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Comparative liver accumulation of dioxin-like compounds in sheep and cattle: possible role of AhR-mediated xenobiotic metabolizing enzymes

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Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR-nuclear translocator; CDNB, 1-chloro-2,4-dinitrobenzene; CHP, cumene hydroperoxide; CYP, cytochrome P450; DCPIP, 2,6-dichlorophenolindophenol; DL, dioxin-like; EFSA, European Food Safety Authority; EROD, 7-ethoxyresorufin *O*-dealkylase; GST, glutathione *S*-transferase; ICG, internal control gene; ML, maximum level; MROD, 7-methoxyresorufin *O*-dealkylase; NDL, non dioxin-like; NQO1, quinone oxidoreductase; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzo-*p*-dioxin; PCDF, polychlorinated dibenzofuran; qRT-PCR, Real-time PCR; TEF, Toxic Equivalent Factor; TCDD, 2,3,7,8 tetrachloro-dibenzo-*p*-dioxin; UGT, uridin-diphospho-glucuronyltransferase; XME, xenobiotic metabolizing enzyme

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ABSTRACT

PCDDs, PCDFs, and PCBs are persistent organic pollutants (POPs) that accumulate in animal products and may pose serious health problems. Those able to bind the aryl hydrocarbon receptor (AhR), eliciting a plethora of toxic responses, are defined dioxin-like (DL) compounds, while the remainders are called non-DL (NDL). An EFSA opinion has highlighted the tendency of ovine liver to specifically accumulate DL-compounds to a greater extent than any other farmed ruminant species. To examine the possible role in such an accumulation of xenobiotic metabolizing enzymes (XME) involved in DL-compound biotransformation, liver samples were collected from ewes and cows reared in an area known for low dioxin contamination. A related paper reported that sheep livers had about 5-fold higher DL-compound concentrations than cattle livers, while the content of the six marker NDL-PCBs did not differ between species. Specimens from the same animals were subjected to gene expression analysis for AhR, AhR nuclear translocator (ARNT) and AhR-dependent oxidative and conjugative pathways; XME protein expression and activities were also investigated. Both AhR and ARNT mRNA levels were about 2-fold lower in ovine samples and the same occurred for CYP1A1 and CYP1A2, being approximately 3- and 9-fold less expressed in sheep compared to cattle, while CYP1B1 could be detectable in cattle only. The results of the immunoblotting and catalytic activity (most notably EROD) measurements of the CYP1A family enzymes were in line with the gene expression data. By contrast, phase II enzyme expression and activities in sheep were higher (UGT1A) or similar (GSTA1, NQO1) to those recorded in cattle. The overall low expression of CYP1 family enzymes in the sheep is in line with the observed liver accumulation of DL-compounds and is expected to affect the kinetics and the dynamics of other POPs such as many polycyclic aromatic hydrocarbons, as well as of toxins (e.g. aflatoxins) or drugs (e.g. benzimidazole anthelmintics) known to be metabolized by those enzymes.

Keywords: Dioxin-like compounds, Xenobiotic metabolizing enzymes, Liver, Sheep, Cattle

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), together defined as *dioxins*, and polychlorinated biphenyls (PCBs) are widespread environmental pollutants that cause serious adverse health effects. Due to their high lipophilicity, they accumulate along the food chain, mainly in fat and in the lipid fraction of organs and tissues (Larsen, 2006), being also transferred into milk and eggs; the consumption of animal products rich in fat represents by far the major source of exposure for humans (Malisch and Kotz, 2014). Among hundreds of congeners, only a few, collectively known as dioxin-like (DL), share a high environmental persistency and bear a coplanar structure that allows for the binding with different affinities to a cytosolic transcription factor, the aryl hydrocarbon receptor (AhR), eliciting common toxic effects (Hoogenboom, 2004). Upon ligand binding, AhR translocates into the nucleus, where it heterodimerizes with the AhR nuclear translocator (ARNT), and subsequently binds to consensus sequences termed "dioxin responsive elements" in the promoter regions of target genes. This mechanism results in the upregulation of the so-called "AhR gene battery" that comprises, among others, genes encoding for a number of enzymes generally involved in xenobiotic biotransformation, including DL-compounds themselves (e.g., cytochrome P450 (CYP) 1A1, CYP1A2, CYP1B1, uridin-diphospho-glucuronyltransferases (UGT)1A, quinone oxido-reductase (NQO1), and glutathione *S*-transferases (GST) A) (Bock, 2013; Inui et al., 2014).

In recent years several monitoring programs have shown that sheep livers often exceed the then existing maximum levels (MLs) for PCDD/Fs and the sum of PCDD/Fs and DL-PCBs set by EC Regulation 1881/2006 (4.5 and 10 pg TEQ/ g fat, respectively), even in the absence of a specific contamination source (Bruns-Weller et al., 2010; Rose et al., 2010). Likewise, the analytical results from 332 liver samples submitted by different Member States and evaluated by the European Food Safety Authority (EFSA) reported mean concentration levels of 14.9 and 26.1 pg TEQ/g fat for PCDD/Fs and the sum of PCDD/Fs and DL-PCBs, respectively, being therefore over the legal

limits in force at that time (EFSA, 2011). In sheep such an accumulation appears to be hepato-specific, since several studies showed much lower DL-compound concentrations in muscle and fat tissues compared to livers of the same individuals (Fernandes et al., 2011; Lindstrom, et al., 2005; Lund et al., 2008). Furthermore, the levels in such tissues were comparable to those found in other food producing species (i.e. cows and pigs), bred under similar contamination conditions, that in any case displayed a lower median TEQ ratio between liver and meat compared to sheep for both PCDD/Fs and the sum of PCDD/Fs and DL-PCBs (Fernandes et al., 2011; Schulz et al., 2005). Although the risk assessment performed by EFSA led to the conclusion that the chronic consumption of sheep liver may be a potential health concern, particularly for children and women of child-bearing age (EFSA, 2011), the official MLs for sheep liver have been recently increased and expressed on a wet weight base, being 1.25 pg TEQ/g ww for PCDD/Fs and the sum of PCDD/Fs and DL-PCBs and 2.00 pg TEQ/g ww for the sum of PCDD/Fs and DL-PCBs (EC Regulation 1067/2013).

According to EFSA, among other physiological factors (e.g. breeding and grazing habits), one of the possible reasons for the hepatic accumulation of DL-compounds in sheep compared to other ruminants such as cattle could be a relatively lower expression and/or activity of phase I enzymes (i.e. CYP1A) responsible for their oxidative biotransformation (EFSA, 2011). Although dioxins and PCBs are in general poorly metabolized in most species, the CYP-mediated hydroxylation is a key event in their metabolic conversion (Murk et al., 1994; Pluess et al., 1987; Tai et al., 1993), in that only the hydroxylated derivatives may undergo phase II reactions -in ruminants mainly glucuronidation- in order to be excreted via the bile or the urine (Grimm et al., 2015; Inui et al., 2014). More to the point, DL-PCBs are metabolized predominantly by CYP1A, while the CYP-mediated hydroxylation of NDL-PCBs is mainly carried out by CY2B and, to a much lesser extent, CYP2A, CYP2C and CYP3A (Grimm et al. 2015; McGraw and Waller, 2009). The generation of hydroxylated derivatives of different DL-PCB congeners has been reported in both cows (Gardner et al., 1976; Safe et al., 1975) and ewes (Berg et al., 2010). While a higher rate of the in vitro

metabolism of CYP1A-dependent substrates has been documented in ovine *vs* bovine liver microsomes (Smith et al., 1984; Szotakova et al., 2004), no further information is available about the possible species-related differences in terms of CYP1A gene and protein expression.

Another hypothesis of DL-compound sequestration in liver relies on the ability of hepatic CYP1A2 to bind some congeners (i.e. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and 2,3,4,7,8-pentachlorodibenzofuran, (4-PeCDF)), making them unavailable for the CYP1A1-mediated hydroxylation. Such a mechanism, linked to the CYP1A2 induction mediated by DL-compounds themselves, has been demonstrated in CYP1A2 knock-out mice. The administration of a single oral dose of TCDD, PeCDF or PCB 153 (a NDL-PCB) resulted in very low liver DL-compound concentration compared to that found in wild type parental strains, despite the occurrence of similar levels in all the other tissues (Diliberto et al., 1997; Diliberto et al., 1999; Hakk et al., 2009). Accordingly, high levels of hepatic CYP1A2, in terms of protein and catalytic activity, have been correlated with high concentrations of dioxins in both rat and human liver (Santostefano et al., 1999; Watanabe et al., 2013). However, the role of CYP1A2 in liver DL-compound sequestration has not been explored so far in ruminants, and only indirect evidence has been provided as to the occurrence of species-related differences in CYP1A2 expression/activity between sheep and cattle (Danielson and Golsteyn, 1996).

A study was therefore designed in sheep and cattle, two ruminant species displaying a different DL-compound accumulation pattern, to i) characterize the AhR signaling pathway and AhR-dependent xenobiotic metabolizing enzymes (XMEs) known to be involved in DL-compound biotransformation or to be induced upon the exposure to such chemicals and ii) examine their possible role in the hepatic sequestration of DL-compounds. A comparative analysis at both gene and protein level was therefore performed in liver samples from ewes and cows reared in selected areas of the Piedmont region (North-western Italy) characterized by similar contamination levels and with no known sources of dioxins or DL-PCBs contamination. The liver accumulation profiles

of dioxins, DL-PCBs and the six marker non-DL (NDL-) PCBs of the investigated animals have been the subject of a separate report (Benedetto et al., 2016).

2. Materials and methods

2.1 Reagents

All the materials for the quantitative gene expression (qRT-PCR) analysis (including RNA extraction and cDNA synthesis) were supplied by Bio-rad (Valencia, CA, USA). The BCA protein assay reagents were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Anti-CYP1A1/2 antibody was purchased from Oxford Biomedical Research (Oxford, MI, USA); anti-GSTα was from Alpha Diagnostic International (San Antonio, TX, USA); anti-β-Actin (AC-15) was from Abcam (Cambridge, UK); anti-Calnexin (H-70), anti-NQO1 (C-19), and anti-UGT1A (H-300) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All the antibodies were polyclonal and reported to cross-react with several species, including sheep and cattle. Horseradish peroxidase-conjugated secondary antibodies and the chemiluminescent substrate for Western blot detection were from Bio-rad. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Sample collection

The sampling program of the ovine and bovine specimens, together with the animal characteristics (sex, age, breed) and the criteria used to select appropriate liver samples (macroscopic analysis) are detailed elsewhere (Benedetto et al., 2016). Briefly, 30 samples from ewes and 10 samples from cows at the end of their productive cycle were included in the study. Specimens for protein expression analysis and catalytic activity assays were immediately frozen in liquid nitrogen, while samples for gene expression analysis were placed in RNAlater® solution for 24 hours. All samples were then transferred at -80°C until they were processed. Liver specimens for chemical determinations were stored at -20°C.

2.2 Analytical determinations

Quantitative determinations of PCDD/Fs, DL-PCBs, and NDL-PCBs were performed as described by Benedetto et al. (2016). In brief, fat extracts from 45 g of lyophilized liver for each sample were analyzed by GC-HRMS, based on the internationally recognized method EPA1613-revision B (EPA,1994) and method EPA 1668-revision C (EPA, 2010). Data were adjusted for the current World Health Organization Toxicity Equivalent factors (TEF-WHO$_{2005}$), as required by the EC Regulation 1259/2011, and expressed as pg TEQ/g ww or pg/g ww (NDL-PCBs), according to the EC Regulation 1067/2013.

2.3 Quantitative gene expression analysis (Real-time PCR)

Total RNA was isolated using the PureZOL™ RNA Isolation Reagent, according to the manufacturer's protocol. RNA purity and quantity were evaluated by absorbance readings using the NanoDrop ND-2000 spectrophotometer (Thermo Fisher 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 Instrument, Bio-Rad). All the samples had a RNA Quality Indicator (RQI) > 7. One μg of total RNA was reverse transcribed into cDNA using iScript cDNA Synthesis kit, according to the manufacturer's instructions, in a final volume of 20 μl.

Primers for bovine AHR, ARNT, CYP1A1, CYP1A2, CYP1B1, NQO1, GSTA1, GSTA2, and GAPDH were according to Girolami et al. (2015). All the other primers were designed on *Bos taurus* and *Ovis aries* GenBank and Ensembl mRNA sequences using Primer 3 Software (version 3.0, Applied Biosystems, Foster City, CA, USA), except for sheep CYP1A2, which was sequenced by a RACE-PCR protocol. In brief, a preliminary *in silico* analysis of CYP1A2 and CYP1A1 RNA sequences, collected from different ruminant and ungulate species, was performed by Clustal W multiple alignment (Bioedit software, ver. 7.0.9). Degenerate primers (forward 5'-GCCCTVTTSAAGCACARYRAG-3' and reverse 5'-GATGGCYARGAAGAGGAAGAYYTCCCA -3') were then designed to amplify a CYP1A2

conserved region. After amplification of ovine liver cDNA samples (50-200 ng) by a 50 µL PCR reaction containing 2x Accuprime Master mix (Thermo Fisher Scientific) and 10 µM of each primer, all amplified fragments were gel purified and sequenced by capillary electrophoresis (ABI 3100 sequencer, BigDye V.1.1 chemistry). The extension of the 3' and 5' ends of candidate fragments of ovine CYP1A2 cDNA was performed by 5'/3' RACE kit 2nd Generation (Roche, Monza, Italy), according to manufacturer's instructions. Sequencing of both 400 bp starting fragments and associated extended 5' and 3' amplicons (1551 bp) revealed a candidate ovine cDNA template with 96% homology (99% coverage) with bovine CYP1A2 sequence and only 93% homology (71% coverage) with ovine CYP1A1 sequence. Further BLAST alignments against last ovine genome assembly (ver. Oar_v4.0) released on 11/20/2015 from Archibald et al. (2010), confirmed the identity of the sequenced cDNA templates as ovine CYP1A2. Primer information (sequences, gene accession numbers and amplicon sizes) of target and candidate internal control genes (ICGs) specific for sheep and cattle are summarized in Supplementary Tables S1 and S2, respectively. Due to the high level of homology (95%) of sheep UGT1A3 and UGT1A4, genes were amplified with the same couple of primers. Each primer set efficiency was evaluated through the dilution method using a pool of all RNA samples for each species, and it was comprised between 95% and 100%.

To identify the most stable couple of ICGs for each species, mRNA levels of a set of candidate genes (B2M, GAPDH, HPRT1, PGK1, SDHA, S24, TFRC, YWHAZ) were measured in ovine and bovine liver samples, and analyzed using three different statistical algorithms - geNorm version 3.5 (Vandesompele et al., 2002), NormFinder version 0.953 (Andersen et al., 2004), and BestKeeper version 1 (Pfaffl et al., 2004) - , according to the developers' recommendations. SDHA/PGK, and SDHA/GAPDH were identified as the best combinations of ICGs for sheep and cows, respectively.

qRT-PCR reactions were performed on 500 ng of cDNA, in a final volume of 20 µl consisting of the 1X iTaq SYBR Green Supermix with ROX and an optimized concentration of each primer set (150-900 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: 30 s 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.

The relative gene expression was calculated with the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001), using the geometric mean of the two best ICGs for each species as a reference (Vandesompele et al., 2002), and was expressed as relative mRNA level.

2.4 Western blot analysis

Hepatic subcellular fractions were isolated by differential ultracentrifugation and stored as detailed by Nebbia et al. (1999). Protein extracts were obtained through RIPA lysis buffer (50 mM TrisHCl, 150 mM NaCl, 0.1 % SDS,1% Tryton X-100, 0.5% sodium deoxycholate) containing 2 mM PMSF and a Protease Inhibitor Cocktail according to manufacturer's instructions. The protein concentration was determined according to the OD at 562 nm using the BCA protein assay kit. Equal amounts of protein (70 µg/lane) were resolved with SDS-PAGE on 10-12% polyacrylamide gels under reducing conditions, and transferred onto a nitrocellulose membrane. After blocking of non-specific binding sites with 10% BSA in TBS, the membranes were incubated for 2 h at room temperature with primary antibodies. β-Actin or Calnexin were used as loading controls for cytosolic and microsomal proteins, respectively. Comparable reactivity of antibodies to sheep and cow proteins was validated through the measurement of amino acid homology between the two species, which resulted >90% for all the investigated proteins. Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, reactive proteins were visualized with an enhanced chemiluminescence system (Clarity™ Western ECL Blotting Substrate) 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 corresponding loading control.

2.5 Enzyme assays

In general, enzyme activities were assayed with substrates that are considered specific for a given CYP- (Ioannides, 2006) or phase II enzyme subfamily (Bock, 2001; Sherratt and Hayes, 2001) in target species or in humans or laboratory species; test conditions were of linearity with respect to protein concentration and incubation time. The *O*-dealkylation rate of either 2 µM ethoxyresorufin (EROD) or 5 µM methoxyresorufin (MROD) were assayed fluorometrically by measuring the amount of the released resorufin as detailed by Nebbia et al. (2003). UGT activity was determined in 0.25% Triton X-100 activated microsomes according to Antoine et al. (1988), using 1-naphthol 0.3 mM, a substrate reported to be specific for the polycyclic aromatic hydrocarbon-inducible UGT1A (Court, 2001). The remaining parameters were assayed in diluted cytosolic fractions. NQO1 was measured following the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm (Lind et al., 1990), while total GST was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) 1 mM as described by Habig et al. (1974). The activity of the GST of the α class was measured using cumene hydroperoxide (CHP) 1.5 mM as the substrate using the method described by Scholz et al. (1981).

2.6 Statistical analysis

Statistical analysis of all data was performed using GraphPad Prism 4.03 (GraphPad Software, Inc., La Jolla, CA, USA). Outlier results identified by the Grubbs' test were omitted from calculations. Differences between mean values were determined by the Mann Whitney test. Statistical significance was assumed at p values of ≤ 0.05.

3. Results

On average, the analytical determinations of PCDD/Fs and DL-PCBs showed that total TEQ levels were approximately 5-fold higher (p<0.0001) in sheep liver compared to cattle (mean values of 0.76 pg TEQ/g ww *vs* 0.15 pg TEQ/g ww). On the other hand, no statistically significant

differences in total NDL-PCBs content were recorded between the two species, with mean concentrations of 1.01 ng/g ww in the ovine samples and 0.87 ng/g ww in the bovine ones. A detailed description of DL-congener patterns in ewe and cow livers may be found in the paper by Benedetto et al. (2016).

The Real-time PCR analysis of the two key AhR signaling pathway members (AhR and ARNT) and of the AhR-dependent XMEs (phase I: CYP1A1, CYP1A2, and CYP1B1; phase II: NQO1, GSTA isoforms and UGT1A isoforms) showed that sheep and cow liver expressed all the investigated genes, except for CYP1B1, which was detectable to a very low extent only in bovine samples (Fig. 1). As far as phase II enzymes are concerned, there were some differences between the two species in the number of the expressed isoforms of the GSTA and UGT1 families, in accordance with the deposited genome sequences. Although sheep UGT1A3 and 4 could not be discriminated by the Real-time PCR analysis due to the high level of homology, ovine liver displayed at least 4 UGT1A isoforms (UGT1A1-3/4-6-9) and only one GSTA isoform (GSTA1), while cattle liver expressed 2 UGT1A (UGT1A1-6), and 2 GSTA isoforms (GSTA1-2).

The comparative analysis of the hepatic mRNA expression levels of the investigated genes in ewes and cows is outlined in Table 1. As regards AhR and ARNT, both genes were expressed to a lower extent (about 2-fold) in the ovine samples compared to the bovine ones (p< 0.001). Likewise, sheep had a lower gene expression of AhR-dependent phase I enzymes compared to cattle (p< 0.001 or less), with approximately a 3- and 9-fold difference for CYP1A1 and CYP1A2, respectively. In contrast, UGT1A6 isoform was expressed at a significant higher level in ewes (11-fold change, p< 0.0001), whereas no statistically significant differences in the mRNA level of the other investigated phase II enzymes (NQO1, GSTA1 and UGT1A) were detected between the two species.

The gene expression results of XMEs were almost completely consistent with the corresponding measured protein level (Fig. 2) and catalytic activities (Table 2). The immunoblot analysis of the CYP1A family enzymes using an anti-rabbit CYP1A1/2 antibody revealed a single band in the

hepatic microsomes from both ruminants, which was less intense in the sheep (2-fold change, p<0.001). Accordingly, the EROD activity was lower in the ovine subcellular preparations compared to the bovine ones (7-fold change, p< 0.0001), while no statistically significant differences in the MROD rate occurred between the two species. As regards the microsomal UGT1A family enzymes, both the protein amount and the rate of 1-naphthol glucuronidation were higher in sheep liver samples compared to cattle ones (p< 0.0001), with approximately 4- and 2-fold difference, respectively. In line with the gene expression analysis results, hepatic NQO1 protein level and activity were comparable between ewes and cows. Conversely, sheep cytosolic fractions exhibited a lower GSTα class protein expression (4-fold change, p<0.0001), despite the lack of difference between the two species in the mRNA level of the GSTA1 isoform. From the catalytic viewpoint, the total GST activity assayed with CDNB as the substrate was more elevated in the ovine samples (about 3-fold change, p<0.0001), while no statistically significant differences in the rate of CHP conjugation were recorded between the two ruminant species.

4. Discussion

In recent years, the tendency of ovine livers to accumulate DL-compounds with respect to cattle and other food producing species has been highlighted by several surveys and an EFSA opinion (Bruns-Weller et al., 2010; EFSA, 2011; Rose et al., 2010). Recently, Benedetto et al. (2016) confirmed such feature on the same animals investigated in this study, reporting an almost 5-fold increase in dioxins and DL-PCBs content in sheep *vs* cattle livers, both collected in areas with no known contamination sources. In line with the results of both an experimental study performed on lambs fed dioxin-contaminated hay (Hoogenboom et al., 2015) and data reported by EFSA (2011), also in our case the hepatic sequestration was strictly limited to DL-compounds and did not involve NDL-PCBs (Benedetto et al. 2016). The aim of the present study was to get further insight into the possible mechanisms of such a species-specific accumulation. Thus, liver samples from the same sheep and cows subjected to DL-compound determination (Benedetto et al. 2016) were analyzed for

the gene/protein expression and the activity of AhR-dependent XMEs involved in the biotransformation of DL-compounds and/or known to be induced upon the exposure to them. In addition, the expression of the AhR and ARNT genes was evaluated in order to assess their contribution to the basal level and the possible inducibility of the related enzymes (mostly CYP1A family) in these two ruminant species (Bock, 2013).

To the best of our knowledge, this is the first report investigating the comparative expression of selected AhR-dependent XMEs at gene and protein level in sheep and cows, as all the studies published so far have dealt only with enzyme activities. In our study, ovine livers exhibited a noticeably lower expression and activity of the CYP1A family when compared to cow livers. The available information about CYP1A family in ruminants concerns the ability of liver microsomes to *O*-dealkylate 7-ethoxyresorufin or 7-methoxyresorufin, two substrates that in the rat are relatively specific for CYP1A1 and CYP1A2, respectively (Nerurkar et al., 1993). In ruminants such a specificity has been established only for bovine CYP1A1 (Pegolo et al., 2010; Sivapathasundaram et al., 2001). In line with the results of comparative studies performed with liver microsomes (Smith et al., 1984; Szotakova et al., 2004) or primary hepatocytes (Vantklooster et al., 1993), the present data confirm the much lesser ability of ovine preparations in performing 7-ethoxyresorufin *O*-dealkylation compared to the bovine ones, which is matched by a 3-fold lower CYP1A1 gene expression. Although the anti-rabbit CYP1A1/2 antibody did not allow discrimination between the two CYP1A isoforms, the resulting single immunoreactive protein was consistently more expressed in bovine liver. In keeping with previous studies (Szotakova et al., 2004), however, liver MROD activity did not differ between the two species, despite that CYP1A2 gene was 9-fold less expressed in sheep than in cattle; this might indicate that 7-methoxyresorufin is not a specific substrate for CYP1A2 in both species. The lower expression of liver CYP1A2 in sheep when compared to cattle is also supported by an indirect *in vivo* experimental evidence. The hepatic clearance of caffeine, an established CYP1A probe (Berthou et al., 1992), occurred at similar rates in ewes and heifers; however, the plasma caffeine metabolite pattern was markedly different, in that paraxanthine

(CYP1A2-dependent) largely predominated over theophylline (CYP1A1-dependent) in heifers, while in sheep the reverse was true; the Authors therefore concluded that CYP1A2 is likely to be expressed to a lesser extent in sheep than in cattle (Danielson and Golsteyn, 1996). Finally, it should be noted that in most animal species CYP1A2 is predominantly expressed in liver, while CYP1A1 is mainly expressed extrahepatically (Ioannides, 2006). According to our results, this does not seem to be the case for the sheep that exhibits a CYP1A2/CYP1A1 liver ratio of about 1.5 and hence much lower than that occurring in cattle (4.9). CYP1B1 is also much more abundant in extrahepatic tissues, but has been reported to be readily inducible by different chlorinated or brominated dioxins in bovine primary hepatocytes (Guruge et al., 2009). In line with the above results, it could be detectable, though at very low levels, in bovine liver only.

As far as the examined AhR-mediated phase II XMEs are concerned, liver NQO1 expression and activity did not vary between sheep and cattle, but no comparative data could be identified in the literature. Limited information is available concerning the comparative conjugative metabolism of xenobiotics in cattle and sheep liver. As mentioned before, the major route of DL-compound phase II metabolism is glucuronidation. In this respect, data from the present work point to a higher expression (at both gene and protein level) and catalytic activity of UGT1A enzymes in liver from ewes compared to cows; our results match those from other studies in which the glucuronidation rate of 1-naphthol, a typical probe of most UGT1A proteins (Tukey and Strassburg, 2000), was found to be up to 5-fold higher in ovine *vs* bovine hepatic microsomes (Smith et al., 1984; Watkins and Klaassen, 1986).

A minor pathway of some (DL)-PCBs comprises the GST-mediated conjugation of epoxides resulting from CYP-dependent biotransformation (Grimm et al., 2015). When using CDNB, a substrate for most GST families, the higher total GST activity recorded in our study in ewe cytosols reproduces what has been outlined in other reports also with respect to the degree of difference between the two species (around 2- and 4-fold) (Smith et al., 1984; Szotakova et al., 2004; Watkins et al., 1987). It is worth noting that the low efficiency in the conjugation of CDNB displayed by

cattle has been described also in comparison with other food-producing species (horse, pig, goat, rabbit and broiler chick) (Gusson et al., 2006; Szotakova et al., 2004). Concerning the GSTα class, which is a target of the AhR-signaling pathway, the lower protein expression recorded in ovine samples is consistent with the occurrence of a single gene isoform (GSTA1) compared to cattle (GSTA1 and GSTA2), although the relative mRNA levels did not show any statistically significant differences. In this respect, the conjugation rate of CHP was almost superimposable in the two tested species. This suggests a limited specificity of such a substrate for the GSTα class in both species, unlike what has been reported in humans (Eaton and Bammler, 1999; Jaitovitch-Groisman et al., 2000).

Based on data generated from this investigation, the lower expression and activity of CYP1 family in the ovine liver with respect to cattle is consistent with the hypothesis of a less efficient hydroxylation rate of DL-compounds by the sheep that could impair their subsequent elimination ultimately resulting in their specific hepatic accumulation. More to the point, in consideration of the remarkably low CYP1A2 expression, it is unlikely that this isoform could be responsible for the specific liver sequestration of such molecules in the sheep, in contrast to what has been previously reported for mice, rats and humans (Hakk et al., 2009, Santostefano et al., 1999; Watanabe et al., 2013). It is worth noting that our results evidence a lower gene expression of AhR and ARNT in sheep, suggesting that AhR-target genes - mostly CYP1A enzymes - could be also less inducible by DL-compounds compared to cattle. Such an event has been demonstrated in human cultured peripheral lymphocytes from healthy donors, where AhR mRNA levels were positively correlated with CYP1A1 inducibility (Lin et al., 2003). As a further hypothesis, it cannot be excluded that a different binding affinity of DL-compounds towards sheep and cow AhR could participate in the lower inducibility of ovine AhR-target genes, as it has been reported for other species (Connor and Aylward, 2006). Overall, results concerning the differences in the expression and activity of AhR-mediated phase II XMEs between the two species suggest that the examined conjugative enzymes are not likely to be involved in the hepatic accumulation of DL-compounds in sheep.

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In conclusion, this work is the first to compare the expression and the catalytic activity of AhR-dependent XMEs in sheep and cows, two ruminant species characterized by a different ability in the hepatic accumulation of DL-compounds. The results have provided further insight into the possible underlying mechanisms, pointing out remarkable differences between the two species, particularly in the expression of both key transcription factors of the AhR signaling pathway and of phase I enzymes participating in the oxidative metabolic conversion of such environmental pollutants, which is a prerequisite for their subsequent elimination. Further research is needed to explore the possible role of other factors in the specific DL-compound accumulation in ovine liver. For example, the *in vitro* inability of human vs. rat preparations in metabolizing model DL-compounds has been reported to reflect structural differences in CYP1A1 protein (Yamazaki et al., 2011). It may be also worthwhile to investigate the involvement of drug transporters (e.g. ABCG2 and MRP2) in the biliary excretion of DL-compounds in ruminants. Finally, the relative low expression of AhR, ARNT, and CYP1A enzymes we found in sheep liver is expected to alter the kinetics and the dynamics of other CYP1A substrates, including other environmental pollutants (e.g. polycyclic aromatic hydrocarbons) (Shimada and Fujii-Kuriyama, 2014), toxins (e.g. aflatoxins) (Dohnal et al., 2014) or drugs (e.g. benzimidazole anthelmintics) (Velík et al., 2005). This will result in probable implications for the sensitivity to certain toxicants or the efficacy and persistence of selected medicines.

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Table 1

Relative mRNA expression levels of AhR signaling pathway members and target genes (phase I and II enzymes) in livers from sheep and cows

	AhR	ARNT	CYP1A1	CYP1A2	NQO1	GSTA1	UGT1A1	UGT1A6
Cows (n = 10)	118.0 ± 15.2	41.4 ± 1.9	745.0 ± 122.0	3512.0 ± 450.0	31.2 ± 6.6	5660.0 ± 553.7	529.4 ± 87.6	137.0 ± 27.5
Sheep (n= 30)	$46.3 \pm 11.0^{**}$	$19.0 \pm 5.3^{***}$	$252.2 \pm 72.6^{***}$	$383.1 \pm 145.2^{***}$	20.6 ± 5.5	4932.4 ± 1182.6	492.1 ± 158.71	$1508.2 \pm 182.0^{***}$

Data are expressed as mean $\pm$ SEM; ** $p<0.001$, *** $p<0.0001$

Table 2

Phase I and phase II metabolic activities measured in subcellular fractions obtained from sheep and cow livers

	CYP1A (EROD)§	CYP1A (MROD)§	NQO1 (DCPIP)†	GST (CDNB)†	GSTα (CHP)†	UGT1A (1-naphtol)†
Cows (n = 10)	354.1 ± 32.1	15.5 ± 1.5	108.7 ± 8.3	182.8 ± 8.3	168.7 ± 7.1	2.7 ± 0.5
Sheep (n = 30)	$47.7 \pm 3.9^{***}$	20.9 ± 2.1	99.1 ± 8.1	$514.8 \pm 22.0^{***}$	156.3 ± 7.6	$5.7 \pm 0.4^{***}$

Data are expressed as mean $\pm$ SEM; § pmol/min/mg protein; † nmol/min/mg protein; *** $p<0.0001$
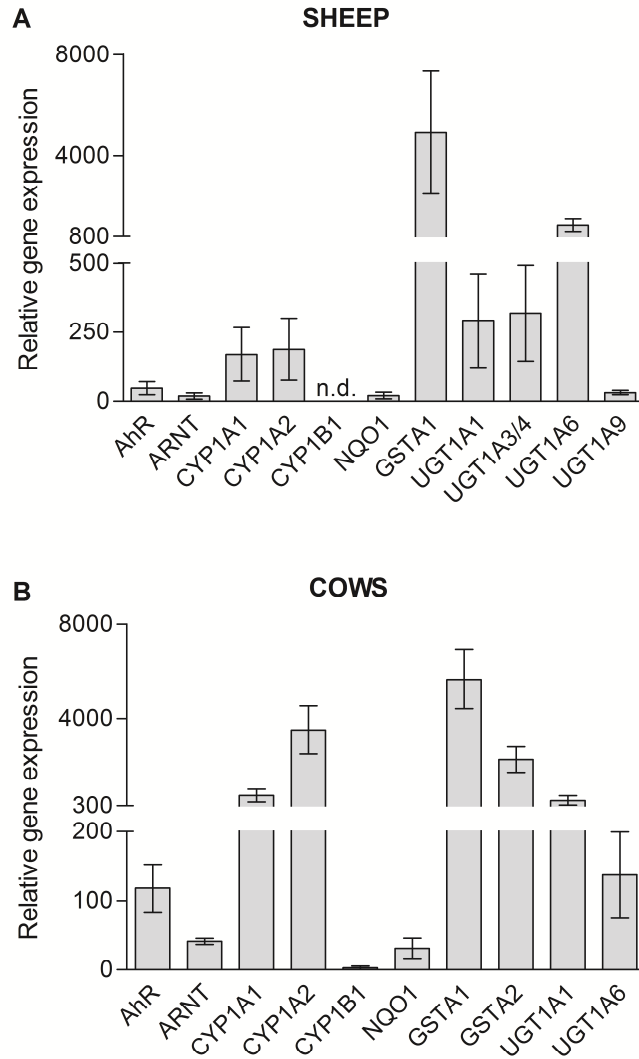
Fig. 1. Relative mRNA expression levels of AhR signaling pathway members (AhR and ARNT) and target genes (phase I and II enzymes) in livers from sheep (n=30) and cows (n=10). Data are expressed as relative mRNA levels compared to the best combination of ICG for each species (SDHA/PGK for sheep, and SDHA/GAPDH for cows), and presented as mean ± 95% confidence interval. UGT1A3/4 have been grouped due to the amplification with the same couple of primers. n.d. = not detectable.

Fig. 2. Protein expression levels of AhR-dependent XMEs in livers from sheep (n=30) and cows (n=10). Western blot analysis was performed on hepatic subcellular fractions. Da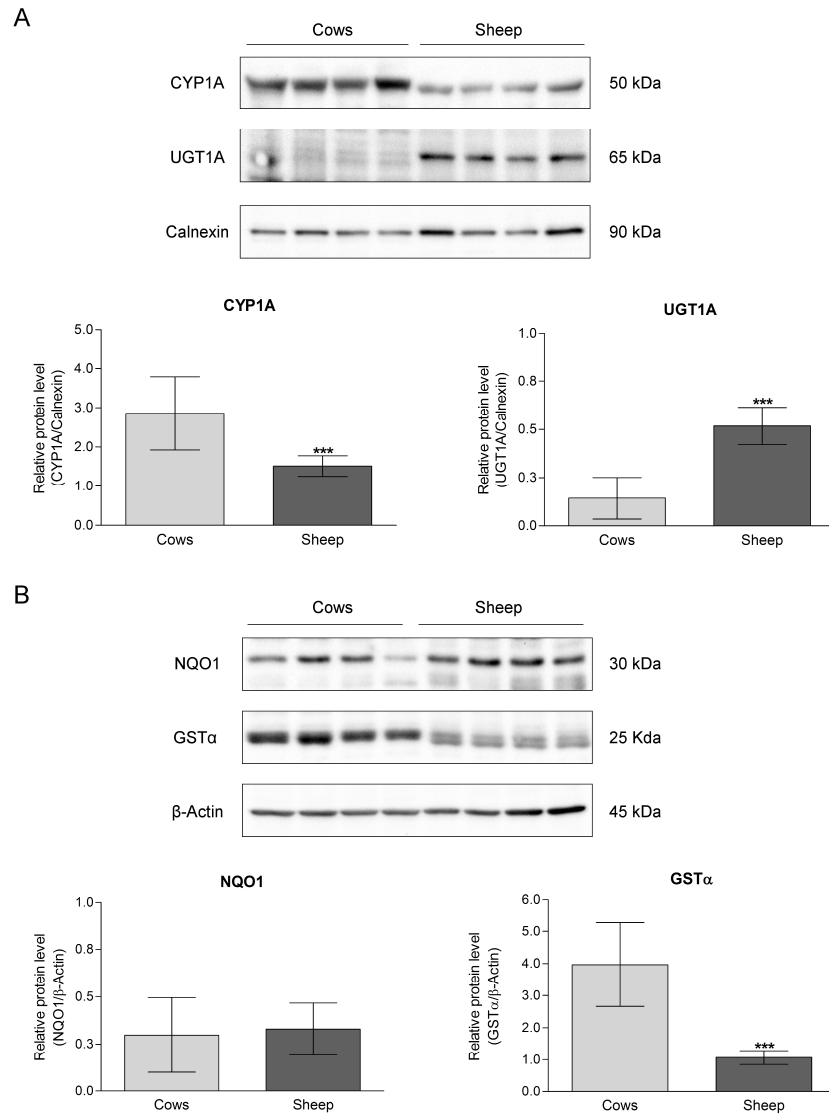ta are expressed as relative protein level using β-Actin or Calnexin as loading controls for cytosolic and microsomal proteins, respectively (mean ± 95% confidence interval). The gels are representative of 4 animals for each species. *** $p < 0.0001$

Table S1

Primers used for Real-time PCR analysis (ovine).

Gene	Accession no.	5'→ 3' sequence	Amplicon size
AHR	XM_004007775	Fwd: GTGCAGAAAACTGTCAAGCCA	172
		Rev: CACTGAGCCTAAGAACGGAAA	
ARNT	XM_004003633	Fwd: GCACCTACAAGCCGTCTTTC	155
		Rev: CTCAGACTGTGGCTGGTTCA	
CYP1A1	NM_001129905	Fwd: CGAGAATGCCAATATCCAGC	174
		Rev: TGCCAATCACTGTGTCCAG	
CYP1A2	XM_012098693	Fwd: CAGTAAGGAGATGCTCAGTG	201
		Rev: CTGTTCTTGTCAAAGTCCTGG	
CYP1B1	XM_004006013	Fwd: CACCAGGTATTCGGAAGTGC	118
		Rev: AAGAAAGGCCATGACGTAGG	
GSTA1	NM_001114766	Fwd: AGAGGGTGTGGCAGATTTGG	141
		Rev: TGGCTCTTCAGCACATTTTCA	
NQO1	XM_004015102	Fwd: AAGAAGGCAGTGCTTTCCAT	124
		Rev: AGCCACAGAAGTGCAGAGTG	
UGT1A1	NM_001205147	Fwd: GGGTCTGTTTGGCTTCTCAG	181
		Rev: CCATTGAGCCCAAAGAGAAA	
UGT1A3/4	NM_001205148	Fwd: TCATTGGGGGCATCAACTGT	111
	NM_001205149	Rev: CCATTGAGCCCAAAGAGAAA	
UGT1A6	NM_001205146	Fwd: CCTCCATTTGGCTGCTAAGA	179
		Rev: CCATTGAGCCCAAAGAGAAA	
UGT1A9	NM_001009189	Fwd: CAGTGGCATCAACTGTCAGAA	113
		Rev: CCATTGAGCCCAAAGAGAAA	
B2M	NM_001009284	Fwd: CTGTCGCTGTCTGGACTGG	86
		Rev: TTTGGCTTTCCATCTTCTGG	
GAPDH	NM_001190390	Fwd: AGATGGTGAAGGTCGGAGTG	117
		Rev: GAAGGTCAATGAAGGGGTCA	
HPRT1	XM_004022693	Fwd: GCCGACCTGTTGGATTACAT	113
		Rev: TCACCTGTTGACTGGTCGTT	
PGK1	NM_001142516	Fwd: CGGAGCTAAAGTTGCAGACA	124
		Rev: GCCAATCTCCATGTTGTTGA	
S24	XM_004018184	Fwd: CATGCGCCTCACTACATCG	100
		Rev: CATCTTCCACTGTTCGCTCA	
SDHA	XM_004017097	Fwd: TAAACCAAATGCTGGGGAAG	115
		Rev: ATGGCTCTGCATCGACTTCT	
TFRC	XM_004003001	Fwd: GCAGTTCTCAAAACTCGGTGT	129
		Rev: CATGGACCAGTTTGCCAGTA	
YWHAZ	NM_001267887	Fwd: TGAAGCCATTGCTGAACTTG	128
		Rev: CTGCTTCAGCTTCGTCTCCT	

Table S2

Primers used for Real-time PCR analysis (bovine).

Gene	Accession no.	5'→ 3' sequence	Amplicon size
AHR	XM_612996	Fwd: GTGCAGAAAACTGTCAAGCC	203
		Rev: GCAACATCAAAGAAGCTCTTG	
ARNT	NM_173993	Fwd: TTTCCTCACTGATCAGGAAC	183
		Rev: TCCAGGATACGCCCTGTC	
CYP1A1	XM_588298	Fw: CGAGAATGCCAATATCCAGC	173
		Rev: TGCCAATCACTGTGTCCAG	
CYP1A2	NM_001099364	Fwd: CAGTAAGGAGATGCTCAGTC	201
		Rev: CTGTTCTTGTCAAAGTCCTGG	
CYP1B1	NM_001192294	Fwd: CACCAGGTATTCGGAAGTGC	118
		Rev: AAGAAAGGCCATGACGTAGG	
GSTA1	NM_001078149	Fwd: AGAGGGTGTGGCAGATTTGG	141
		Rev: TGGCTCTTCAGCACATTTTCA	
GSTA2	NM_177515	Fwd: TTACCACTGTGCCCACCTGAT	112
		Rev: CTTGTCCGTGATTCTTCAGCAC	
NQO1	NM_001034535	Fwd: CGGAATAAGAAGGCAGTGCT	130
		Rev: AGCCACAGAAGTGCAGAGTG	
UGT1A1	NM_001105636	Fwd: TGGGTCTGTCTGGATTCTCA	195
		Rev: GGAATCTCCGAGACCATTGA	
UGT1A6	NM_174762	Fwd: CAACACGGTCCTCATCGGA	115
		Rev: GCCCAAAGAGAAAACCACAA	
B2M	NM_173893	Fwd: ATCGGAGCAGTCAGACCTGT	154
		Rev: CAGGTCTCGATCCCACTTAAC	
GAPDH	NM_001034034	Fwd: GGAGAAACCTGCCAAGTATGAT	125
		Rev: GAGTGTCGCTGTTGAAGTCG	
HPRT1	NM_001034035	Fwd: CACTGGGAAGACAATGCAGA	102
		Rev: ACACTTCGAGGGGTCCTTTT	
PGK1	NM_001034299	Fwd: TGACAAGAATGGCGTGAAGA	139
		Rev: CTCAGGACCACAGTCCAAGC	
S24	NM_001025339	Fwd: AACACAGGCTTGCGAGACAT	154
		Rev: CCTTCTGTTGTCCAATCTCCA	
SDHA	NM_174178	Fwd: CTTCAAGGAGAGGGTTGACG	101
		Rev: CGTAGGAGAGCGTGTGCTT	
TFRC	NM_001206577	Fwd: ATGCTGCTTTCCCTTTCCTT	149
		Rev: ACGTGCCACTCTGTTCAACT	
YWHAZ	NM_174814	Fwd: TGAAGCCATTGCTGAACTTG	128
		Rev: CTGCTTCAGCTTCGTCTCCT	