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Constitutive expression of the AHR signaling pathway in a bovine mammary epithelial cell line and modulation by dioxin-like PCB and other AHR ligands

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ABSTRACT

Environmental pollutants, such as dioxin-like (DL) PCBs, benzo(a)pyrene [B(a)P], and flavonoids are aryl hydrocarbon receptor (AHR) ligands and may be excreted in dairy milk. The expression of AHR-target genes, particularly those involved in xenobiotic biotransformation, and their modulation by two DL-PCBs, [B(a)P], and β-naphthoflavone was investigated in a bovine mammary epithelial cell line (BME-UV). As assessed by quantitative PCR, BME-UV cells expressed a functional AHR signaling pathway. All the AHR ligands induced a concentration-related increase in the transcription of cytochrome P450 1A1 and 1B1, known to be implicated in the bioactivation of several xenobiotics. Conversely, genes encoding for antioxidant and detoxifying enzymes, like quinone oxidoreductase or glutathione S-transferase A2, were not affected or even depressed. This study demonstrates the occurrence and the modulation by different AHR-ligands of genes involved in xenobiotic metabolism in BME-UV cells, with the potential generation of (re)active metabolites that may damage mammary tissue and/or affect animal or human health via the contaminated milk.

Keywords: Bovine Mammary Epithelial Cell; AHR-agonist; Dioxin-like PCB; Gene Expression; Xenobiotic Metabolizing Enzyme

Abbreviations: AHR, aryl hydrocarbon receptor; AHRR, AHR-repressor; ARNT, AHR-nuclear translocator; B[a]P, benzo[a]pyrene; β-NAF, β-naphthoflavone; CYP, cytochrome P450; cDNA, complimentary DNA; DL, dioxin-like; GST, glutathione S-transferase; NQO1, quinone oxidoreductase; Nrf2, NF-E2-related factor 2; PAH, polycyclic aromatic hydrocarbon; q-PCR, Real-time PCR; TEF, Toxic Equivalent Factor; TEQ, Toxic Equivalent; TCDD, 2,3,7,8 tetrachloro-dibenzo-*p*-dioxin; XME, xenobiotic metabolizing enzyme; XRE, xenobiotic-responsive element.

1. Introduction

Among the persistent organic environmental pollutants, PCDDs, PCDFs, and dioxin-like (DL) PCBs – collectively known as DL-compounds – are of great health concern because of the well known wide array of adverse effects on the immune, endocrine, and reproductive systems, as well as owing to their mutagenic, carcinogenic and teratogenic properties (Mandal, 2005). Although a number of non genomic effects have been recently described (White and Birnbaum, 2009), it is generally accepted that the aryl hydrocarbon receptor (AHR) is the major cellular target of DL-compounds as it mediates most of the toxic effects elicited by such chemicals. For regulatory purposes, DL-compounds have been classified according to their Toxic Equivalent Factors (TEFs), representing the binding affinity toward AHR, which is maximal for the 2,3,7,8 tetrachloro-dibenzo-*p*-dioxin (TCDD) and conventionally set to 1 (Van den Berg et al., 2006). Upon ligand binding, the AHR is translocated into the nucleus, where it heterodimerizes with the AHR-nuclear translocator (ARNT) protein and subsequently binds to the xenobiotic-responsive elements (XREs) in gene promoter regions; the whole process is regulated by the AHR-repressor (AHRR), a feedback modulator inhibiting AHR transcriptional activity (Abel and Haarmann-Stemmann, 2010). On the one hand, microarray studies have demonstrated that hundreds of genes may be modulated, involving multiple signaling pathways and cellular regulatory factors, also revealing wide species- and ligand-specific differences (Dere et al., 2011). On the other hand, the exposure to DL-compounds generally results in the up-regulation of the so called "AHR gene battery", which comprises a number of enzymes involved in endo- and xenobiotic biotransformations (e.g. cytochrome P450 [CYP] 1A1, CYP1A2, CYP1B1, uridin-diphospho-glucuronyltransferases 1A, quinone oxidoreductase [NQO1], glutathione S-transferases [GSTA] 1/2) not only in liver (Bock and Kohle, 2006; Guruge et al., 2009), but also in some extrahepatic tissues (Pavek and Dvorak, 2008). Many other structurally diverse natural and synthetic compounds can bind to AHR and activate the AHR signaling cascade, including for instance flavonoids and polycyclic aromatic hydrocarbons (PAHs) like the environmental and food contaminant benzo[a]pyrene (B[a]P). The absolute requirement of AHR for B[a]P to exert its toxic properties has been demonstrated (Shimizu et al., 2000).

For dairy ruminants, milk represents an important excretion route of DL-compounds, which may be ingested through contaminated feedstuffs and soil; the carry over rate ranges from 1 to about 52% and is inversely related to the degree of chlorination of each compound (Rychen et al., 2008). To protect human health, maximum limits for dioxins and the sum of dioxins and DL-PCBs (Toxic Equivalent, TEQ) in foodstuffs have been laid down in an

annex of the Commission Regulation (EU) 1881/2006; based on new occurrence data, limits have been recently lowered by the Commission Regulation (EU) 1259/2011 for certain food commodities, including “food for infant and young children”. In fact, although a general decrease in the degree of food contamination has been noticed over the last decade in Europe, a number of field outbreaks of dioxin contamination of dairy herds have occurred in recent years not only in Italy (Girolami et al., 2013; Perucatti et al., 2006), but also in France (ANSES, 2012) and in The Netherlands (Lascano Alcoser et al., 2011). This is of particular concern as raw milk and dairy products represent the major contributors to total exposure to DL-compounds in toddlers and children (EFSA, 2012; Rauscher-Gabernig et al., 2013). Opposed to DL-compounds, PAHs are subjected to extensive oxidative and conjugative biotransformations and are less prone to be transferred as such in dairy milk (Lapole et al., 2007).

Further to its secretory functions, the mammary gland participates in the biotransformation of many endogenous and exogenous compounds. The capital role played by breast xenobiotic metabolizing enzymes (XMEs) in the bioactivation/detoxification of a large array of chemicals, including many carcinogenic compounds, has been well documented (Williams and Phillips, 2000). While the expression of both the AHR-driven gene machinery and the related proteins have been thoroughly investigated in the mammary epithelial cells of rodent species and humans owing to their implication in breast cancer (Larsen et al., 2004), only scant information is available for cattle (McFadden and Corl, 2010; Peng et al., 2008). As regards BME-UV cells, a clonal cell line established from primary bovine mammary epithelial cells and widely used as a model for physiological and pharmacological investigations (Accornero et al., 2009), only indirect evidence has been provided as to the presence of AHR-dependent XMEs (Caruso et al., 2009).

In consideration of the widespread environmental contamination from DL-compounds and PAHs and their well demonstrated carry over in milk, the primary aim of this work was to characterize the basal expression of the AHR signaling pathway (AHR, ARNT, AHRR), and of selected AHR-mediated genes involved in xenobiotic biotransformations (CYP1A1, CYP1A2, CYP1B1, NQO1, GSTA1/2) in BME-UV cells. The modulation of the above genes by DL-compounds was investigated using two different DL-PCBs. The first one, PCB126, is the most toxic congener (TEF = 0.1) and has been reported to account for the larger contribution to the TEQ in dairy milk (Focant et al., 2013; Turrio-Baldassarri et al., 2009); in addition, it has been recently classified as a human carcinogen (IARC, 2012) and, among the DL-PCBs, is characterized by one of the highest carry-over and the slowest elimination rate

from dairy milk (Huwe and Smith, 2005). The second one, PCB77, was selected to estimate the modulation of the pathway by a non-*ortho* congener displaying different receptor affinity, being characerized by a TEF value lower by a factor of thousand (Van den Berg et al., 2006). Finally, β-naphtophlavone (β-NAF) and B[a]P, a compound normally used as guideline value for PAHs contamination in milk due to its high toxicity (Garcia Londono et al., 2013), were selected as model non DL-ligands.

2. Materials and methods

2.1 Cell culture and chemical reagents

The BME-UV bovine mammary epithelial cell line was kindly provided by Prof. Mario Baratta (Dept. of Veterinary Sciences, University of Torino, Italy) and grown in 10-cm dishes. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1000 units /mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B and kept in a humidified chamber at 37 °C and 5% CO_2. All cell culture reagents were purchased from Sigma–Aldrich (Milan, Italy). Cells were trypsinized every 3–4 days for subculturing. Before each experiment, cells were seeded at 3 X 10^6 cells/dish and cultured for 24 hours until they reached 50% confluence. The medium was then replaced with 10 mL of fresh medium, and cells were treated with DMSO alone (used as control) or with the different AHR-ligands (PCB126, PCB77, β-NAF or B[a]P) dissolved in DMSO, whose final concentration in the growth medium did not exceed 0.1% (v/v). DMSO, β-NAF and B[a]P were purchased from Sigma–Aldrich. PCB126 and PCB77 were supplied by LabService Analytica S.r.l. (Anzola Emilia, BO, Italy).

2.2 RNA extraction and Real-time PCR (q-PCR)

All the materials for the q-PCR analysis (including RNA extraction and cDNA synthesis) were purchased from Qiagen (Valencia, CA). Total RNA was isolated using QIAzol Lysis Reagent, according to the manufacturer's protocol. RNA purity and quantity was evaluated by absorbance readings using the NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Illkirch Cedex, France). The ratio of the optical densities measured at 260 and 280 nm were >1.9 for all RNA samples. One μg of total RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit, according to the manufacturer's instructions, in a final volume of 20 μl. Sufficient cDNA was prepared in a single run to perform the q-PCR experiments for all the selected genes. Primers for AHR, ARNT, AHRR, CYP1A1, CYP1A2,

CYP1B1, NQO1 and GAPDH were according to (Girolami et al., 2011), whereas primers for GSTA1 and GSTA2 were designed on *Bos Taurus* GenBank and Ensembl mRNA sequences using Primer 3 Software (version 3.0, Applied Biosystems, Foster City, CA). Oligonucleotides were designed to cross the exon/exon boundaries to minimize the amplification of contaminant genomic DNA, and were analyzed with the NetPrimer tool (available at http://www.premierbiosoft.com/netprimer/index.html) for hairpin structure and dimer formation. Primer specificity was verified with BLAST analysis against the genomic NCBI database. Table 1 summarizes primer information including sequences, gene accession numbers and amplicon sizes. Each primer set efficiency was comprised between 95% and 100%. GAPDH was selected as the reference gene since its expression was not influenced by any of the treatments. q-PCR reactions were performed on 100 ng of cDNA, in a final volume of 20 µl consisting of the 1X QuantiFast SYBR Green PCR Master Mix and an optimized concentration of each primer set (300-900 nM range). PCR amplification was run on an ABI 7500 Real-Time PCR System (Applied Biosystems) using 96-well optical plates under the following conditions: 5 min at 95 °C for polymerase activation, and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Each reaction was run in triplicate, and a no-template control was included using water instead of cDNA.

2.3 Western blot analysis

Whole-cell protein extracts were obtained through a lysis-buffer consisting of 80 mM TrisHCl, pH 6.8, and 2.7 % SDS, supplemented with 2 mM PMSF and a Protease Inhibitor Cocktail (Sigma-Aldrich) according to manufacturer's instructions. The protein concentration was determined according to the OD at 562 nm using the BCA protein assay reagent kit (Pierce, Rockford, IL). Equal amounts of protein (50 µg/lane) were resolved with SDS-PAGE on 10% polyacrylamide gels under reducing conditions, and transferred onto a nitrocellulose membrane (Bio-rad, Hercules, CA). After blocking of non-specific binding sites with 10% BSA in TBS, the membranes have been incubated for 2 h at room temperature with primary antibodies against rabbit CYP1A1/1A2 (Oxford Biomedical Research, Oxford, MI). The specificity of the CYP1A antibody against the bovine protein has been previously ascertained (Virkel et al., 2010). A primary antibody against β-tubulin (Santa Cruz Biotechnology, CA) was used as loading control. Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, reactive proteins were visualized with the enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate, Pierce) according to manufacturer's instructions. Immunoblot bands were visualized by

means of the ChemiDoc MP System (Bio-rad) and protein expression was quantified by densitometry using Imagelab software version 4.1 (Bio-rad). The relative density of each individual protein band was normalized to that of the β-tubulin band.

2.4 Data analysis

Three fully independent biological replicates were obtained for each experiment. Basal gene expression data were calculated with the $2^{-\Delta Ct}$ method and were expressed as relative mRNA level. The modulation of gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method and data were expressed as fold-change compared to control samples (Livak and Schmittgen, 2001); a 2.0 fold-change cut-off was selected. Differences between each treatment and the controls were determined by one-way ANOVA followed by Dunnett's *post hoc* test using GraphPad Prism software (4.03 version, La Jolla, CA). Statistical significance was assumed at *P* values < 0.05 or less. Concentration-response analysis was performed with GraphPad Prism software using a sigmoidal dose-response (variable slope) equation that generates Hill slope, EC50 and R^2. Starting values for the regression analyses were generated by subtracting the control response from the response at each concentration level and then dividing each data set by the maximum increase in response (Girolami et al., 2011).

3. Results and discussion

3.1 Constitutive expression of the main AHR pathway genes in BME-UV cells

Besides liver metabolism, breast metabolism in lactating dairy ruminants may be a determinant of the overall kinetics of drugs, pesticides, and environmental pollutants to which animals could be exposed. Toxic metabolites (e.g. aflatoxin M1) may be generated by (inducible) breast XMEs and excreted into the milk raising health concerns for suckling offspring as well as for the consumers. In other instances, chemicals may be bioactivated to reactive metabolites (e.g. catecholestrogens, aflatoxin B1 epoxides) with the potential for *in situ* cellular toxic damage. The biotransformative capacity toward aflatoxin B1 of the bovine mammary gland has been only recently tested in a cell line (BME-UV) employed as an *in vitro* model (Caruso et al., 2009).

In the present study, the constitutive gene expression of AHR pathway members (AHR, ARNT and AHRR) and of AHR-dependent XMEs (phase I: CYP1A1, CYP1A2, and CYP1B1; phase II: NQO1, GSTA1 and GSTA2) was investigated in non stimulated BME-UV cells, harvested after 24 h of culturing under standard conditions. All the examined genes

but CYP1A2 were detectable by q-PCR (Figure 1). In particular, AHR and CYP1B1 transcript levels were the most elevated, while AHRR and GSTA1 exhibited the lowest expression; CYP1A1, NQO1 and GSTA2 displayed an intermediate expression.

To the best of our knowledge the present study, although performed in a cell line, reports for the first time the presence of the AHR signaling pathway in the bovine mammary epithelium. So far, the basal gene expression of AHR, AHRR and ARNT in the normal and carcinogenic mammary gland has been demonstrated in rodents and dogs, as well as in humans (Collins et al., 2009; Giantin et al., 2013; Larsen et al., 2004; Spink et al., 1998; Tsuchiya et al., 2003).

Concerning the phase I AHR-dependent XMEs, the only available information about the expression of the CYP1 family in the bovine mammary gland refers to the presence of the CYP1A1 protein in the microsomal fractions from a lactating dairy cow (Peng et al., 2008). Besides, the gene expression of the same enzyme has been reported in a study about the role of liver X receptor in the regulation of fatty acid synthesis in bovine mammary epithelial cells (McFadden and Corl, 2010). As mentioned before, an indirect evidence for the expression of CYP1A1 has been provided in BME-UV cells treated with aflatoxin B1 and tested for the generation of its hydroxylated milk metabolite, aflatoxin M1, whose production is commonly mediated by that enzyme (Caruso et al., 2009). On the other hand, no data about CYP1B1 expression in bovine breast tissue have been reported so far. Thus, our work characterizes for the first time the constitutive CYP1 family profile in the bovine mammary epithelium. In particular, the lack of expression of CYP1A2, which is known to be almost exclusively present in liver (Ioannides, 2006), and the higher presence of CYP1B1, which was twice as expressed as CYP1A1, closely resembles the human breast CYP1 profile (Williams and Phillips, 2000).

The AHR gene battery also comprises enzymes mainly implicated in the antioxidant defense, such as NQO1 and GSTs. The former is a flavoprotein that reduces reactive quinones arising from the biotransformation of both exogenous (e.g. PAHs, benzene) and endogenous compounds (e.g. catecholamines, 17β-estradiol), preventing them from triggering cellular stress damage (Dinkova-Kostova and Talalay, 2010). In women, NQO1 is constitutively expressed in the mammary epithelium and affords protection against the quinone estrogens-mediated induction of breast cancer (Gaikwad et al., 2007). Aside from their key role in detoxifying a wide variety of electrophilic compounds by means of conjugation with glutathione, GST enzyme classes (Alpha, Mu, Pi, and others) are also involved in several endogenous pathways such as, for instance, steroid and prostaglandin biosynthesis (Hayes et

al., 2005). Based on the occurrence of XREs in rat GSTA1 and GSTA2 and in human GSTA2 (Alpha class GSTs) promoter regions, both genes were included in our study. In line with the physiological role mentioned above, Rabahi et al. (1999) reported on the high expression of GSTA1 and GSTA2 in several bovine steroidogenic tissues, including granulose cells, corpus luteus, placental cotyledons, testis and adrenals, but did not examine the mammary gland. We report for the first time the presence of NQO1, GSTA2 and, to a much lesser extent, GSTA1 in BME-UV cell line.

3.2 Different modulation of AHR target genes by selected ligands in BME-UV cells.

BME-UV cells were exposed to two DL-PCBs characterized by different TEF values (PCB126, TEF = 0.1, and PCB77, TEF = 0.0001). Two other well-known AHR agonists, i.e. the flavonoid, β-NAF, and the PAH, B[a]P, were employed for comparative purposes. All the ligands were used at the concentration of 100 nM, and the gene expression analysis was performed at 2 - 4 - 8 - 24 - 48 and 72 h from the treatment initiation.

Among the AHR pathway members, only AHRR was slightly induced by all ligands at 8 h (between 3- and 5-fold change), and the induction persisted up to 24-48 h only upon DL-PCBs stimulation (Figure 2). Likewise, the *in vitro* treatment of a human breast carcinoma cell line with either TCDD, the most effective AHR agonist, or 3-methylchoranthrene induced the AHRR mRNA by almost 2-fold (Tsuchiya et al., 2003). On the other hand, AHR and ARNT mRNA expression was not affected by any of the treatments (data not shown), although a decrease in AHR protein expression has been reported in mammary tissue from TCDD-treated mice or in rodent cell lines exposed to the same agonist (Collins et al., 2009; Larsen et al., 2004). Nevertheless, the lack of AHR and ARNT mRNA down-regulation noticed in our and in other investigations (Beedanagari et al., 2010; Giannone et al., 1998), although performed in different cell lines, is consistent with the observed transcriptional up-regulation of AHRR, which is known to initiate the signal for the proteasomal degradation of the receptor eliciting a reduction of AHR at protein level (Pollenz, 2002).

The two most induced AHR-dependent XME transcripts were CYP1A1 and CYP1B1, with CYP1A1 being the most responsive gene with all ligands, and PCB126 the most potent inducer (Figure 3). Similarly to AHRR, the CYP1A1 and CYP1B1 expression response was characterized by a different time-course pattern between the two DL-PCBs and the other AHR agonists. In particular, the treatment with PCB126 or PCB77 up-regulated the expression of both genes up to 72 h, with a peak at 8h for CYP1B1 (about 20- and 28-fold change with PCB77 and PCB126, respectively), and at 24h for CYP1A1 (about 45- and 112-fold change

with PCB77 and PCB126, respectively) (Figure 3A). By contrast, the induction generated by β-NAF or B[a]P peaked at 8 hours for both genes (about 23- and 49-fold change with β-NAF and B[a]P, respectively, for CYP1A1; about 24 and 17-fold change with β-NAF and B[a]P, respectively, for CYP1B1) and then rapidly decreased, turning negligible (less than 2-fold change) by 24 and 48 h with β-NAF and B[a]P, respectively (Figure 3B). Such results were substantially confirmed at protein level for CYP1A1. Western blotting was performed using an anti-CYP1A1/1A2 antibody in BME-UV cells stimulated with each ligand at the same concentration (100 nM) and harvested after 16 - 24 - 48 - 72 h. As reported in Figure 4, BME-UV cells displayed a single band, corresponding to CYP1A1, only in the presence of all the tested AHR agonists, while control cells did not show any specific signal. In particular, PCB126 and β-NAF resulted as the most and the least potent inducer, respectively. Moreover, both DL-PCBs up-regulated the CYP1A1 protein more persistently than the other AHR ligands, whose effect at protein level peaked at 16 h and then rapidly decreased. The persistency of the transcriptional effect elicited by DL-PCBs reflects the general refractoriness of such compounds to the metabolic degradation (Larsen, 2006).

The concentration-dependent changes in CYP1A1 and CYP1B1 expression were measured in the presence of increasing logarithmic concentrations (from 10^{-2} to 10^{3} nM) of all ligands at the peak time-points (8 h for β-NAF and B[a]P, and 24 h for PCB77 and PCB126, respectively). The range of concentrations used was selected on the basis of dairy milk contamination levels reported for PCB126 (0-543 pg/g fat) (Esposito et al., 2009; Turrio-Baldassarri et al, 2009) and B[a]P (0-0.28 µg/Kg dry mass) (Garcia Londono et al., 2013; Girelli et al., 2014; Naccari et al., 2011). Figure 5A shows that both DL-PCBs induced a concentration-dependent increase in both CYP1A1 and CYP1B1 mRNA levels compared to control cells. The maximal response to the PCB126 treatment was achieved for both genes at the concentration of 10 nM, whereas PCB77 was most effective at the concentration of 1µM. The calculated EC50 values for CYP1A1 induction by PCB126 and PCB77 were 0.29 and 107.1 nM, respectively; as to CYP1B1, they were 0.13, and 50.07 nM, respectively (Supplementary data). As expected, the sharp difference in TEF values between PCB126 and PCB77 is matched by the much higher induction potency of the former, as reflected by the differences in the concentration at which the maximal response was reached (100 folds), the respective calculated EC50 values (350 to 380 folds), and the lowest concentration capable of increasing gene transcription (0.01 nM for PCB126, and 1 nM for PCB77). It is noteworthy that the minimal effective concentration of PCB126 is lower almost by a factor of 10 than the upper limit of the range of milk contamination reported above. Concerning the other tested

AHR ligands, β-NAF and B[a]P induced CYP1A1 starting from the concentration of 1 nM (corresponding for B[a]P to approximately 0.28 µg/Kg dry mass of milk), while the up-regulation of CYP1B1 was noticeable only from the concentration of 10 nM with either ligand; the peak of induction for both genes was reached at the concentrations of 100 nM and 1 µM with B[a]P and β-NAF, respectively (Figure 5B). The calculated EC50 values for CYP1A1 induction by B[a]P and β-NAF were 16.67 and 134 nM, respectively; as to CYP1B1, they were 53.03, and 153.01 nM, respectively (Supplementary data).

In agreement with what observed in the present study, a concentration- and time-dependent CYP1A1 and CYP1B1 mRNA increase, with CYP1A1 being the most responsive gene, was detected in primary cultures from bovine liver and circulating lymphocytes, treated with comparable concentrations of TCDD and PCB126, respectively (Girolami et al., 2011; Guruge et al., 2009). The inducibility of the pathway, at protein level only, has been recently demonstrated in primary cultures of normal bovine mammary epithelium; the exposure to 10 nM TCDD resulted in an up to 60-fold increase in CYP1 enzyme activity (measured by the 7-ethoxyresorufin O-deethylase assay) after 72 h (Halwachs et al., 2013). At the same time, several evidences demonstrate the up-regulation of CYP1A1 and CYP1B1 in human and rodent mammary cell lines challenged with different DL-compounds (Chen et al., 2004), as well as in both mouse and rat mammary tissue following the *in vivo* treatment with TCDD or β-NAF, respectively (Collins et al., 2009; Larsen et al., 2004). As regards B[a]P, an increase in the levels of CYP1A1 and CYP1B1 mRNA has been reported in either human normal mammary epithelial and breast cancer cells after 12 and 6 h incubation, respectively (Kemp et al., 2006; Keshava et al., 2005a). Such data are consistent with the B[a]P-mediated time-course induction of CYP1A1 and CYP1B1 reported herein; however, the ligand concentrations employed in those studies were much higher than ours (5 µM *vs* 100 nM), suggesting that the bovine mammary cells might be more sensitive than their human counterparts to CYP1 family enzyme up-regulation upon B[a]P exposure.

Finally, upon the incubation with all the tested AHR-ligands up to 72 h, all the examined phase II XMEs failed to exhibit the expected increase in gene transcription levels (data not shown), GSTA2 being even lowered by both DL-PCBs, starting from 24 h and lasting up to 48 h with PCB77 and 72 h with PCB126, respectively (Figure 6). Again, the most effective ligand was PCB126, which modulated GSTA2 expression already at the concentration of 0.01 nM (data not shown), similarly to CYP1A1. In line with our results, NQO1 and GSTA1 transcripts were not affected in mouse embryonic stem cells treated with TCDD up to 100 nM and 48 hr (Neri et al., 2008) or in mammary epithelial cells from healthy human breast tissue

incubated with 4 µM B[a]P for up to 24 h (Keshava et al., 2005b). In contrast, the administration of TCDD led to a remarkable enhancement of NQO1, and NQO1 and GSTA1 liver gene expression in rats (Brauze et al., 2006) and mice (Yeager et al., 2009), respectively. In all cases, the CYP1A1 and/or CYP1B1 gene expressions were consistently increased by the treatments. Therefore, it appears that the regulation of certain enzymes belonging to the AHR gene battery may be tissue specific. For instance, the induction by AHR ligands of enzymes involved in the antioxidant defense, including NQO1, GSTs and uridin-diphospho-glucuronyltransferases, requires the transcription factor NF-E2-related factor 2 (Nrf2), which recognizes the Antioxidant Response Elements (ARE) in the promoter region of target genes (Wang et al., 2013; Yeager et al., 2009). Since such genes have been reported to be strongly modulated following an experimental intramammary bacterial infection in the bovine species (Moyes et al., 2010), the lack of NQO1 and GSTA induction recorded in our study would point to a defective expression/functionality of the Nrf2 signaling pathway in the BME-UV cell line, at least under the adopted culturing conditions. The observed DL-PCB-mediated down-regulation of GSTA2 in the used cell line is difficult to interpret. In this respect, it is well acknowledged that the exposure to TCDD and other DL-compounds entails an increase in cellular oxidative stress (Reichard et al., 2005), which has been linked to a fall in the liver activity of both GST accepting 1-chloro-2,4-dinitrobenzene as the substrate and glutathione peroxidase in TCDD-treated mice (Shon et al., 2002). Further research is needed to confirm our data and to unravel the molecular mechanism(s) underlying the observed DL-PCB-mediated down-regulation of GSTA2.

In conclusion, results from the present study confirm the constitutive expression of the AHR-mediated gene machinery and its partial inducibility by DL- and non DL-ligands in the BME-UV cell line, which may therefore serve as a tool for assessing the contribution of the bovine mammary gland to the overall biotransformation of xeno- and endobiotics, particularly of those being substrates of the CYP1 family and of other XMEs belonging to the AHR gene battery. It is worth noting that they comprise not only widespread environmental food and feed contaminants such as DL-compounds, PAHs, and aflatoxins, but also steroid hormones and drugs widely used in the bovine practice like benzimidazole anthelmintics (Velik et al., 2004), fluoroquinolones (Fu et al., 2011), and partly avermectins (Zeng et al., 1996). If confirmed by further *in vivo* studies, the remarkable concentration-related increase in the expression of CYP1A1 and CYP1B1 brought about mainly by DL-PCBs and B[a]P may be therefore of concern not only in view of the likely influence on drug kinetics, but also due to the potential for such CYPs to activate several xenobiotic and endogenous molecules to toxic

or carcinogenic metabolites that could affect animal and human health (Williams and Phillips, 2000).

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1. Constitutive AHR-dependent pathway gene expression levels in BME-UV cells. Data are expressed as relative mRNA levels compared to GAPDH (mean ± SEM of three independent biological replicates). a.u. = arbitrary units. n.d. = not detectable.

Fig. 2. Time-course profiles of AHRR gene induction in BME-UV cells treated with 100 nM of PCB126 or PCB77 (A), and β-NAF or B[a]P (B). Data are expressed as fold change compared to control samples treated with DMSO (mean ± SEM of three independent biological replicates). * = $P < 0.05$ or less.

Fig. 3. Time-course profiles of CYP1A1 and CYP1B1 gene induction in BME-UV cells treated with 100 nM of PCB126 or PCB77 (A), and β-NAF or B[a]P (B). Data are expressed as fold change compared to control samples treated with DMSO (mean ± SEM of three independent biological replicates). * = $P < 0.05$ or less.

Fig. 4. Effects of PCB126 or PCB77 (A), and β-NAF or B[a]P (B) on CYP1A1 protein expression in BME-UV cells. Western blot analysis was performed on total extracts from control (DMSO) and treated cells (100 nM) at different time-points. Data are expressed as relative density using β-tubulin as a loading control (mean ± SD). The gel is representative of three independent biological replicates.

Fig. 5. Dose-response profiles of CYP1A1 and CYP1B1 gene induction in BME-UV cells treated with increasing logarithmic concentrations (from 10^{-2} to 10^{3} nM) of PCB126 or PCB77 (A), and β-NAF or B[a]P (B). Fold change levels compared to control samples (DMSO) are expressed as mean ± SEM of three independent biological replicates. * = $P < 0.05$ or less.

Fig. 6. Time-course profile of GSTA2 gene down-regulation in BME-UV cells treated with 100 nM of PCB126 or PCB77. Data are expressed as fold change compared to control samples treated with DMSO (mean ± SEM of three independent biological replicates). * = $P < 0.05$ or less.

Table

Primers for real-time PCR analysis

Gene	Accession no.	5'→ 3' sequence	Amplicon size
AHR	XM_612996	F: GTGCAGAAAACTGTCAAGCC	203
		R: GCAACATCAAAGAAGCTCTTG	
AHRR	NM_001077982	F: TGGAGTCTCTCCACGGCTTC	58
		R: GCGTAGAAGATCATCCCTTCC	
ARNT	NM_173993	F: TTTCCTCACTGATCAGGAAC	183
		R: TCCAGGATACGCCCTGTC	
CYP1A1	XM_588298	F: CGAGAATGCCAATATCCAGC	173
		R: TGCCAATCACTGTGTCCAG	
CYP1A2	NM_001099364	F: CAGTAAGGAGATGCTCAGTC	201
		R: CTGTTCTTGTCAAAGTCCTGG	
CYP1B1	NM_001192294	F: CACCAGGTATTCGGAAGTGC	118
		R: AAGAAAGGCCATGACGTAGG	
NQO1	NM_001034535	F: CGGAATAAGAAGGCAGTGCT	130
		R: AGCCACAGAAGTGCAGAGTG	
GSTA1	NM_001078149	F: AGAGGGTGTGGCAGATTTGG	141
		R: TGGCTCTTCAGCACATTTTCA	
GSTA2	NM_177515	F: TTACCACTGTGCCCACCTGAT	112
		R: CTTGTCCGTGATTCTTCAGCAC	
GAPDH	NM_001034034	F: GAGAAACCTGCCAAGTATGAT	125
		R: GAGTGTCGCTGTTGAAGTCG	

Fig. 1

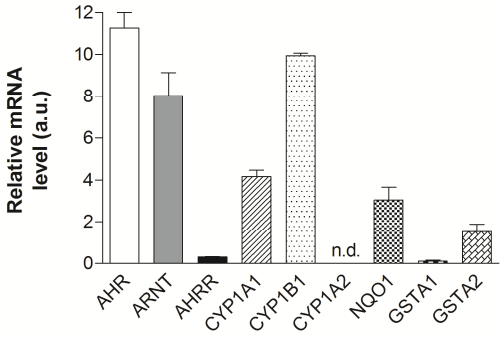

Relative mRNA level (a.u.)
12
10
8
6
4
2
0
n.d.
AHR
ARNT
AHRR
CYP1A1
CYP1B1
CYP1A2
NQO1
GSTA1
GSTA2

Fig. 2

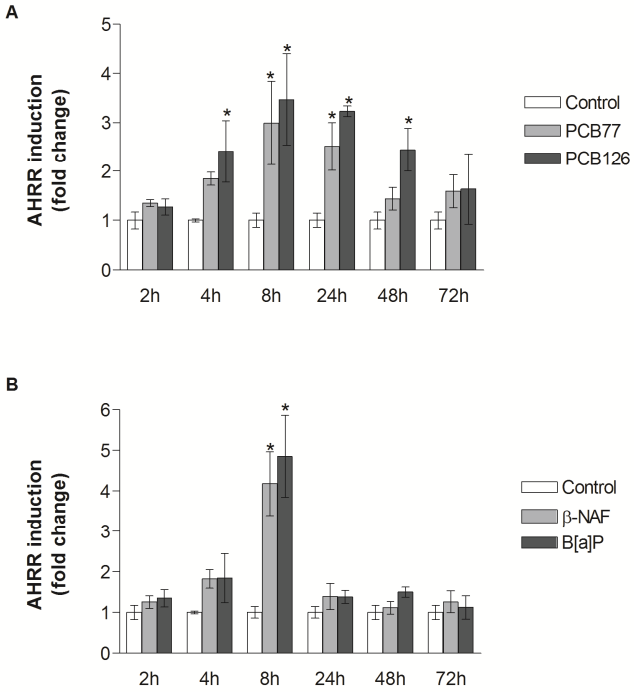

A
AHRR induction (fold change)
5
4
3
2
1
0
2h
4h
8h
24h
48h
72h
Control
PCB77
PCB126
B
AHRR induction (fold change)
6
5
4
3
2
1
0
2h
4h
8h
24h
48h
72h
Control
β-NAF
B[a]P

Fig. 3

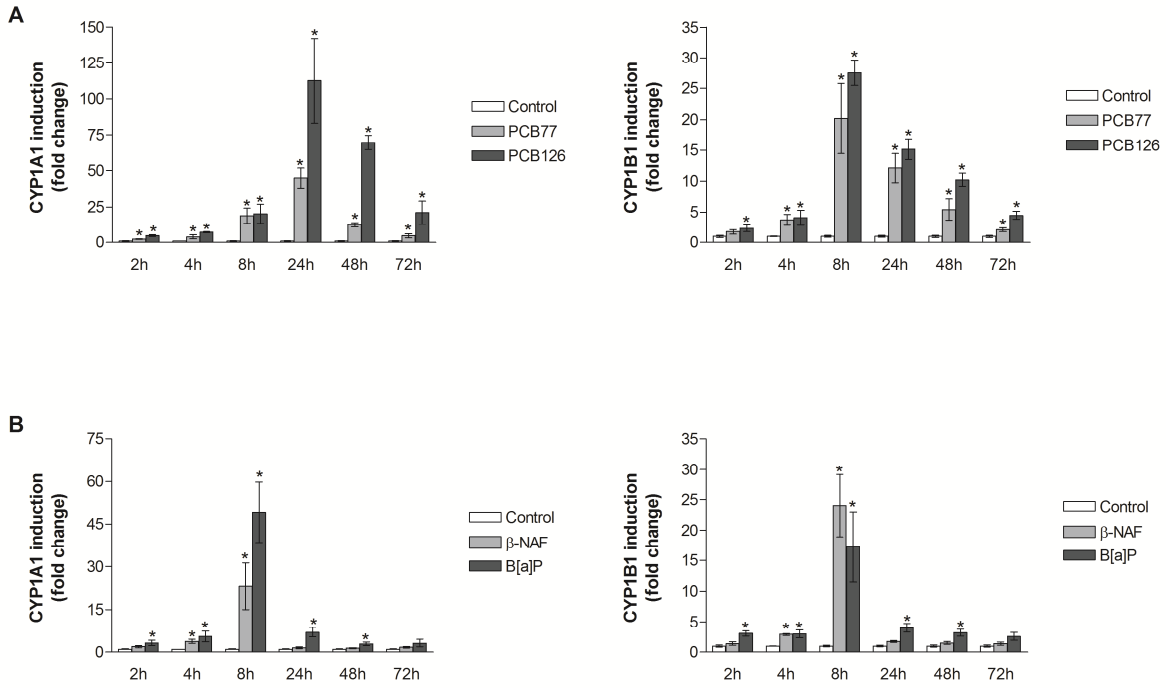

A
CYP1A1 induction (fold change)
150
125
100
75
50
25
0
2h
4h
8h
24h
48h
72h
Control
PCB77
PCB126
CYP1B1 induction (fold change)
35
30
25
20
15
10
5
0
B
CYP1A1 induction (fold change)
75
60
45
30
15
0
Control
β-NAF
B[a]P
CYP1B1 induction (fold change)

Fig. 4

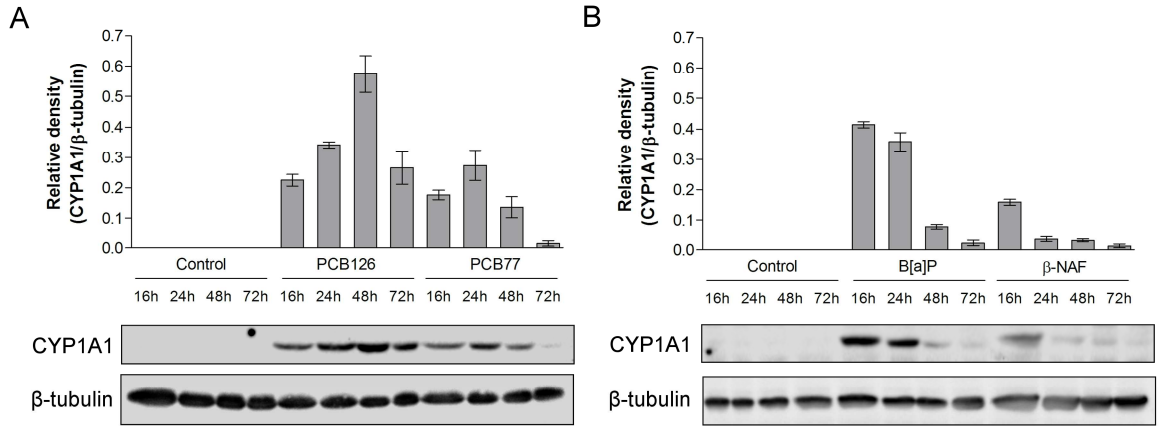

A
Relative density (CYP1A1/β-tubulin)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
PCB126
PCB77
16h 24h 48h 72h 16h 24h 48h 72h 16h 24h 48h 72h
CYP1A1
β-tubulin
B
Relative density (CYP1A1/β-tubulin)
Control
B[a]P
β-NAF
16h 24h 48h 72h 16h 24h 48h 72h 16h 24h 48h 72h
CYP1A1
β-tubulin

Fig. 5

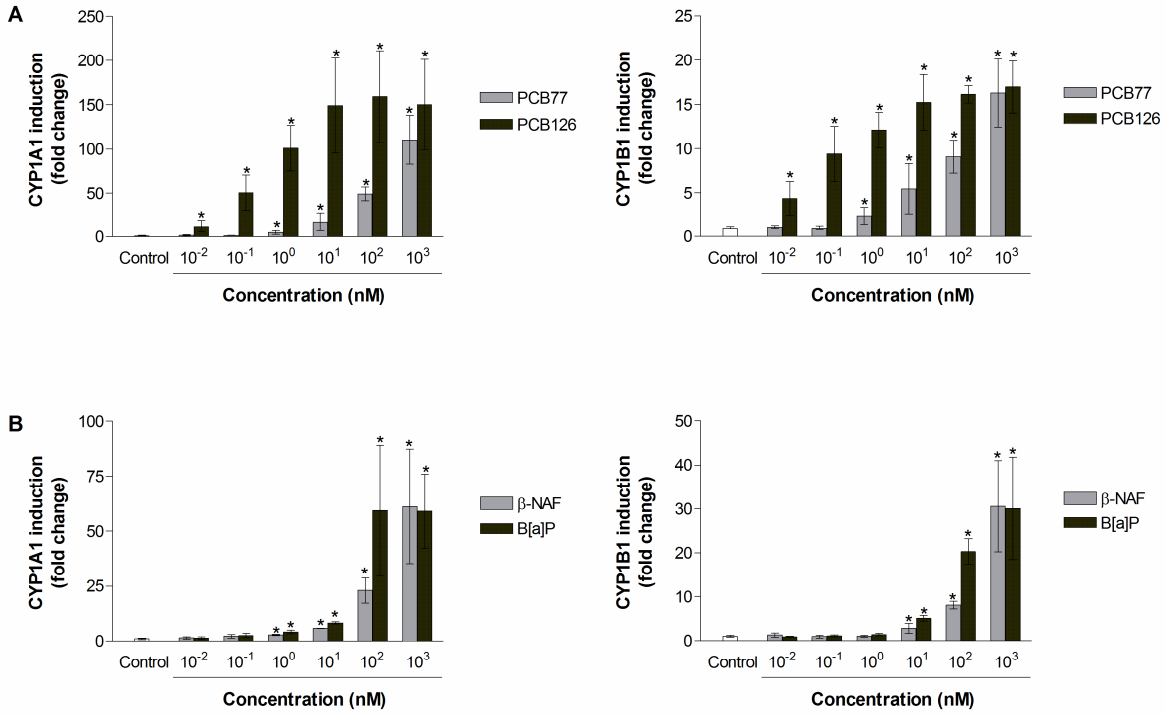
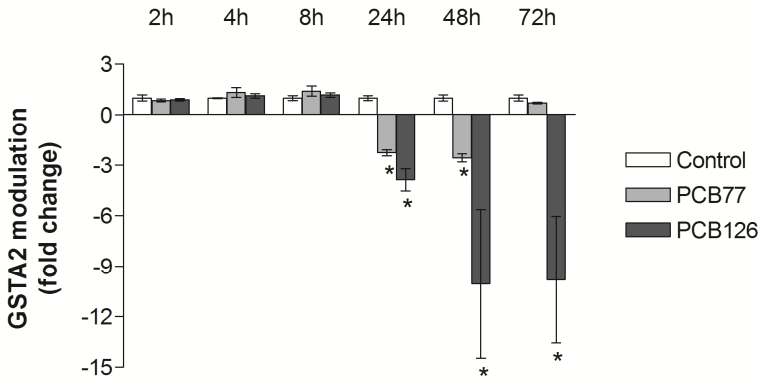

A
CYP1A1 induction (fold change)
CYP1B1 induction (fold change)
PCB77
PCB126
Control
Concentration (nM)
B
β-NAF
B[a]P

Fig. 6


2h
4h
8h
24h
48h
72h
GSTA2 modulation (fold change)
Control
PCB77
PCB126