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Gene expression and inducibility of the aryl hydrocarbon receptor-dependent pathway in cultured bovine blood lymphocytes

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Abstract

The exposure to dioxin-like (DL) compounds, an important class of persistent environmental pollutants, results in the altered expression of target genes. This occurs through the binding to the aryl hydrocarbon receptor (AhR), the subsequent dimerization with the AhR nuclear translocator (ARNT), and the binding of the complex to DNA responsive elements. A number of genes are up-regulated, including, among others, the AhR repressor (AhRR) and several biotransformation enzymes, such as the members of CYP1 family and NAD(P)H-quinone oxidoreductase (NQO1). The expression and the inducibility of the above genes were investigated in mitogen-stimulated cultured blood lymphocytes from cattle, which represent a notable source of DL-compound human exposure through dairy products and meat. As assessed by real-time PCR, all the examined genes except CYP1A2 and NQO1 were detected under basal conditions. Cell exposure to the DL-compounds PCB126 or PCB77 in the 10^{-6} - 10^{-9} M concentration range resulted in a 2- to 4-fold induction of CYP1A1 and CYP1B1, which was antagonized by α -naphthoflavone or PCB153. This study demonstrates for the first time the presence and inducibility of the AhR pathway in easily accessible cells like bovine peripheral lymphocytes and prompts further investigations to verify whether similar changes could occur under *in vivo* conditions.

Keywords: Aryl hydrocarbon receptor, Dioxin-like compounds, Lymphocytes, Cattle, Real-time PCR

Abbreviations: AhR, aryl hydrocarbon receptor; AhRR, aryl hydrocarbon receptor repressor; ARNT, AhR nuclear translocator; CYP, cytochrome P450; cDNA, complementary DNA; DL-, dioxin-like; DMSO, dimethylsulfoxide; Hsp90, heat shock protein 90 kD; α -NAF, α -naphthoflavone; β -NAF, β -naphthoflavone; NQO1, NAD(P)H-quinone oxidoreductase; PBMC, peripheral blood mononuclear cell; PCB, polychlorobiphenyl; PCDD, polychlorodibenzo-*p*-dioxin; PCDF, polychlorodibenzofuran; PHA, phytohemagglutinin; q-PCR, real-time PCR; TCDD, 2,3,7,8 tetrachloro-bibenzo-*p*-dioxin; TEF, toxic equivalency factor; XAP2, hepatitis B virus X-associated protein; XME, xenobiotic metabolizing enzyme; XRE, xenobiotic responsive element.

1. Introduction

Polychlorodibenzo-*p*-dioxins (PCDDs), polychlorodibenzofurans (PCDFs) and polychlorobiphenyls (PCBs) are chlorinated aromatic hydrocarbons long recognized as persistent and widespread environmental pollutants of great concern to human health. The prolonged exposure to such contaminants causes a broad spectrum of adverse effects, including tumor promotion and teratogenesis, as well as perturbation of the immune, endocrine, and nervous systems (Mandal, 2005). Among hundreds of congeners, only a few, collectively identified as dioxin-like (DL) compounds, are capable of binding, albeit with different affinities, to a specific cytosolic receptor, the aryl hydrocarbon receptor (AhR), which is responsible for most of their toxic effects. The accumulation of DL-compounds at the top soil level, following combustion fallout or organic fertilization with sewage sludges, represents an important source of contamination for food producing animals at the farm level (Brambilla et al., 2004). Due to the high degree of lipophilicity, these substances tend to build up to a significant extent in animal productions (eggs, fat, meat, milk and dairy products), which represent the main source of exposure for humans (Donato et al., 2006; Hirako, 2008), and are therefore included in the monitoring procedures for risk assessment and management.

The AhR is an intracellular transcription factor, which, in the absence of a ligand, is stabilized in a cytosolic protein complex with heat shock protein 90 kD (Hsp90), hepatitis B virus X-associated protein (XAP2) and p23. Ligand binding leads to nuclear translocation, release of the chaperon proteins, and formation of a heterodimer with its partner molecule, the AhR nuclear translocator (ARNT). The AhR/ARNT complex interacts with xenobiotic responsive elements (XREs) located in the regulatory region of a number of target genes encoding for phase I and phase II biotransformation enzymes, including cytochrome P450 (CYP) 1A1, 1A2, and 1B1, and NAD(P)H-quinone oxidoreductase (NQO1), as well as proteins involved in the regulation of development, proliferation and differentiation (Abel and Haarmann-Stemann, 2010). It is worth noting that CYP1A1 (Olson et al., 1994) and, to a lesser extent, CYP1B1 (Santostefano et al., 1997) are likely involved in the oxidative biotransformation of DL-compounds yielding -OH derivatives, which may be subsequently glucuronidated or sulphated and then excreted via the biliary and urinary routes. In rodents and in humans CYP1A1 and CYP1B1 are predominantly expressed in extra-hepatic tissues, while CYP1A2 is constitutively expressed to a notable extent in the liver (Ioannides, 2006). Finally, the activation of the AhR-dependent pathway induces the up-regulation of the AhR

repressor (AhRR), which inhibits the AhR transcriptional activity in a negative feedback loop manner (Hahn et al., 2009).

Although the liver plays a capital role in the biotransformation of foreign compounds, many xenobiotic metabolizing enzymes (XMEs) are also expressed in extrahepatic organs or tissues (Ding and Kaminsky, 2003; Pavek and Dvorak, 2008), including circulating lymphocytes, where they may be involved in the bioactivation of a number of pro-carcinogens, like polycyclic aromatic hydrocarbons (Fung et al., 1999) and aflatoxin B1 (Wilson et al., 1995). As blood cells are readily accessible from living animals, they have been proposed as surrogate for XME expression in liver or other target organs (Furukawa et al., 2004). In particular, several reports have addressed the expression of genes involved in the AhR signaling pathway in leukocytes from laboratory species and humans (Saurabh et al., 2010; Shah et al., 2009; Siest et al., 2008), and changes in their pattern of expression and/or inducibility have been found in circulating lymphocytes from 2,3,7,8 tetrachloro-dibenzo-*p*-dioxin (TCDD)-exposed individuals approximately 20 years after Seveso's (Italy) accident (Landi et al., 2003).

The modulation of AhR-dependent gene transcription caused by the exposure to DL-compounds has been mainly characterized in liver cell lines (Beedanagari et al., 2010; Kim et al., 2006) and in hepatocyte primary cultures from laboratory animals and humans (Budinsky et al., 2010; Le Vee et al., 2010); by contrast, only scant information is available for food producing species (Guruge et al., 2009; Kennedy et al., 1996; Landi et al., 2003). As regards cattle, which have been implicated in several outbreaks of environmental contamination from DL-compounds (Brambilla et al., 2004), both CYP1A1 and CYP1B1 transcripts have been detected in calf liver and found to be induced upon the exposure to TCDD and other PCDDs and PCDFs (Guruge et al., 2009). However, there are no data about bovine blood cells. Here we report for the first time the expression of a number of AhR-dependent genes and their *in vitro* inducibility in cattle peripheral blood lymphocytes. It is anticipated that data generated from this study will be applied to monitor the changes in target gene expression in circulating mononuclear cells from cattle reared in dioxin-contaminated areas.

2. Materials and methods

2.1 Reagents

Histopaque-1077, dimethylsulfoxide (DMSO), β -naphthoflavone (β -NAF), α -naphthoflavone (α -NAF) and all cell culture reagents were purchased from Sigma-Aldrich (St.

Loius, MO, USA). PCB77, PCB126 and PCB153 were supplied by LabService Analytica S.r.l. (Anzola Emilia, BO, Italy). All the materials for the real-time PCR (q-PCR) analysis (including RNA extraction and reverse transcription) were from Qiagen (Valencia, CA, USA).

2.2 Blood lymphocyte isolation and culture

For each experiment, approximately 300 ml of peripheral venous blood was collected from a healthy cow in a transfusion bag containing citrate-phosphate-dextrose-adenine (CPDA-1) as the anticoagulant preservative. Lymphocytes were isolated with the method described by Spalenza et al. (2010) with slight modifications. In brief, all the washes were performed with RPMI-1640 medium, and all the procedures were conducted at room temperature.. Although these cell preparations are frequently termed as peripheral blood mononuclear cells (PBMCs) for the presence of monocytes, in our case it has been demonstrated that the majority of them (90%) are lymphocytes (Spalenza et al., 2010). Thus, for ease of nomenclature we will refer to them exclusively as lymphocytes. After isolation, cells 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, 2mM L-glutamine and 10 μ g/ml phytohemagglutinin (PHA), at 37°C and 5% CO₂.

2.3 Chemical treatments

All the ligands were dissolved in DMSO, whose final concentration in the growth medium did not exceed 0.1% (v/v). After 2 h of pre-incubation, lymphocytes were treated with the solvent alone (used as control) or with the test compounds under the following conditions. For the time-course experiments, cells were exposed to 100 nM β -NAF or PCB126 and lysed after 2, 4, 16, 24 and 48 h. For the dose-response experiments, lymphocytes were treated with increasing logarithmic concentrations (from 0.1 nM to 1 μ M) of PCB126 or PCB77 for 2 h. Finally, α -NAF and PCB153 treatments were performed exposing cells to 100 nM for 1 h before the incubation with 100 nM PCB126 for 2h. Lymphocyte viability, assessed by the trypan blue exclusion test, was not affected by any of the employed chemical compounds at any examined concentrations.

2.4 RNA extraction and reverse transcription

Total RNA was isolated using RNeasy Mini Kit, 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 complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit, according to the manufacturer's instructions, in a final volume of 20 μl . The cDNA was subsequently diluted in nuclease-free water and stored at $-20\text{ }^{\circ}\text{C}$. Sufficient cDNA was prepared in a single run to perform the q-PCR experiments for all the selected genes.

2.5 Real-time PCR

Primers for target and reference genes 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 dimers formation. Primer specificity was verified with BLAST analysis against the genomic NCBI database and by agarose gel electrophoresis. Table 1 summarizes primer information including sequences, gene accession number 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 25 μl consisting of the 1 X QuantiFast SYBR Green PCR Master Mix and an optimized concentration of each primer set (300-900 nM range). PCR amplification was run on a ABI 7500 Real-Time PCR System (Applied Biosystems) using 96-well optical plates under the following conditions: 5 min at $95\text{ }^{\circ}\text{C}$ for polymerase activation, and 40 cycles of 15 s at $95\text{ }^{\circ}\text{C}$ and 60 s at $60\text{ }^{\circ}\text{C}$. Each reaction was run in triplicate, and a no-template control was included using water instead of cDNA.

2.6 Data analysis

To enable statistical analysis, all the experiments were performed independently three times, each using blood cells from different cows. Basal gene expression data were calculated with the $2^{-\Delta\text{Ct}}$ method and were expressed as relative mRNA level, while induction of gene expression was calculated with the $2^{-\Delta\Delta\text{Ct}}$ method and data were expressed as fold-change

compared to control samples (Livak and Schmittgen, 2001). Biological replicates were standardized as described by Willems et al. (2008), and the statistical significance was determined by calculating of the 95% CI ($p < 0.05$).

Concentration-response analysis was performed with GraphPad Prism software (4.03 version) 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.

3. Results and discussion

To begin this study, we determined the basal gene expression profiles of some AhR pathway members (AhR, ARNT and AhRR) and AhR target molecules (CYP1A1, CYP1B1, CYP1A2 and NQO1) in blood lymphocytes isolated from healthy cows. In cells cultured for 24 h in the presence of PHA, all the investigated genes were detectable, with the exception of CYP1A2 and NQO1 (Fig. 1). In particular, AhR and ARNT were the most expressed, while CYP1A1 resulted as the least expressed. To the best of our knowledge, this is the first report showing the presence of the AhR signaling pathway in bovine blood cells, as the only related study published so far deals with calf hepatocyte primary cultures (Guruge et al., 2009). Our data are comparable to those obtained for human lymphocytes, where the mRNA levels of CYP1A1 and CYP1B1 are clearly detectable in primary cultures and considerably lower than those of AhR and ARNT (Landi et al., 2003; van Duursen et al., 2005). Likewise, CYP1A2 has never been detected in either fresh or cultured blood cells, confirming its quite strictly liver-specific expression (Krovat et al., 2000; Siest et al., 2008).

The activation of the AhR signaling pathway was explored stimulating lymphocytes with a DL-compound, PCB126, and a well-known AhR agonist, β -NAF, used as a positive control, both at the concentration of 100 nM. The gene expression analysis, performed at different time points from the initiation of the treatment, showed a significant increase in CYP1A1 and CYP1B1 levels in the presence of either ligand compared to the DMSO control (Fig.2). On the other hand, the expression of AhR, ARNT and AhRR were not significantly modulated by any of the treatments at any time point (data not shown). The results about AhR and ARNT are consistent with earlier studies performed on human lymphocytes (Lin et al., 2003), whereas the lack of induction of AhRR, a known target of the pathway (Baba et al., 2001), has been described in some human-tissue derived cell lines and in peripheral mononuclear

cells from infants, stimulated with TCDD or 3-methylcholantrene (Tsuchiya et al., 2003; Yamamoto et al., 2004). Thus, our data suggest that the inducibility of AhRR mRNA, unlike that of the CYP1 family, may be influenced by cell type, age, and possibly species.

The peak of CYP1A1 and CYP1B1 induction was reached already at 2 h, followed by a decrease in mRNA expression up to 16 h, as reported for the lymphocytes from certain mouse and rat strains upon TCDD treatment (Nohara et al., 2006). Interestingly, CYP1A1 and CYP1B1 expression started to augment again at 24 h up to 48 h. This biphasic inducibility pattern may be explained by a non transcriptional down-regulation of the receptor. Indeed, it has been reported that in several cell lines the levels of AhR protein decreased following ligand exposure (Pollenz, 2002). In particular, the incubation with TCDD resulted in a down-regulated state of the receptor up to 72 h, while the use of less stable ligands, such as 3-methylcholantrene or β -NAF, caused a transitory reduction that is almost completely recovered by the 72 h time-point. Finally, in line with data about human lymphocytes (Landi et al., 2005; Lin et al., 2003), CYP1A1 proved to be more inducible than CYP1B1, as the average induction folds at 2h were 7.89 and 5.39 for CYP1A1, versus 5.33 and 3.77 for CYP1B1, upon stimulation with PCB126 and β -NAF, respectively (Fig. 2).

The concentration-dependent changes in CYP1A1 and CYP1B1 expression were measured in the presence of increasing logarithmic concentrations of PCB126 and PCB77 at the peak time (2 h) identified in the previous experiments. The use of PCB77, a DL-compound with a toxic equivalency factor (TEF) lower than that of PCB126 by three orders of magnitude (0.0001 versus 0.1) (Van den Berg et al., 2006), allowed to better characterize the modulation of the AhR pathway in bovine lymphocytes. Fig. 3 shows that both DL-compounds induced a dose-dependent increase of both CYP1A1 and CYP1B1 mRNA levels compared to the DMSO control. The maximal response to PCB126 treatment was achieved for both genes at the concentration of 100 nM, whereas PCB77 was maximally effective at the concentration of 1 μ M. The calculated EC50 values for CYP1A1 induction by PCB126 and PCB77 were 1.73 and 21.25 nM, respectively; as to CYP1B1, they were 0.83, and 66.32 nM, respectively. Under the experimental conditions adopted in this study, the induction potency of the two PCBs, meant as the capability to activate CYP1A1 and CYP1B1 transcription, only partially reflected the large difference in their respective TEF values. A similar behavior was observed in primary mouse aortic endothelial cells treated with PCB126 and PCB77 in a study aimed at investigating their effects on the expression of genes involved in atherosclerosis (Han et al., 2010). Taken together, such findings suggest that the binding affinity to the AhR

as expressed by TEF does not strictly reflect the degree of inducibility of target genes, which may be influenced also by the transcription capacity of the specific cell type.

To gain further insight into the specificity of the gene expression response of bovine lymphocytes to DL-compounds, cells were incubated with either α -NAF, a known AhR antagonist, or PCB153, a non-DL congener, prior to PCB126 stimulation. As shown in Fig. 4, pre-treatment with 100 nM α -NAF or PCB153 significantly antagonized CYP1A1 and CYP1B1 induction by 100 nM PCB126; conversely, no changes in target gene expression were detected after exposure to α -NAF or PCB153 alone, as expected. These observations clearly confirm that the transcription modulation induced by PCB126 in bovine lymphocytes was AhR-dependent.

In conclusion, we have demonstrated for the first time that the AhR signaling pathway is present, functional and inducible in bovine peripheral lymphocytes. As a result, our data are expected to promote further investigations aimed at studying the effects of dioxins and DL-compounds on the immune system of this species. In this respect it should be noted that PCB126, one of the DL-compound used in our experiments, is one of the most abundant congeners found in feed and in food of animal origin (EFSA, 2010). As a further consideration, previous studies conducted on the rate of PCDDs and PCDFs elimination in cows (Van den Berg et al., 1994) and the 4-4' substitution pattern of PCB126 rendering such molecule relatively resistant to the oxidative biotransformation (McLachlan, 1993), point to a low metabolic clearance of such compound (s) in cattle. Although it is always difficult to extrapolate results obtained under *in vitro* conditions to those occurring in practice, data from the present study suggest that the measurement of CYP1A1 and CYP1B1 mRNA expression in these easily accessible cells could be employed to monitor the exposure of cattle to dioxins and DL-compounds under field conditions, as already proposed for humans (Landi et al., 2005). Preliminary data from analyses performed in bovine lymphocytes from cows reared in contaminated areas would be in line with this hypothesis (unpublished results).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Table 1

Primers for real-time PCR

Gene	Accession no.	5'→3' sequence	Amplicon size
AhR	XM_612996	F: GTGCAGAAAACGTCAAGCC R: GCAACATCAAAGAAGCTCTTG	203
AhRR	NM_001077982	F: TGGAGTCTCTCCACGGCTTC R: GCGTAGAAGATCATCCCTTCC	58
ARNT	NM_173993	F: TTTCTCACTGATCAGGAAC R: TCCAGGATACGCCCTGTC	183
CYP1A1	XM_588298	F: CGAGAATGCCAATATCCAGC R: TGCCAATCACTGTGTCCAG	173
CYP1A2	NM_001099364	F: CAGTAAGGAGATGCTCAGTC R: CTGTTCTTGTCAAAGTCCTGG	201
CYP1B1	NM_001192294	F: CACCAGGTATTCGGAAGTGC R: AAGAAAGGCCATGACGTAGG	118
NQO1	NM_001034535	F: CGGAATAAGAAGGCAGTGCT R: AGCCACAGAAGTGCAGAGTG	130
GAPDH	NM_001034034	F: GAGAAACCTGCCAAGTATGAT R: GAGTGTCGCTGTTGAAGTCG	125

Figures

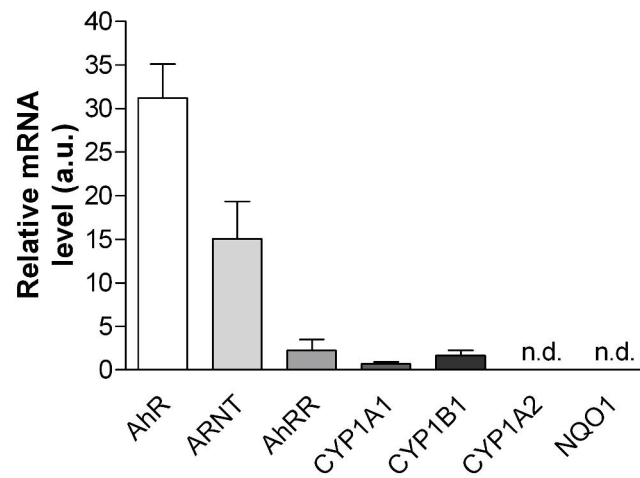


Fig. 1

AhR-dependent pathway genes expression levels in bovine primary cultured blood lymphocytes. Data are expressed as relative mRNA levels compared to GAPDH (mean \pm S.D. of three independent biological replicates).

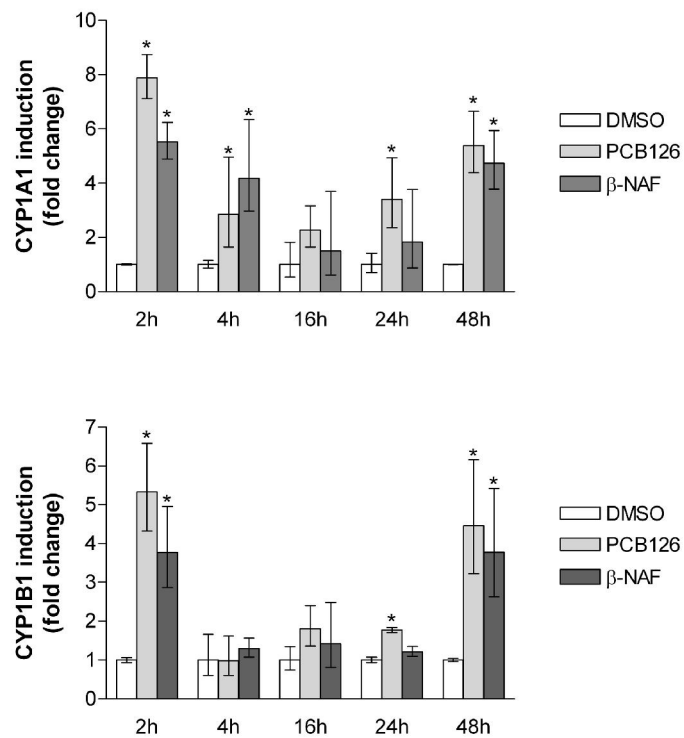


Fig. 2

Time-course profiles of CYP1A1 and CYP1B1 induction in bovine primary cultured blood lymphocytes treated with β -naphthoflavone or PCB126. Data are expressed as fold change

compared to control samples (mean \pm 95% C.I. of three independent biological replicates). *: $p < 0.05$ compared to DMSO.

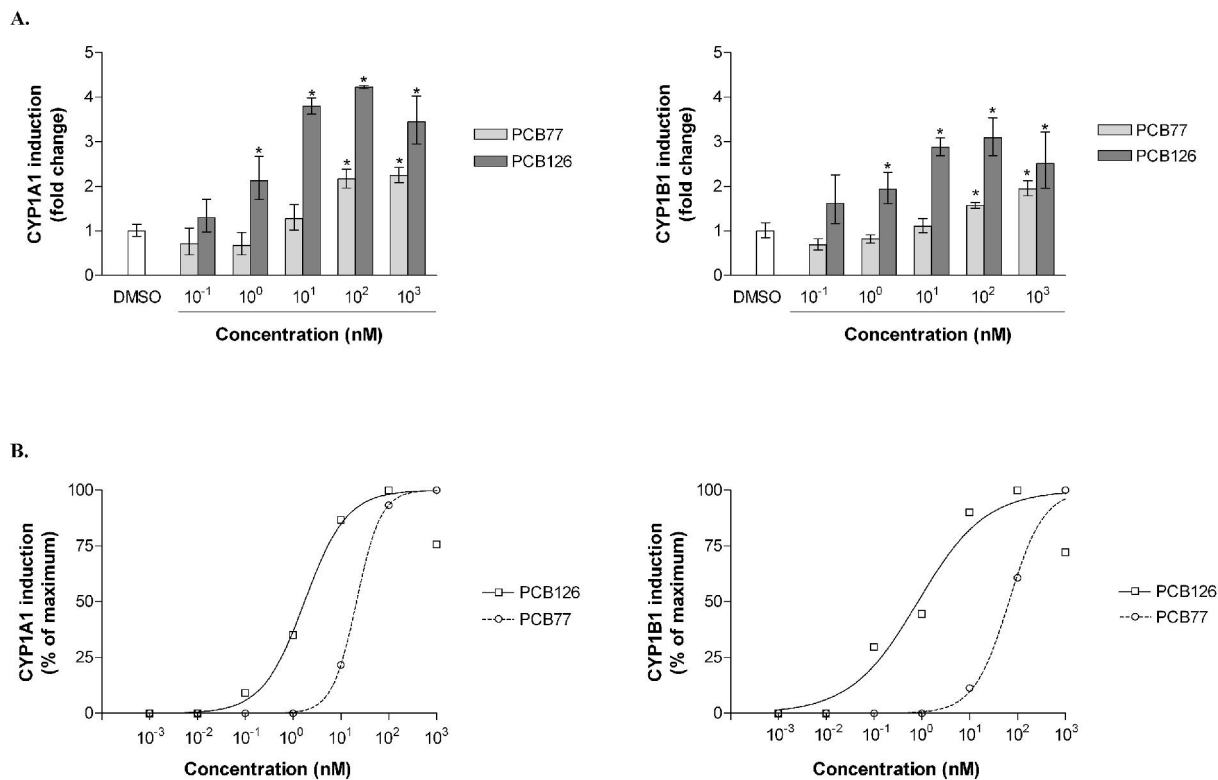


Fig. 3

Dose-response profiles of CYP1A1 and CYP1B1 induction in bovine primary cultured blood lymphocytes treated with PCB126 or PCB77. (A) Fold change levels compared to control samples are expressed as mean \pm 95% C.I. of three independent biological replicates. *: $p < 0.05$ compared to DMSO. (B) Sigmoidal concentration-dependent curves are expressed as the percentage of maximum induction calculated by the mean of three independent biological replicates.

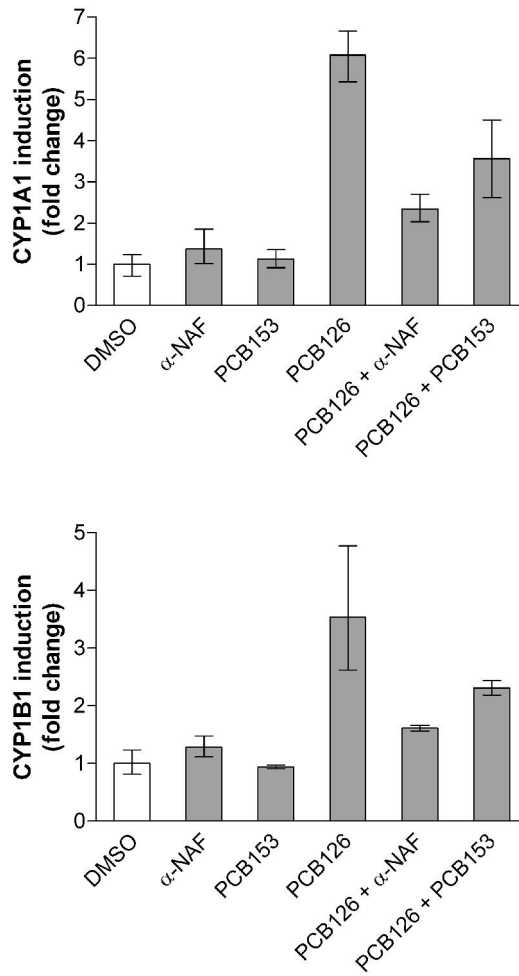


Fig.4

Antagonism by α -naphthoflavone or PCB153 of PCB126- mediated increase in CYP1A1 and CYP1B1 mRNA expression in bovine primary cultured blood lymphocytes. Data are expressed as fold change compared to control samples (mean \pm 95% C.I. of three independent biological replicates).