformation of pollutants may be altered due to environmental factors (e.g. temperature, sedimentation)[2]. Changes in such factors may also lead to increased vulnerability of marine organisms to pollutants by influencing the response mechanism regarding toxic injury or by modifying their predation behaviours[3]. High pollutant levels in marine mammals may cause severe effects such as morphological changes of organs and may lead to an increased susceptibility to infectious diseases[4, 5, 6]. A set of biomarkers can help elucidate relationships between pollutant levels and health effects at the population level[7]. One group of such biomarkers comprises proteins which are up-regulated by xenobiotics (e.g. proteins which are involved in the detoxification and biotransformation processes). The *in vitro* approach described in this paper was designed to reveal proteins up-regulated by polychlorinated biphenyls (PCBs), which are linked to severe effects in seals, such as infertility and intestinal ulcers, but also induce more subtle effects including endocrine disruption and immunosuppression[7]. Proteomics-based approaches used in an ecotoxicological context[8, 9] focus on pollutant-induced modifications in protein expression in appropriate blood or tissue samples which can be traced back unambiguously to the pollutant source. While samples from feeding experiments with organisms from lower trophic levels (e.g. mussels[10], fish) are readily accessible in sufficient number, sampling of and experimental work with free ranging marine mammals presents a challenge. Exposure experiments with isolated cells of marine mammals can help overcome the problem. A similar proteomics-based strategy uses blood cell cultures from seals[11].

In this paper we report first results from proteome analysis of primary hepatocytes from harbour seals (*Phoca vitulina)* which were incubated with PCBs. For isolating primary cells from wild-ranging seals in the North Sea, we developed a technique using tissues from freshly dead animals.

Materials and methods

Primary seal hepatocytes were isolated from a harbour seal (*Phoca vitulina*, FTZ lab code PV 3615, male, 8 months old, found 03.01.2007 at Eidersperrwerk, Schleswig-Holstein/Germany) approx. 1 h after death with a non-perfusion method[12]. The animal had to be euthanized by a designated seal ranger due to severe illness. After sampling the liver, a full necropsy examination[13] was performed at the FTZ. Suspensions of the isolated hepatocytes were seeded in culture flasks (Cell$^+$, Sarstedt) using GIBCO™ HepatoZYME serum free medium (6 x 10^6 cells/flask). After 24 h of incubation in a 5% CO_2 incubator at 37 °C, a 1:1:1 mixture of Aroclor 1254, 1260 and 1262 in isooctane (Supelco) was added (according to a final concentration of 1, 50 or 100 µM

Aroclor). Negative controls were treated with the solvent only. All treatments were performed in triplicate. After 24 h the cells were harvested and each pellet was extracted by sonication in rehydration buffer (8 M urea, 2% CHAPS, Bio-Lyte® buffer, bromophenol blue), to which dithiothreitol (DTT) and the protease inhibitor pepstatin A were added. IPG strips (linear pH 3-10, 24 cm) were rehydrated overnight at 50 V with the samples (according to approx. 300 µg protein) in 450 µl rehydration buffer and isoelectrically focused (1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, 1 h at 10000 V, and up to 40000 V-hr at 10000 V). The focused IPG strips were equilibrated in two steps (15 min each) in sample buffer (6 ml; 0.375 M Tris-HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol; 2% (w/v) SDS; bromophenol blue) supplemented with 20 mg/ml dithiothreitol (DTT) and 25 mg/ml iodoacetamide (IAA), respectively. The second dimension was carried out after 1 h at 1000 V in 26 h at 80 V using homogeneous acrylamide gels (12% T, size 250 x 205 x 10 mm) and a SDS-buffer system (125 mM Trizma® base, 960 mM glycine, 0.5% (w/v) SDS). Gels were stained with colloidal Coomassie® Brilliant Blue G 250. 2D gel image analysis and statistical comparisons between individual protein abundances in the scanned gels were performed with the software Delta2D 3.6 (Decodon, Greifswald, Germany). Spots were selected if their spot volume met the following criteria: two-fold up- or down-regulation; relative standard deviation (rsd) within one sample group: $\leq$30%; significance level in t-Test $\geq$95%. Selected spots were excised manually, destained with acetonitrile, reduced with DTT (10 mM in 400 µl 100 mM NH_4HCO_3) for 1 h at 56 °C, alkylated with IAA (55 mM in 400 µl 100 mM NH_4HCO_3) and subjected to in-gel digestion with trypsin using Trypsin Singles Proteomics Grade (Sigma) according to Shevchenko[14]. The peptide mixtures were acidified with 1 µl trifluoracetic acid (TFA) each and prepared on a MALDI target with α-cyano-4-hydroxycinnamic acid (HCCA) as matrix. Peptides were analyzed using an ultraflex II MALDI-TOF(-TOF) mass spectrometer and Biotools software, version 2.0 (both Bruker Daltonik) as well as MASCOT queries (Matrix Science, Oxford, UK).

Results and discussion:

Proteome analysis of incubation experiments

Incubation of primary seal hepatocytes with contaminants should reflect the *in vivo* response in the animals and therefore provide a means to examine species-specific effects and corresponding molecular structures as potential biomarkers. Figure 1 illustrates the *in vitro* approach comprising the comparison of protein expression levels in cells incubated with three pollutant concentrations with those in negative controls from the same cell batch. With the software Delta2D, about 160 spots/gel were detected all over the pI and MW ranges of the gels. After warping with Delta2D to eliminate slight differences in spot positions, the gel images were fused and the hereby detected spots transferred to all gel images of the sample batch. The intensities of all protein spots were quantified by normalizing the spot volumes, before statistical analyses were applied for identifying significant differences of protein abundances. For eleven spots the changes of the average values exceeded two-fold up- or down-regulation due to incubation with 50 µM and 100 µM of the Aroclor mixtures (see Table 1). The greatest changes in expression levels were detected for the up-regulated protein-spots (up to 11- fold) in the 100 µM incubation experiments.

Principal component analysis

We applied principal component analysis (PCA) to confirm the significance of modification in protein expression levels of these eleven proteins. PCA clearly distinguishes all the gels within three groups separated by the first component (see Figure 2). The negative controls as well as those from the 1 µM incubation experiment are located on the negative side of the x-axis, while the samples from the 100 µM incubation experiment are found on the positive side. The gels of the 50 µM and the 100 µM incubation experiment were separated by the second component. No gel was clustered separately.

Identification of proteins with contaminant-induced modification of expression level

Characterisation of these protein-spots was achieved by MALDI-TOF mass spectrometry. Results from the peptide mass fingerprint (PMF) approach of selected up- or down-regulated protein-spots including homology searches within protein sequences from other mammalian species are given in Table 1. Cytochrome P450 enzymes are a group of monooxygenases which are responsible for detoxification mainly by hydroxylation and epoxidation of hydrophobic substances. The cytochrome P450 pattern varies for different organs, individuals and species, making species-specific investigations, such as those described here necessary. Investigations of

cytochrome P450 1A (CYP1A) expression level in liver and skin biopsies of captive harbour seal pups as well as in skin biopsies of free-ranging seals indicated that CYP1A in skin may be used as a biomarker of contaminant exposure[15]. Many heat shock proteins (HSPs) assist the protein folding due to their function as molecular chaperones and are up-regulated by heat or chemical stress as a response to the denaturation of proteins[16, 17].

For a complete characterization, *de novo* sequencing of the proteins is essential, as only a few protein sequences from marine mammals are known to date. The PMF for spot 486, for example, was confirmed by MS/MS data in a peptide fragmentation fingerprint (PFF) approach.

Conclusion and outlook

The pilot study with primary hepatocytes from *Phoca vitulina* showed that the modification in protein expression levels affects only a manageable number of around ten proteins from approx. 160/gel. Principal component analysis confirmed that the protein expression pattern makes it possible to discriminate between incubated cells and negative controls. The incubating experiments described herein were performed using polychlorinated biphenyls (PCBs) with a hepatotoxic mode of action reported in other species[18]. Some of the up-regulated proteins are putatively identified to belong to the group of cytochrome P450 enzymes which are known to play a central role in biotransformation of pollutants. The potential identification of this type of enzyme supports the applicability of our *in vitro* approach. Further incubation experiments with other substance classes are currently under way. This species-specific set of proteins, for which expression levels are modified when exposed to pollutants has to be reconfirmed in tissue and blood samples. The set should contribute to the optimization of an effect-oriented monitoring strategy of the influence of pollutants on marine mammals.

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Figure 1: Workflow of the *in vitro* approach

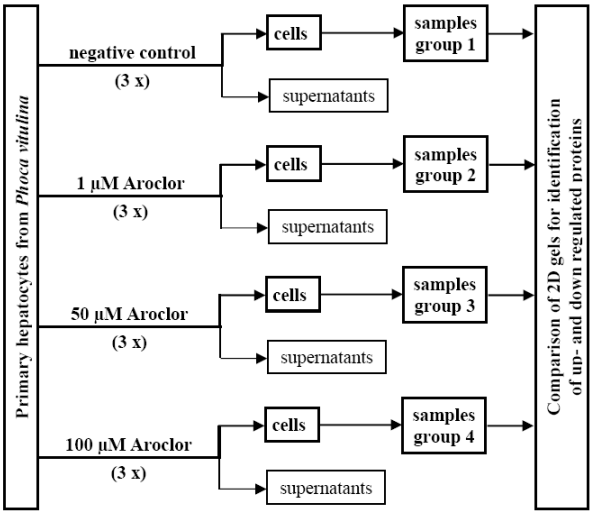

Figure 2: Principal component analysis for clustering of the 2D GE maps

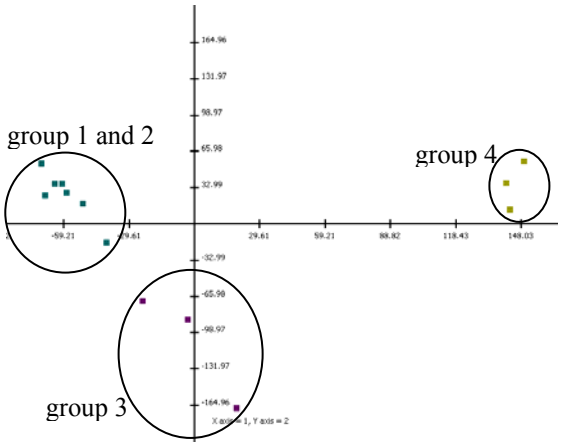

Table 1: Differential spot intensity and putative identification of spots in the 2D GE maps by MALDI-TOF-MS

	observed data			Differential spot intensity [x-fold][b]			Putative identification by PMF[c] (peptide mass fingerprint)				
Spot no.	**pI**	**M_r [kDa]**	**rsd [%][a]**	**2**	**3**	**4**	**Highest score**	**score**	**Best match with M_r**	**score**	
461	6.3	50	13.1	1.1	2.0	11.2	Roundabout homolog 4 precursor (Human)	44	GTP- binding protein (Human)	35	
486	6.5	65	2.4	1.3	1.8	8.9	60 kDa heat shock protein (Orangutan)	48	60 kDa heat shock protein (Orangutan)	48	
501	6.5	55	20.5	0.5	1.4	8.3	Cystathionine beta-synthase (Human)	48	Cytochrom P450 3A1 (Rat)	37	
540	6.9	50	6.2	1.0	0.7	2.3	Serum Albumin precursor (Bovine)	59	Protein HEXIM1 (Rat)	36	↑
559	6.9	47	6.7	1.1	0.8	3.0	DNA topoisomerase 2-alpha (Pig)	49	Cytochrom P450 11B1 (Golden hamster)	33	
564	6.9	67	6.8	1.3	1.4	9.9	Cytochrom P450 3A1 (Rat)	41	Neurexin-3-beta (Human)	32	
622	7.1	60	27.3	-0.8	-1.2	-4.5					
624	7.4	45	28.3	-0.9	-1.1	-2.5					
652	7.2	48	29.0	-0.7	-1.1	-3.2	Glia-activating factor (Human)	49	Islet cell autoantigen 1-like protein (Rat)	34	↓
689	7.1	48	29.6	-1.0	-1.2	-8.3					
743	7.5	50	5.9	-1.1	-2.2	-5.8					

[a] rsd relative standard deviation; [b] ratios of corresponding spot volumes;
[c] search against the Swiss-Prot database