Science of the Total Environment 640–641 (2018) 22–30

Contents lists available at ScienceDirect

Science of the Total Environment

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

Identification of candidate biomarkers of the exposure to PCBs in contaminated cattle: A gene expression- and proteomic-based approach

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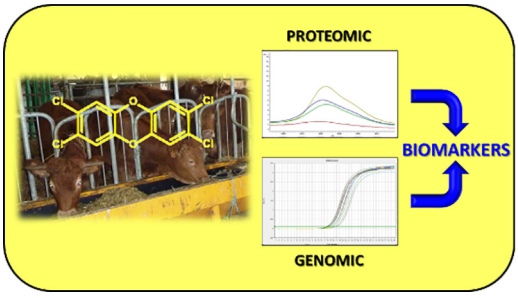
HIGHLIGHTS

- Blood gene expression and protein profiling in PCB exposed heifers were evaluated.
- mRNA levels of interleukin 2 and CYP1B1 were correlated with contamination.
- MALDI-TOF-MS serum profiling identified 7 differentially represented polypeptides.
- A dioxin-responsive network of proteins and genes could serve as biomarker of contamination.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 29 March 2018
Received in revised form 23 May 2018
Accepted 23 May 2018
Available online xxxx

Editor: Adrian Covaci

Keywords:
Cattle
Polychlorinated biphenyls
Biomarkers
AhR genes
MALDI-TOF-MS serum profiling
TEQ

ABSTRACT

Dioxins and polychlorinated biphenyls (PCBs) are widespread and persistent contaminants. Through a combined gene expression/proteomic-based approach, candidate biomarkers of the exposure to such environmental pollutants in cattle subjected to a real eco-contamination event were identified. Animals were removed from the polluted area and fed a standard ration for 6 months. The decontamination was monitored by evaluating dioxin and PCB levels in pericaudal fat two weeks after the removal from the contaminated area (day 0) and then bimonthly for six months (days 59, 125 and 188). Gene expression measurements demonstrated that CYP1B1 expression was significantly higher in blood lymphocytes collected in contaminated animals (day 0), and decreased over time during decontamination. mRNA levels of interleukin 2 showed an opposite quantitative trend. MALDI-TOF-MS polypeptide profiling of serum samples ascertained a progressive decrease (from day 0 to 188) of serum levels of fibrinogen β-chain and serpin A3-7-like fragments, apolipoprotein (APO) C-II and serum amyloid A-4 protein, along with an augmented representation of transthyretin isoforms, as well as APOC-III and APOA-II proteins during decontamination. When differentially represented species were combined with serum antioxidant, acute phase and proinflammatory protein levels already ascertained in the same animals (Cigliano et al., 2016), bioinformatics unveiled an interaction network linking together almost all components. This suggests the occurrence of a complex PCB-responsive mechanism associated with animal contamination/decontamination, including a cohort of protein/polypeptide species involved in blood redox homeostasis, inflammation and lipid

Abbreviations: ACN, Acetonitrile; AhR, Aryl hydrocarbon receptor; APO, Apolipoprotein; CNRQ, Calibrated normalized relative quantity; DL, Dioxin-like; ESI-Q, Electrospray ionization quadrupole; GPX, Glutathione peroxidase; HP, Haptoglobin; IL-2, Interleukin 2; LIT, Linear ion trap; MALDI-TOF, Matrix-assisted laser desorption ionization-time of flight; MS, Mass spectrometry; NDL, Non dioxin-like; PCB, Polychlorinated biphenyl; PCDD, Polychlorinated dibenzo-p-dioxin; PCDF, Polychlorinated dibenzofuran; q-PCR, Quantitative RT-PCR; SOD, Superoxide dismutase; TCDD, 2,3,7,8 tetrachloro-dibenzo-p-dioxin; TEQ, Toxic equivalent; TFA, Trifluoroacetic acid; 2-DE, Two-dimensional gel electrophoresis; TNF-α, Tumor necrosis factor-α; TTR, Transthyretin.

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https://doi.org/10.1016/j.scitotenv.2018.05.284

transport. All together, these results suggest the use in combination of such biomarkers for identifying PCB-contaminated animals, and for monitoring the restoring of their healthy condition following a decontamination process.

1. Introduction

Polychloro-dibenzo-*p*-dioxins (PCDDs), polychloro-dibenzo-furans (PCDFs), and polychlorinated biphenyls (PCBs) are hazardous and highly persistent environmental pollutants of anthropogenic origin. A large array of adverse effects have been reported in animals and humans as the result of the prolonged exposure to such contaminants, including tumor promotion and teratogenicity, and perturbation of the immune, endocrine and nervous systems (Tavakoly Sany et al., 2015). For the so-called "dioxin-like" (DL) compounds, it is believed that most of the above effects are mediated by the interaction with the aryl hydrocarbon receptor (AhR). This triggers the transcription of the "AhR gene battery", which comprises genes encoding for a number of biotransformation enzymes (*e.g.* CYP1A1, CYP1B1, UGT1A, and GSTA1), as well as proteins involved in the regulation of proliferation and differentiation, and in the homeostasis of the immune system (*e.g.* interleukin 2, IL-2) (Bock and Koehle, 2009). PCBs not able to bind to the AhR receptor are defined as non-DL (NDL), and are mainly involved in neurotoxicity, immune suppression and endocrine disruption (Hamers et al., 2011).

Due to their high lipophilicity, PCDDs, PCDFs and PCBs are reported to accumulate along the food chain and to contaminate animal products (meat, milk and dairy products), which represent by far the most important non-occupational source of contamination for humans (Malisch and Kotz, 2014). Analytical methods for screening and confirmation of DL-compounds in animal products are rather expensive and time consuming, thereby limiting the number of tests to be carried out in the frame of the National Control Plans on food and feed. Thus, there is a growing interest in the development of faster and cost effective complementary screening methods able to identify the contaminated animals. A promising approach is the search for biomarkers resulting from biological events ensuing the binding of DL-compounds to AhR in target cells or tissues that should be easily collectable in living organisms. In this respect, different expression profiles between waste incineration workers and controls were reported upon the gene microarray analysis of peripheral blood mononuclear cells (Kim et al., 2004). Accordingly, circulating lymphocytes from individuals exposed to 2,3,7,8 tetrachloro-dibenzo-*p*-dioxin (TCDD) during the Seveso disaster displayed significant differences in AhR-dependent gene expression and inducibility compared to unexposed subjects (Landi et al., 2003). In line with the findings in humans, we have recently demonstrated that the gene expression of CYP1B1 in lymphocytes is significantly upregulated in dairy cows naturally exposed to DL-compound contaminated feedstuffs, as assessed by bulk milk Toxic Equivalent (TEQ) values (Girolami et al., 2013).

Another readily available biological matrix is serum or plasma, which contains a complex array of proteins that may be correlated to biological events occurring in the entire organism (Villanueva et al., 2004), including the exposure to DL-compounds (Joo et al., 2003). Proteomic methods based on two-dimensional gel electrophoresis (2-DE) coupled with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and/or electrospray ionization quadrupole (ESI-Q)-TOF mass spectrometry (MS) have been applied to plasma samples to identify differentially represented proteins in workers at municipal incinerators (Kang et al., 2005; Kim et al., 2004), and in rats experimentally treated with TCDD (Son et al., 2003). More recently, the serum protein profiling of TCDD-exposed rats through magnetic bead separation and MALDI-TOF mass spectrometry showed several peaks (*i.e.* peptides) having differential representation between treated and control animals (Chen et al., 2012). A similar approach has already been employed in the bovine species for the detection of the treatment with illegal growth promoting agents in veal calves (Della Donna et al., 2009); however, no data are available about the use of serum protein profiling for the study of DL-compound contamination in food producing animals.

The present study has been carried out in Limousine heifers accidentally exposed to a forage contaminated by the emissions of an industrial plant specialized in PCB treatment. Subsequently, the animals underwent a decontamination procedure based on the removal from the polluted area and the feeding of a controlled diet for 6 months. We have already described the changes of specific indices of blood redox homeostasis and inflammation associated with the decontamination (Cigliano et al., 2016). Here we report on gene expression and protein changes in corresponding lymphocytes and serum as determined by transcript and protein profiling procedures, respectively.

2. Materials and methods

2.1. Animals and sampling

All the information regarding the animals included in the study and the sampling program, together with the source of contamination, the decontamination procedure (*i.e.* location and diet) and the evaluation of the contamination extent were detailed elsewhere (Cigliano et al., 2016). Briefly, eight 1-year old PCB contaminated heifers were removed from the contaminated area and housed in an experimental facility located far away this area for 6 months. The animals were weighed, blood sampled and submitted to a pericaudal biopsy to get fat samples 2 weeks after their arrival in the experimental facility (day 0, sampling A), and then bimonthly during the decontamination period (59, 125 and 188 days after starting the decontamination; samplings B, C, and D, respectively). Fat samples were used for the measurement of TEQ values (DL-PCB + PCDD/F), and of NDL-PCB concentrations. Blood samples were divided in dry or in EDTA tubes for serum and lymphocytes separation, respectively. After clotting, serum was separated by centrifugation at 1272*g* for 15 min at 25 °C, divided into aliquots and stored at −80 °C pending MALDI-TOF-MS protein profiling. Circulating lymphocytes were isolated by Histopaque-1077 gradient centrifugation (Spalenza et al., 2011), and stored at −80 °C until gene expression analysis.

2.2. DL-compound and NDL-PCB determinations

Quantitative determination of PCDD/F,DL-PCB and NDL-PCB in pericaudal fat biopsies was reported in Cigliano et al. (2016). Samples were analysed by GC-HR-MS, according to the requirements of the quality assurance parameters of the Commission Directive 2002/69/EC and 2002/70/EC of July 2002 laying down the sampling methods and the methods of analysis for the determination of PCBs in foodstuffs and feeding stuffs, respectively. Analytical methods were accredited to the ISO 17025 standard.

2.3. Quantitative RT-PCR on blood lymphocytes

Total RNA was isolated using QIAzol Lysis Reagent (Qiagen, Valencia, CA), according to the manufacturer's protocol. RNA purity and quantity were evaluated by absorbance readings using the NanoDrop ND-2000 spectrophotometer (Thermo Scientific); the ratio of the optical densities measured at 260 and 280 nm were > 1.9 for all RNA samples. RNA integrity was assessed using an automated electrophoresis station (Experion