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Modulation of aryl hydrocarbon receptor target genes in circulating lymphocytes from dairy cows bred in a dioxin-like PCB contaminated area

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HIGHLIGHTS

- The expression of AHR-target genes in blood bovine lymphocytes was evaluated.
- The lymphocyte CYP1B1 expression appears to be related to bulk milk TEQ values.
- Blood lymphocytes from dairy cows might represent a matrix for dioxin biomonitoring.

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ABSTRACT

Animal productions (i.e. fish, eggs, milk and dairy products) represent the major source of exposure to dioxins, furans, and dioxin-like (DL) polychlorobiphenyls for humans. The negative effects of these highly toxic and persistent pollutants are mediated by the activation of the aryl hydrocarbon receptor (AHR) that elicits the transcriptional induction of several genes, including those involved in xenobiotic metabolism. Previously we demonstrated the presence and functioning of the AHR signaling pathway in primary cultures of bovine blood lymphocytes. The aim of the present study was to investigate by real time PCR the expression and the inducibility of selected target genes (i.e. AHR, AHR nuclear translocator (ARNT), AHR repressor, CYP1A1 and CYP1B1) in uncultured cells from dairy cows naturally exposed to DL-compounds. The study was carried out on two groups of animals bred in a highly polluted area and characterized by a different degree of contamination, as assessed by bulk milk TEQ values, and a control group reared in an industry free area. Bovine lymphocytes expressed only AHR, ARNT and CYP1B1 genes to a detectable level; moreover, only CYP1B1 expression appeared to be correlated to TEQ values, being higher in the most contaminated group, and decreasing along with animal decontamination. Finally, lymphocytes from exposed cows displayed a lower inducibility of both CYP1A1 and CYP1B1 after the in vitro treatment with a specific AHR ligand. In conclusion, our results indicate that DL-compound contaminated cows may display significant changes in AHR-target gene expression of circulating lymphocytes.

1. Introduction

Polychlorodibenzo-*p*-dioxins (PCDDs), polychlorodibenzofurans (PCDFs) and polychlorobiphenyls (PCBs) are persistent environmental pollutants of main anthropogenic origin. A wide array of pathological effects has been reported following the exposure to such contaminants, including cancer and developmental defects, as well as perturbation of the endocrine, reproductive, and immune systems (Mandal, 2005). Only a relatively small number of congeners, collectively referred to as dioxin-like (DL), display the property to bind with different affinities to the cytosolic aryl hydrocarbon receptor (AHR), which is considered a key event in DL-compound toxicity (Denison et al., 2011). After translocating into the nucleus, the complex first binds to the AHR nuclear translocator (ARNT) protein and subsequently to xenobiotic-response elements (XREs), resulting in the transactivation of a number of target genes encoding for biotransformation enzymes (e.g. CYP1A1, CYP1A2, CYP1B1, UGT1A, GSTA1, and NQO1), for the AHR repressor (AHRR) regulating the expression of AHR itself, and for proteins involved in cell growth, death, and migration (Hahn et al., 2009).

The AHR-dependent pathway appears to be well conserved across species and widely expressed not only in the liver, but also in several

Abbreviations: PCDDs, polychlorodibenzo-p-dioxins; PCDFs, polychlorodibenzofurans; PCBs, polychlorobiphenyls; DL, dioxin-like; AHR, aryl hydrocarbon receptor; ARNT, AHR nuclear translocator; XREs, xenobiotic-response elements; AHRR, AHR repressor; TCDD, 2,3,7,8 tetrachloro-dibenzo-*p*-dioxin; TEQ, toxic equivalent; DMSO, dimethylsulfoxide; qRT-PCR, quantitative reverse transcription PCR; cDNA, complementary DNA; CNRQ, calibrated normalized relative quantity; FC, fold-change; WHO, World Health Organization; EROD, 7-ethoxyresorufin O-deethylase.

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extrahepatic tissues and cell types, such as the skin, spleen and lymphoid tissues, lung, ovary, testis, and prostate (Harper et al., 2006; Mason and Okey, 1982). In recent years, a considerable body of knowledge has accumulated about the presence of the AHR pathway in circulating blood cells, and namely in peripheral lymphocytes. In particular, the constitutive expression of target genes such as CYP1A1 and CYP1B1, and their positive modulation brought about by several AHR ligands, including DL-compounds, has been reported in mouse, rat, and human lymphocytes under both in vitro and in vivo conditions (Nohara et al., 2006). More to the point, CYP gene expression comparable to that found in the liver was detected in human blood mononuclear cells (Furukawa et al., 2004) and quantitative evidence for similarities in the AHR-mediated CYP regulation suggests that circulating lymphocytes could serve as a surrogate for studying AHR-dependent changes in tissue CYP expression (Saurabh et al., 2010).

Owing to their high lipophilicity and to a general resistance to CYP-mediated biotransformation, DL-compounds tend to accumulate in the food chain being stored in fatty tissues of animals and humans and excreted in dairy milk and eggs. Food of animal origin is generally the principal route of human exposure to such pollutants, cow's milk and dairy products, bovine and porcine fats, eggs, and fish being identified as the main contributors (Bernard et al., 1999; Larsen, 2006). The monitoring of food producing species exposure to DL-compounds is therefore a critical step in the risk management procedures. Indeed, in the last decade the accidental contamination of certain feed ingredients has been the cause of veritable alimentary crises as was the case for the Belgian (1999), Irish (2008), and German (2010) incidents (Casey et al., 2010; Kupferschmidt, 2011). Episodes of more limited extent have been also recently recorded in Italy and France, as the result of either the pollution of pastures due to the emissions of certain industrial settings, or the illegal practice of dumping or burning solid wastes releasing PCDD-, PCDF-, and DL-PCBs (Diletti et al., 2004; Marchand et al., 2010).

Analytical methods for screening and confirmation of DL-compounds in animal productions 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, as well as their usefulness in the case of contamination outbreaks. A number of different approaches are therefore being developed, including bioassays like CALUX test (Denison et al., 2004) or devices based on a biological response such as biosensors (Chobtang et al., 2011). Landi et al. (2003) reported on differences in AHR-dependent gene expression and inducibility in circulating lymphocytes from individuals exposed to 2,3,7,8 tetrachloro-dibenzo-*p*-dioxin (TCDD) during Seveso's accident occurring 20 years before, making therefore peripheral leukocytes potential candidates for use as easily accessible biomarkers of exposure to DL-compounds in living organisms. As regards cattle, while the constitutive expression and the ligand-mediated modulation of the AHR pathway have been recently characterized in cultured blood lymphocytes (Girolami et al., 2011), no data pertaining living animals are available on this topic. This study was undertaken to gain further insight into the regulation of the AHR signaling pathway in circulating lymphocytes of dairy cows involved in a recent episode of DL-compound contamination occurring in the Susa Valley (northern Italy). Cows had been fed contaminated forage and hay collected nearby a high-temperature steel production plant, resulting in unacceptable levels of PCDD/Fs and DL-PCBs in bulk milk. An increased chromosomal fragility (Di Meo et al., 2011) and plasma changes suggestive of a marked oxidative stress (Spagnuolo et al., 2012) were also reported in the cows subject of the present investigation.

2. Materials and methods

2.1. Reagents

Histopaque-1077, dimethylsulfoxide (DMSO), and all cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO). PCB126 was supplied by LabService Analytica S.r.l. (Anzola Emilia, BO, Italy). All the materials for the quantitative (q) reverse transcription (RT) PCR analysis (including RNA extraction) were from Qiagen (Valencia, CA).

2.2. Animals and sampling

The study was performed in the Susa Valley (northern Italy) on 60 dairy cows, 40 of which came from two different farms (A and B, 20 animals each) located near a DL-compound contaminated area; the remaining 20 animals, reared in a non-contaminated area of the same valley, were used as controls (C). Cows from farms A and B were mainly Piedmontese × Valdostana cross-breeds, while control animals were Valdostana breed. The housing conditions were similar in all the farms. The animals were kept inside during the cold season (4 to 5 months/year), being offered hay and unifeed, while for the rest of the year cows were allowed to graze on pastures on areas that for farms A and B were located nearby the source of contamination. The extent of the DL-compound exposure was defined by measuring TEQ values of PCDDs, PCDFs, and DL-PCBs in bulk milk samples collected by the Regional Veterinary Services. The official chemical analyses were carried out by Istituto Zooprofilattico Sperimentale of Piemonte, Liguria, e Valle d'Aosta using a validated High Resolution Gas Chromatography Mass Spectrometry method. Milk sampling from the contaminated farms was performed twice at an 8-month interval (sampling I and II, respectively), in order to monitor the decrease of animal contamination, which was achieved by bringing the cows on less contaminated pastures and providing non-contaminated hay even during the outdoor season. TEQ values are summarized in Table 1.

Peripheral venous blood from each animal was collected by jugular venipuncture in EDTA tubes approximately 2 months after milk sampling I and II, respectively. In particular, blood sampling I was performed in April, and blood sampling II was performed in January. Due to farm management problems, blood sampling II was performed on 18 cows from farm A, 19 from farm B, and only 12 from farm C. Three months after blood sampling II, an additional lymphocyte collection was performed from a selected number of the already sampled cows (11 from farm A, and 11 from farm C) to establish primary cultures and to assess their in vitro inducibility by DL-compounds, as reported by Girolami et al. (2011).

2.3. Blood lymphocyte isolation, culture and treatment

Peripheral blood lymphocytes were isolated by Histopaque-1077 gradient centrifugation, using the method described by Spalenza et al. (2011). In certain experiments, lymphocytes were counted with a hemocytometer and viability was assessed with the trypan blue exclusion test (>90%). Cells were seeded at 2×10^6 cells/ml into 10-cm dishes and cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated horse serum, 2 mM L-glutamine and 10 μg/ml phytohemagglutinin, at 37 °C and 5% CO_2. After 2 h of pre-incubation, lymphocytes from each animal were treated with the solvent (DMSO) alone (used as control) or with PCB126 100 nM, and lysed after 2 h. Although the EC50 for

Table 1
Dioxin and DL-PCB levels in bulk milk from dairy cows reared in different areas of Susa Valley (northern Italy) at two time-points.

	Sampling I			Sampling II		
	A	B	C	A	B	C
WHO-PCDD/PCDF-TEQ (pg/g of fat)	1.66	1.11	1.2	0.53	0.69	n.d.
WHO-PCB-TEQ (pg/g of fat)	16.09	7.45	0.55	8.12	4.15	n.d.
WHO-PCDD/PCDF-PCB-TEQ (pg/g of fat)	17.75	8.56	1.75	8.65	4.84	n.d.

WHO: World Health Organization; TEQ: toxic equivalents; PCDD: polychlorinated dibenzo-*p*-dioxins; PCB: polychlorinated biphenyls; PCDF: polychlorinated dibenzofurans; n.d.: not determined.
Allowed levels of WHO-PCDD/F-TEQ and WHO-PCDD/F-PCB-TEQ in force at the time of the study were 3.0 and 6.0 pg/g of fat, respectively.