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Short communication

Transcriptomic analysis of bottlenose dolphin (*Tursiops truncatus*) skin biopsies to assess the effects of emerging contaminants

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ABSTRACT

Chemicals discovered in water at levels that may be significantly different than expected are referred to as *contaminants of emerging concern* (CECs) because the risk to environmental health posed by their occurrence/frequency is still unknown. The worldwide distributed compounds perfluorooctanoic acid (PFOA) and bisphenol A (BPA) may fall into this category due to effects on endocrine receptors.

We applied an *ex vivo* assay using small slices of bioptic skin from the bottlenose dolphin, *Tursiops truncatus*, cultured and treated for 24 h with different PFOA or BPA concentrations to analyze global gene expression. RNA was labeled and hybridized to a species-specific oligomicroarray. The skin transcriptome held information on the contaminant exposure, potentially predictive about long-term effects on health, being the genes affected involved in immunity modulation, response to stress, lipid homeostasis, and development. The transcriptomic signature of dolphin skin could be therefore relevant as classifier for a specific contaminant.

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1. Introduction

The health decline of the marine ecosystem can be mostly related to anthropogenic impacts such as overfishing, coastal habitat destruction, deep sea mining, oil and gas exploration, and ocean acidification, but it is also strictly correlated to industrial application and the release of chemical contaminants and pollutants. The exposure to those named as *contaminants of emerging concern* (CECs, http://water.epa.gov/scitech/cec/) is unavoidable: after their release into the environment, they can be transported through air, water and soil, and be a threat to both ecosystem and human health. Several of these chemical products are classified as endocrine disruptors (EDs), since they can interfere modifying the synthesis, circulating levels, and peripheral action of hormones (Casals-Casas and Desvergne, 2011).

Among the CECs is bisphenol A [2,2 bis(4-hydroxyphenyl) propane; BPA], an ubiquitous, high-volume-production (>2.5×10^6 kg year^{-1}) monomer used in the manufacture of polycarbonate plastics (Casals-Casas and Desvergne, 2011). BPA has been shown to leach out of products such as plastic containers, utensils, toys, water bottles and fax paper, and high levels of monomer have been identified in human and animal samples (McLachlan, 2001). In humans, high urinary concentrations of BPA are associated with an increased occurrence of cardiovascular disease, diabetes, and liver enzyme abnormalities (Lang et al., 2008) and a large body of evidence links BPA to adverse health effects in perinatal, childhood and adult (Rochester, 2013). It can bind estrogen receptors and promote both agonist and antagonist activities. BPA binds to aryl hydrocarbon receptors and has diverse endocrine effects on mammalian and non-mammalian health (Kharrazian, 2014). The contaminant concentration for BPA already measured in the environment is 1 µg/ml (http://www.epa.gov/oppt/existingchemicals/pubs/actionplans/bpa.html).

Another CEC is the perfluorooctanoic acid (PFOA), one of the

Abbrevations: ADIRF, adipogenesis regulatory factor; BCAP31, B-cell receptor-associated protein 31; BPA, bisphenol A; CDC42, cell division cycle 42; CECs, contaminants of emerging concern; EDs, endocrine disruptors; f, fold; GADPH, glyceraldehyde 3-phosphate dehydrogenase; GAP, GTPase-activating protein; GO, Gene Ontology; GPCR, G protein-coupled receptor; MTSS1, metastasis suppressor 1; PFCs, perfluorinated compounds; PFOA, perfluorooctanoic acid; qPCR, quantitative real-time polymerase chain reaction; RGS2, regulator of G-protein signaling 2; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

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most important synthetic perfluorinated compounds (PFCs). The chemical structure of PFCs gives them unique properties, such as thermal stability and the ability to repel both water and oil, that make them useful components in a wide variety of consumer and industrial products including paper, leather and fire-fighting foam. Due to its persistence and bioaccumulation PFOA has been listed as emerging persistent organic pollutant in 2009 Stockholm Convention (Wang et al., 2009). High production volumes led to widespread distribution in the environment, and once absorbed PFOA does not undergo biotransformation, distributing primarily in the liver and plasma, and to a lesser extent in the kidneys and lungs (Kudo and Kawashima, 2003). PFOA has been found to exert acute and sub-chronic toxic effects with the liver as a primary target organ in mice and rats, rabbits and birds (Kannan et al., 2002; Betts, 2007). Several recent studies detected PFOA in a variety of wildlife animals including fresh water species, marine mammals and shellfish, and suggested that can be biomagnified at the top levels of the food chain (Lau et al., 2004). PFOA concentrations up to 10 ng/ml have been detected worldwide in human serum (Haug et al., 2009). PFOA can be considered an ED, since the exposure of rodents led to serious impact on phospholipid metabolism, reductions in serum cholesterol (Peng et al., 2013), hormonal perturbations with decreased testosterone and increased estradiol levels (Shi et al., 2007).

Some CECs can bioaccumulate through the food chain, leading to highest levels of exposure in predator species. The bottlenose dolphin (*Tursiops truncatus*) spends its life in marine coastal environment, feeds at a high trophic level and is exposed to contaminants of human concern. Due to their long lifespan, dolphins could be chronically exposed to CECs and as top-level predators concentrate the contaminants in their body, being also endowed with large blubber stores that can serve as depots for anthropogenic chemicals and toxins. In addition, its populations display high site fidelity for coastal locations thus have the potential to be sentinel species for emerging contamination in that areas (Aguirre and Tabor, 2004; Wells et al., 2004; Moore, 2008; Bossart, 2011; Kucklick et al., 2011).

Here we report about the effects of BPA and PFOA on bottlenose dolphin skin biopsies analyzed by transcriptomics. This tool allows the response to treatment of thousands of genes to be examined simultaneously, providing a comprehensive information on the pathways modulated. The analysis of genes in the cellular context under controlled conditions would allow identifying those differentially expressed and classifying as exposure biomarkers. This approach could be informative not only of the impact of CECs on dolphins (and marine mammals in general), but also on the threats posed to the marine ecosystem and have a practical outcome due to the development of real-time tools for rapid detection of contaminant exposure.

2. Methods

2.1. Bottlenose dolphin samples

Skin samples were obtained 4 h after death of a juvenile female (length 144 cm, weight 31.6 Kg) stranded on Tyrrhenian shores (Italy) in October 2011 (CITES permit: Nat. IT025IS, Int. CITES IT 007 issued to Accademia dei Fisiocritici and University of Siena). No relevant pathology, parasites and lesions were detected at *post mortem* examination and the cause of death is unknown.

2.2. Ex vivo assay

Slices (about 2 mm-thick) spanning the epidermis and dermis were cut from skin samples of the stranded specimen immediately after collection, to set up the organotypic cultures exposure experiments in 5 ml culture tubes, as previously described (Godard et al., 2004). Distinct slices were separately incubated for 24 h at room temperature (24–28 °C) in cell culture media (Fossi et al., 2006) containing BPA (0.1 or 1 μg/ml), or PFOA (0.1 or 1 μg/ml), or the vehicle (final concentrations: 0.01% ethanol for BPA and 0.1% methanol for PFOA) in a final volume of 3 ml. Thereafter, they were homogenized using a Tissue Lyser (Qiagen) and RNA was extracted using the Aurum™ Total Fatty and Fibrous Tissue kit (Bio-Rad) following the manufacturer's instructions.

2.3. Dolphin microarray hybridization and gene expression analysis

The microarray used was a species-specific, custom 4 × 44K Agilent oligo array representing 24,418 unigene sequences (Mancia et al., 2015).

All RNA labeling and microarray hybridizations were performed according to the manufacturer's instructions in the One-Color Microarray-Based Gene Expression Analysis manual (Agilent Technologies, Santa Clara, CA). Five hundred nanograms of RNAs from each treated slice were hybridized. One-color gene expression was performed according to the manufacturer's procedure. Briefly, total RNA fraction was obtained from samples by using the RNeasy kit (Qiagen). RNA quality was assessed by the use of Agilent 2100 Bioanalyzer. Low quality RNAs (RNA integrity number <6) were excluded from microarray analyses. Fluorescent complementary RNA (cRNA) was synthesized from 200 ng of total RNA using the Low Input Quick-Amp Labeling Kit, one color (Agilent) in the presence of cyanine 3-CTP. Hybridizations were performed at 65 °C for 17 hs in a rotating oven. Images at 5 μm resolution were generated by Agilent scanner and the Feature Extraction 10.7.3.1 software (Agilent) was used to obtain the microarray raw-data. The microarray build and hybridization data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO accession numbers GSM1712791- GSM1712796 (www.ncbi.nlm.nih.gov/geo/).

Microarray results were analyzed using GeneSpring GX v.12 software (Agilent). Data transformation was applied to set all the negative raw values at 1. Data were normalized using quantile normalization. A filter on low gene expression was used to keep only the probes expressed in at least one sample (flagged as detected). Then, samples were grouped in accordance to their treatment status. BPA- and PFOA-treated samples were analyzed compared with samples incubated in cell media containing the specific vehicles. Differentially expressed genes were selected as having a 1.5-fold expression difference (geometrical mean) between the groups of interest and a statistically significant p-value (<0.05) at moderated t-test statistic, followed by the application of the Benjamini and Hoechberg correction for false positives reduction. Differentially expressed genes were employed for cluster analysis of samples using the Manhattan correlation as a measure of similarity. Gene Ontology (GO) analyses were carried out using Blast2go pro software (www.blast2go.com).

2.4. Quantitative real time PCR: validation of microarray data

Results from the microarray analysis were validated by qPCR by measuring mRNA expression of four selected genes in all the treated samples. *GADPH* (glyceraldehyde 3-phosphate dehydrogenase) and *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) genes were used as internal controls.

Relative mRNA levels were determined on a CFX Connect (Bio-Rad) with specific primers designed using *Tursiops truncatus* sequences on the arrays and in the NCBI public database. Each primer

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