Constitutive expression and phenobarbital modulation of drug metabolizing enzymes and related nuclear receptors in cattle liver and extra-hepatic tissues

UNIVERSITÀ DEGLI STUDI DI TORINO

Xenobiotica

42:1096-1109;

doi:10.3109/00498254.2012.694493

CONSTITUTIVE EXPRESSION AND PHENOBARBITAL MODULATION OF DRUG METABOLIZING ENZYMES AND RELATED NUCLEAR RECEPTORS IN CATTLE LIVER AND EXTRA-HEPATIC TISSUES

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Running head: cattle hepatic and extrahepatic DMEs, NRs and PB

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Keywords

Phenobarbital; cattle; drug metabolizing enzymes; nuclear receptors; gene expression; extrahepatic tissues.

Abstract

1. In humans and rodents, phenobarbital (PB) induces hepatic and extra-hepatic drug metabolizing enzymes (DMEs) through the activation of specific nuclear receptors (NRs). In contrast, few data about PB transcriptional effects in veterinary species are available.
2. The constitutive expression and modulation of PB-responsive NR and DME genes, following an oral PB challenge, were investigated in cattle liver and extra-hepatic tissues (duodenum, kidney, lung, testis, adrenal and muscle).
3. Likewise to humans and rodents, target genes were expressed to a lower extent compared to the liver with few exceptions.
4. Phenobarbital significantly affected hepatic CYP2B22, 2C31, 2C87, 3A and UDP-glucuronosyltransferase 1A1-*like*, glutathione *S*-transferase A1-*like* and sulfotransferase 1A1-*like* (SULT1A1-*like*) mRNAs and apoprotein amounts; in extra-hepatic tissues, only duodenum showed a significant down-regulation of SULT1A1-*like* gene and apoprotein. Nuclear receptor mRNAs were never affected by PB.
5. Presented data are the first evidence about the constitutive expression of foremost DME and NR genes in cattle extra-hepatic tissues, and data obtained following a PB challenge are suggestive of species-differences in drug metabolism; altogether, these information are of value for the extrapolation of pharmacotoxicological data among species, the characterization of drug-drug interactions as well as the animal and consumer's risk caused by harmful residues formation.

Introduction

In mammals, the cytochrome P450 (CYP) superfamily of drug metabolizing enzymes (DMEs) plays a key role in the oxidative metabolism of xenobiotics and relevant endogenous compounds (i.e., steroids, bile acids). The liver is the organ mostly endowed of CYP isoforms, but these DMEs are also expressed in extrahepatic tissues like gastrointestinal tract, kidney, lung and upper airways, brain, adrenal gland, gonads, heart and skin (Martignoni et al., 2004; Graham and Lake, 2008; Pelkonen et al., 2008). To date, limited information about DMEs constitutive expression, regulation and function in extra-hepatic tissues have been published in veterinary species and particularly in cattle, which represent a relevant food-producing species worldwide; the few available data refer to gastrointestinal mucosa (Kawalek and el Said, 1994; Virkel et al., 2010), kidney (Darwish et al., 2010; Merlanti et al., 2007), lung (Darwish et al., 2010), olfactory tract (Longo et al., 1997), testis (Lopparelli et al., 2010), tongue (Yang et al., 2003) and coronary arteries (Grasso et al., 2005).

The cytochrome P450 induction is a known phenomenon influencing CYP-dependent drug metabolism, pharmacokinetics and drug-drug interactions, the carcinogenicity of xenobiotics as well as the activity and disposition of endogenous hormones. Barbiturates, dexamethasone, rifampicin, polycyclic aromatic hydrocarbons and ethanol are considered as classical CYP inducers; what's more, in the past decade specific xenobiotic-sensor receptors (nuclear receptors, NRs) have been shown to be involved in CYP transcriptional activation and the resulting protein induction (Handschin and Meyer, 2003; Xu et al., 2005; Tolson and Wang, 2010).

Phenobarbital (PB) is a hypnotic and antiepileptic drug causing pleiotropic effects in the liver; in addition, PB induces CYP2A, 2B, 2C and 3A subfamilies and also some transferases (Honkakoski and Negishi, 1997). Some compounds (PB-*like* compounds) show this same behavior, although most of them have no evident structural relationship with PB

itself or each other (Audet-Walsh et al., 2009). In human and rodents, much is known about the molecular mechanism by which PB and PB-*like* compounds induce CYPs: target genes transcriptional activation is essentially mediated by the constitutive androstane receptor (CAR, whose gene symbol is NR1I3) and the pregnane X receptor (PXR, NR1I2: Handschin and Meyer, 2003; Timsit and Negishi, 2007).

On a comparative basis, a PB-dependent transcriptional up-regulation of hepatic CYPs has been documented in pig (Puccinelli et al., 2010), rabbit (Marini et al., 2007), chicken (Goriya et al., 2005) and dog (Graham et al., 2006; Makino et al., 2009). No data are actually available for cattle. Aim of the present study was to provide a first overview about the constitutive expression of foremost PB-responsive DMEs and related xenosensors (NRs) in cattle extra-hepatic tissues; then, to evaluate the transcriptional effects of PB upon these same target genes. For this purpose, a quantitative Real Time polymerase chain reaction (qPCR) approach was chosen, and target tissues were represented by typical sites of absorption/metabolism/excretion (duodenum, lung, liver and kidney) as well as by testis, adrenal gland and muscle. Whenever a significant modulation of gene expression occurred, confirmatory post-translational investigations were executed by using the immunoblotting technique. This study is part of a project aiming to assess the transcriptional and post-translational effects of PB upon cattle DMEs, NRs and drug transporters, whose just preliminary liver post-translational data have been published thus far (Cantiello et al., 2006); nonetheless, in a wider context this study intends to increase knowledge about species-differences in drug metabolism. Data obtained might be useful either for the extrapolation of pharmacotoxicological data among the different veterinary species or the extension of veterinary drug licenses from major to minor or exotic species; the characterization of potentially and clinically relevant drug-drug interactions; finally, the evaluation of cattle and consumers' risk caused by the formation and consumption of harmful xenobiotic residues.

Materials and Methods

Chemicals and reagents

Phenobarbital sodium salt was from Sigma-Aldrich (St. Louis, MO). Chloroform, isopropyl and ethyl alcohol were obtained from Thermo Electron Corporation (Waltham, MA), whereas TRIzol® reagent and agarose from Invitrogen (Carlsbad, CA). High Capacity cDNA Reverse Transcription Kit, RNAlater® solution and Power SYBR® Green PCR Master Mix were from Applied Biosystems (Foster City, CA). Oligonucleotide primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). The Power SYBR®Green PCR Master Mix was from Applied Biosystems (Foster City,CA). The Quant-iT® Protein Assay Kit was from Invitrogen (Eugene, OR). NuPAGE® Novex® 4-12% Bis-Tris Gels and iBlot™ Gel Transfer Stack, Nitrocellulose, Mini were from Invitrogen (Eugene, OR). The rabbit anti-human CY2B6 was purchased from LifeSpan BioSciences Inc. (Seattle, WA); the rabbit anti-human CYP2C8/9/19 and anti-human UDP glucuronosyltransferase 1A1 (UGT1A1) were from Millipore (Temecula, CA); the rabbit anti-human CYP3A4, anti-human glutathione *S*-transferase alpha 1 (GSTA1) and anti-human sulfotransferase 1A1 (SULT1A1) were purchased from GeneTex Inc. (Irvine, CA). The peroxidase-conjugated goat anti-rabbit IgG and the molecular weight marker ChemiBlot™ were obtained from Millipore (Temecula, CA). The chemiluminescence kit SuperSignal West Pico Chemiluminescent Substrate was from Pierce Chemical (Rockford, IL).

Animal phase and tissues sampling

The experiment was run in an authorized facility located nearby the Faculty of Veterinary Medicine of Turin, according to the European Community Directive 86/609, recognized and

adopted by the Italian Government (DLgs 116/92). The experimental plan was approved by the Italian Ministry of Health.

Seven healthy male Friesian cattle (about 300 kg, 10 months old) were divided, on a weight basis, into two groups of three and four animals. The former one served as control (CTRL), while individuals of the second group (PHEN) were administered with PB. The barbiturate (dissolved in water) was administered by oral gavage at 18 $mg \cdot kg^{-1} \cdot body\ weight^{-1} \cdot day^{-1}$ and for 7 days. Cattle were slaughtered the day after the suspension of PB administration. After the exsanguinations step, aliquots (about 200 mg each) of liver, duodenum, kidney, lung, adrenal gland, testis and skeletal muscle were sampled, immediately snap frozen in liquid nitrogen and stored at -80°C until use.

Total RNA extraction and reverse-transcription

Total RNA was isolated by using the TRIzol® reagent and according to the manufacturer's instruction. The ribonucleic acid concentration and quality were determined by using the Nanodrop ND-1000 spectrophotometer (Labtech France, Paris, France), while its integrity was confirmed by denaturing gel electrophoresis and visualization of 18S and 28S rRNA bands. The reverse transcription of 2 μg RNA was performed by using the High Capacity cDNA Reverse Transcription Kit and random primers (in a final assay volume of 20 μL) following the purchaser's protocol.

Quantitative Real Time RT-PCR

Oligonucleotide primers of internal control genes (ICGs) and target DMEs and NRs were taken from previously published studies, except for the β2-microglobulin (B2M) and the hepatocyte nuclear factor 4 alpha (HNF4α, NR2A1), that were designed *ex novo*. Briefly, *Bos taurus* coding sequences were obtained from GenBank website [http://ncbi.nlm.nih.gov/] and

best primer pairs for amplification were chosen by using the Primer Express™ Software 3.0 (Applied Biosystems, Foster City, CA, USA). This same software as well as OligoAnalyzer 3.1 [http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx] were used to confirm the absence of primer dimers and hairpin formation. A validation of primer pairs was made to be sure that only the target gene sequence was amplified. Primers specificity was checked *in silico* by using the NCBI Nucleotide Basic Local Alignment Search Tool and Primer- Nucleotide Basic Local Alignment Search Tool [http://blast.ncbi.nlm.nih.gov/Blast.cgi]; furthermore, one of the two primers was designed spanning an exon-exon junction, to avoid amplification of potentially contaminating genomic DNA. Finally, the presence of specific amplification products was confirmed by agarose gel electrophoresis and a dissociation curve after the qPCR reaction. Primer pairs were optimized in the 300-900 nM range, to identify those primers concentration providing the highest sensitivity. Calibration curves were obtained following the amplification of decreasing amounts of different cDNA pools. For this purpose, two pools were prepared according to preliminary information on gene amplification in target tissues (data not shown). The former comprised liver, duodenum, adrenal gland, and testis; the second one kidney, lung and skeletal muscle. To avoid inaccurate results, tissues in which target genes were not constitutively expressed or, alternatively, showed mean cycle threshold (C_t) values over 30 or very close to the limit of quantification of the thermal cycler were not considered. Pooled cDNA, diluted at 3- or 4-fold intervals, were used to evaluate qPCR performances, i.e. the PCR efficiency (E_x), determined by using the equation $E_x = 10^{-1/\text{slope}}$, the assay sensitivity as well as the test of linearity correlation. Only E_x values comprised between 1.9 (90% of efficiency) and 2.1 (110% of efficiency) plus high correlation coefficients ($0.970 \leq R^2 \leq 1.000$) were considered acceptable. Primer references or oligonucleotide sequences of ICGs, target DMEs and NRs are listed in Table 1. The internal control genes here used, namely β-

actin (ACTB), B2M and ribosomal protein, large P0 (RPLP0) were chosen within a set of candidates genes that were processed by using the geNorm^{PLUS} algorithm [http://www.biogazelle.com/mybiogazelle/], and the geometric mean of their C_t values was used for data normalization.

The qPCR was performed on 5 μL of 100-fold diluted cDNA, in a final volume of 20 μL, by using the Power SYBR®Green PCR Master Mix and an ABI-Prism 7000 thermal cycler (Applied Biosystems, Foster City, CA) under standard qPCR conditions. The $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) was used to analyze data.

Immunoblotting

Subcellular fractions were isolated from CTRL and PHEN tissue aliquots by differential centrifugation according to Pegolo et al. (2010), and then stored at -80°C until analysis. Cytosolic and microsomal protein amounts were determined by using the Qubit®Fluorometer and the Quant-iT® Protein Assay Kit. Proteins (50 μg) were initially separated on NuPAGE® Novex® 4-12% Bis-Tris Gels by using the XCell SureLock™ Mini-Cell electrophoresis system (Invitrogen, Eugene, Oregon); then, transferred onto nitrocellulose filters through the iBlot™ Dry Blotting System, consisting in the iBlot™ Gel Transfer Stack, Nitrocellulose, Mini and the iBlot™ Gel Transfer Device (Invitrogen™, Eugene, Oregon). Further than CTRL and PHEN samples, a positive control (liver microsomal proteins obtained from a PB-induced rat) and a molecular marker were loaded on each minigel. Next, membranes were firstly incubated with rabbit anti-human CYP2B6 (1:1000 final dilution), CYP2C8/9/19 (1:7500), CYP3A4 (1:1500), UGT1A1 (1:3000), GSTA1 (1:1000) and SULT1A1 (1:1000) polyclonal antibodies; then, with peroxidase-conjugated goat anti-rabbit IgGs (1:6000 final dilution for membranes probed with anti-CYP2B6, CYP3A4, UGT1A1, GSTA1 and SULT1A1 antibodies, and 1:10000 for the one probed with anti-CYP2C8/9/19). Peroxide conjugates

were detected by using a chemiluminescence kit according to the manufacturer's instructions. Immunopositive bands were captured by the Photo Studio 5 version 5.0.0.53 software for the Canon CanoScan Lide 20 (LED indirect exposure) scanner, and their optical density was analyzed by the ImageJ 1.44p image analysis software.

Statistical analysis

Data on the constitutive expression of target genes in extra-hepatic tissues and their modulation by PB (relative quantification values, arithmetic means ± standard deviation, SD) were expressed as fold changes normalized to $\Delta\Delta C_t$ mean values of liver (constitutive expression) and CTRL group (PB challenge), to whom an arbitrary value of 1 was assigned. Immunoblotting densitometric data (mean arbitrary units, a.u. ± SD) were normalized to the mean value of CTRL integrated density, to whom an arbitrary value of 1.00 was assigned.

The presence of statistically significant differences in constitutive gene expression was checked by the analysis of variance (ANOVA), followed by Tukey's post test, while CTRL and PHEN gene expression and immunoblotting data were analyzed by using the Student's *t*-test (GraphPad InStat 3, San Diego, California, USA). In both cases, a $p< 0.05$ value was considered as statistically significant.

Results

Validation of qPCR assays

Quantitative real-time RT-PCR primers gave rise to specific amplicons; furthermore, a distinct dissociation peak and single bands of the expected size in agarose gel electrophoresis were obtained. Efficiency values comprised between 1.931 and 2.092 and high linear

regression coefficients ($0.972 \leq R^2 \leq 0.999$) were recorded for each qPCR assay (see Table 2).

Constitutive expression of DMEs and NRs in cattle extra-hepatic tissues

Extra-hepatic mRNA levels of target DMEs and NRs were compared with liver ones, to help the reader's understanding. Results are reported in Table 3.

Among NRs, both NR1I2 and the retinoid X receptor alpha (RXRα, NR2B1) were constitutively expressed at analyzable levels in target tissues; highest NR1I2 mRNA amounts were found in the liver ($p<0.01$ *vs* duodenum and kidney; $p<0.001$ *vs* lung, testis, adrenal gland and skeletal muscle), while the NR2B1 gene was for the most part distributed in the lung ($p<0.05$ *vs* duodenum, testis, adrenal gland and $p<0.01$ *vs* skeletal muscle) and muscle ($p<0.001$ *vs* liver, duodenum, kidney, testis and adrenal gland). The constitutive androstane receptor and NR2A1 showed a comparable pattern of gene expression. The former gene was detected in liver ($p<0.05$ *vs* duodenum), duodenum and kidney; likewise, NR2A1 was expressed in liver ($p<0.01$ respect to kidney and testis), duodenum ($p<0.001$ *vs* kidney), kidney ($p<0.001$ *vs* testis) and testis.

As expected, all target CYPs were expressed to a greater extent in the liver except for CYP2B22, for which higher mRNA levels were noticed in the lung ($p<0.001$ *vs* liver, duodenum, kidney, testis, adrenal gland and skeletal muscle). Apart from the liver, the duodenum was the tissue endowed with the larger number of CYPs (CYP2B22, 2C87, 2C88 and 3A), followed by lung (CYP2B22, 2C31 and 2C88), testis (CYP2B22, 2C31 and 3A), kidney (CYP2B22 and 2C87), adrenal gland (CYP2B22 and 2C31) and skeletal muscle (CYP2B22 only).

As regards conjugative DMEs, UGT1A1- *like* was predominantly expressed in the liver ($p<0.001$ *vs* duodenum, testis and adrenal gland). The same behavior was shown by

SULT2A1-*like* gene ($p<0.001$ *vs* all the extra-hepatic tissues). In contrast, the SULT1A1-*like* gene was by far the gene more constitutively expressed in extra-hepatic tissues and particularly in the lung, where its mRNA levels were comparable to those measured in the liver ($p<0.01$ *vs* kidney and $p<0.001$ *vs* duodenum, testis, adrenal gland and skeletal muscle). No statistically significant differences in GSTA1-*like* tissue distribution were ever noticed.

Effects of PB upon target gene mRNAs

Phenobarbital did not cause a transcriptional effect upon target NRs (see Table 4); by contrast, a modulation of a number of genes coding for oxidative and conjugative DMEs was observed in the liver and, just for SULT1A1-*like*, in the duodenum (see Figg. 1-2). No differences in candidate gene expression profiles were ever noticed between CTRL and PHEN in the remaining extra-hepatic tissues here considered (Table 5).

Hepatic CYP2B22, 2C31, 2C87 and 3A mRNA levels were significantly up-regulated by PB ($p<0.001$, $p<0.001$, $p<0.01$ and $p<0.001$, respectively). As a result, CYP2B, 2C and 3A apoprotein amounts were increased, too ($p<0.01$, $p<0.001$ and $p<0.01$, respectively: see Fig. 1).

With regard to conjugative DMEs, GSTA1- and UGT1A1-*like* mRNAs were significantly increased by PB in the liver ($p<0.05$ and $p<0.01$, respectively); on the other hand, the SULT1A1-*like* gene was significantly inhibited in duodenum ($p<0.05$). Immunoblotting investigations confirmed either the hepatic UGT1A1-*like* gene up-regulation ($p<0.01$) than the duodenal SULT1A1-*like* mRNA decrease ($p<0.05$; see Figure 2).

Discussion

Cattle is a relevant food-producing species worldwide, but the knowledge about cattle DMEs expression and their regulatory mechanisms is still incomplete and largely based on post-

transcriptional approaches, likewise to other veterinary species (Ioannides, 2006; Fink-Gremmels, 2008; Antonovic and Martinez, 2011). In the present study, the constitutive expression of PB-responsive DMEs and NRs was initially investigated in foremost cattle extrahepatic tissues, thereby increasing the knowledge on comparative drug metabolism; on a second instance, the transcriptional effects of a prototypical CYP inducer like PB were explored in these same tissues and target genes.

Nuclear receptors are ubiquitous transcription factors involved in the regulation of DMEs; mechanistically speaking, they make possible the regulation of gene expression at the transcriptional level, following the exposure to a xenobiotic or an endogenous ligand (Tolson and Wang, 2010). In this study, major interest was given to NRs that, following the exposure to PB and PB-*like* compounds, transactivate CYP2B, 2C and 3A promoters (Xu, 2005; Bell and Michalopoulos, 2006; Chen and Goldstein, 2009). If we exclude the liver, three target NRs (NR1I2, NR1I3 and NR2A1) were chiefly expressed in duodenum and kidney. In remaining extra-hepatic tissues, lower or unquantifiable mRNA amounts were noticed for NR1I2 (I~K>M>Ln>A~T), NR1I3 and NR2A1 genes. Moreover, the renal NR2A1 gene expression profile was higher than the liver one (K>Lv>I). Altogether NR1I2, NR1I3 and NR2A1 data are consistent with those previously published in humans, rodents and pig or cattle itself (Baes et al., 1994; Drewes et al., 1996; Zhang et al., 1999; Jiang et al., 2003; Nishimura et al., 2004; Bell and Michalopoulos, 2006; Nannelli et al., 2008; Xu et al., 2009; Lopparelli et al., 2010), even though species- and tissue-differences in the extra-hepatic constitutive expression of NR1I3 and NR2A1 have been noticed, too (Chirulli et al., 2005; Dean et al., 2010). In contrast, the heterodimerizing partner NR2B1 was undoubtedly more expressed in cattle extra-hepatic tissues, and higher mRNA amounts were observed in skeletal muscle and lung (M>Ln>Lv>K>I~T~A). If the large interindividual variation

noticed in the lung prevent us to make further consideration, skeletal muscle data substantially agree with human ones (Nishimura et al., 2004).

Extra-hepatic CYPs are usually associated with a specific physiological role, but their modulation may affect the local disposition of xenobiotic or endogenous compounds, thus altering their effects (Seliskar and Rozman, 2007; Pavek and Dvorak, 2008). The cytochrome P450 2B is constitutively expressed and inducible in human liver and extra-hepatic tissues (Nishimura et al., 2003; Bièche et al., 2007). In cattle, CYP2B22 was mostly expressed in lung and liver, followed by duodenum and kidney; limited amounts were noticed in muscle, adrenal and testis (Ln>Lv>I~K>M~T~A). Moreover, pulmonary CYP2B22 mRNA levels were about 20-fold higher than the liver ones. Such a result was not astonishing. Previous comparative data indicate CYP2B as the most abundant CYP isoform in pulmonary tissue of mammals, whose amounts are equal or even higher the hepatic ones (Hukkanen et al., 2001; Chirulli et al., 2005; Bièche et al., 2007; Nannelli et al., 2008; Darwish et al., 2010). Concerning CYP2B22 expression in the other extra-hepatic tissues subject of investigation, present data agree with those previously reported in humans (Nishimura et al., 2003; Bièche et al., 2007) and, albeit limited to kidney and testis, in cattle (Darwish et al., 2010; Lopparelli et al., 2010). Four CYP2C isoforms (CYP2C8, 2C9, 2C18, and 2C19) have been described in humans, and these ones are expressed to a variable extent in gut, kidney, testis, muscle, brain, and cardiovascular tissues, while contradictory results have been published for the lung (Klose et al., 1999; Ferguson et al., 2002; Nishimura et al., 2003; Bergheim et al., 2005; Bièche et al., 2007; Chen and Goldstein, 2009). Cattle CYP2C31, 2C87 and 2C88 (corresponding to human CYP2C19, 2C9, and 2C18, respectively: Zancanella et al., 2010) were basically found in the liver, likewise to humans. On the other hand, conflicting results were obtained at the extra-hepatic level. The cytochrome P450 2C87 gene was detected only in duodenum and kidney (K>I), and this finding only partially agree with human data. In fact,

CYP2C9 is for the most part expressed in intestine and kidney (I>K), but also in skeletal muscle, adrenal, and testis (Klose et al., 1999; Nishimura et al., 2003; Bièche et al., 2007). As regards CYP2C31 and 2C88, the former gene was primarily expressed in testis, lung and adrenal (T>Ln>A), the second one merely in lung and duodenum (Ln>I). By contrast, CYP2C18 and 2C19 are mostly distributed in the gut and, to a lower extent, in kidney, lung, muscle, adrenal and testis (Nishimura et al., 2003; Bièche et al., 2007). Nevertheless, CYP2Cs exhibit species-differences in their extra-hepatic pattern of gene expression; furthermore, contrasting results about the extra-hepatic distribution of human CYP2Cs have been published; finally, it has been recently shown that human CYP2Cs are not orthologous to the corresponding cattle sequences (Klose et al., 1999; Tsao et al., 2001; Nishimura et al., 2003; Bièche et al., 2007; Chen and Goldstein, 2009; Zancanella et al., 2010). To date, four human CYP3A genes (CYP3A4, 3A5, 3A7 and 3A43) have been identified. The former one represents the most abundant CYP3A isoenzyme in liver and small intestine (Burk and Wojnowski, 2004; Thelen and Dressman, 2009); on the contrary, higher CYP3A5 and 3A43 amounts may be found in some extra-hepatic tissues, even if genetic interindividual variations may affect CYP3A gene expression at the extra-hepatic level (Burk and Wojnowski 2004; Daly, 2006).

Cattle CYP3A gene was typically distributed in liver, duodenum and testis, in line with human and preceding (albeit limited to the gonad) cattle data (Burk and Wojnowski, 2004; Thelen and Dressman, 2009; Lopparelli et al., 2010). No detectable CYP3A mRNA amounts were ever found in kidney, lung and adrenal. These findings disagree with the few studies about CYP3A expression in lung (Larsson et al., 2003; Raunio et al., 2005), kidney (Wojnowski, 2004; Bièche et al., 2007; DiMaio Knych et al., 2010) and adrenal gland (Nishimura et al., 2003; Bièche et al., 2007; Nishimura et al., 2009; Shang et al., 2009; Uno et al., 2009). As a whole, definitive conclusions cannot still be drawn for cattle.

Glucuronidation represents the primary phase II reaction and an essential detoxification pathway in humans. Most of UGTs are expressed in the liver; in contrast, some isoforms (e.g., UGT1A7, 1A8 and 1A10) are exclusively distributed in extra-hepatic tissues, particularly in the gut (Mohamed and Frye, 2011). In human and rat, the UGT1A1 gene is expressed to a greater extent in tissues contributing either to xenobiotic first pass metabolism or excretion (liver, intestine and kidney, respectively: Fisher et al., 2000; Shelby et al., 2003; Gregory et al., 2004; Nishimura and Naito, 2006; Nakamura et al., 2008; Bellemare et al., 2011). Cattle UGT1A1-*like* gene was mainly expressed in the liver and, to a lower extent, in duodenum, testis and adrenal. No UGT1A1-*like* mRNA was detected in the kidney, in which another UGT1A isoform (UGT1A6) has been shown to be strongly expressed (Iwano et al., 2001). Collectively, cattle UGTs tissue distribution agree with the aforementioned comparative data.

Glutathione transferases catalyze the conjugation of glutathione to electrophilic metabolites, usually yielding a product with a decreased reactivity (Forkert et al., 1999). According to previous published data (Rowe et al., 1997; Nishimura and Naito, 2006; Darwish et al., 2010; Lopparelli et al., 2011). Cattle GSTA1-*like* gene was mostly expressed in the liver and, to a lower degree, in extra-hepatic tissues (K>Ln>I~T~A~M). Present data would confirm human ones (Rowe et al., 1997; Nishimura and Naito, 2006), but disagree with those recently published in cattle and concerning kidney and testis, in which GSTA1-*like* mRNA amounts higher than liver ones were detected (Darwish et al., 2010; Lopparelli et al., 2011). The sulphotransferase superfamily consists of five families of conjugative DMEs (SULT1-5: Strott, 2002). The sulfotransferase 1A1 gene is primarily expressed in the liver, while species-differences in extra-hepatic tissue distribution have been noticed; overall, gastrointestinal tract (small intestine or colon), lung and kidney express SULT1A1 (Dunn and Klaassen, 1998; Tsoi et al., 2002; Alnouti and Klaassen, 2006; Riches et al., 2009). In cattle,

the SULT1A1-*like* gene was distributed in liver and target extra-hepatic tissues, with highest amounts observed in lung and liver (Ln>Lv>K>A~I~M>T). Present data would suggest that lung and kidney are involved in cattle sulfation pathway, likewise to other species; further studies are needed to clarify the SULT1A1-*like* gene expression in gut. The sulfotransferase 2A1-*like* gene was constitutively expressed either in liver than in extra-hepatic tissues (Lv>K>Ln>I~T~A~M). Present results corroborate data obtained in human and rodents (Alnouti and Klaassen, 2006; Riches et al., 2009).

Collectively, the tissue distribution of known PB-responsive DMEs and NRs was investigated, for the first time, in cattle extra-hepatic tissues. Gene abundances were, with few exceptions, lower than liver ones. On a second instance, the constitutive expression of target genes mirrored that of human and/or rodents, although some potential species-differences were noticed.

The term induction denotes a dose-dependent increase in DMEs expression (gene/protein) and function (catalytic activity). Phenobarbital and PB-*like* compounds induce a number of hepatic DMEs, and the up-regulation of target gene transcription is mostly driven by NR1I3, NR1I2 or maybe NR2A1, although such a xenobiotic-dependent NR activation in different animal species is still subject of debate (Pustylnyak et al., 2005; Bell and Michalopoulos, 2006; Kodama and Negishi, 2006; Yamada et al., 2006; Audet-Walsh et al., 2009; Tamasi et al., 2009). Phenobarbital induction occurs in many species, from bacteria to humans. In vertebrates, induction largely takes place in liver, intestine and, to a lower extent, in other extra-hepatic tissues (i.e., kidney and lung: Kakizaki et al., 2003; Denison and Whitlock, 1995; Goriya et al., 2005). Actually, few papers about the *in vivo* PB-dependent transcriptional up-regulation of DMEs in veterinary species have been published, and none in cattle (van't Klooster et al., 1993; Chirulli et al., 2005; Goriya et al., 2005; Marini et al., 2007; Makino et al., 2009).

In the present study PB never affected target NRs gene expression both in liver and extra-hepatic tissues, even though it has been hypothesized that CYP2B induction is strictly related to the amount of NR1I3 mRNA present in liver and extra-hepatic tissues (Pustylnyak et al., 2009). Nevertheless, these results were not astonishing. Species-differences in CYP2B response to PB and some PB-*like* compounds may occur, basically through variations in NR ligand-binding domain sequences or differences in NR1I3/NR1I2-dependent transactivation of target genes (Kiyosawa et al., 2008; Pustylnyak et al., 2009; Kojima et al., 2011). Likewise, the presence of tissue-differences in DMEs regulation has been hypothesized (Marini et al., 2007). Finally, dose- and time-dependent effects of PB upon NR1I3 and NR1I2 mRNAs have been observed (Lambert et al., 2009; Zheng et al., 2011); in particular, while NR1I3 mRNA increases with PB concentration, NR1I2 gene expression is unchanged, even at high barbiturate concentrations (Lambert et al., 2009). Similarly, species- and dose-dependent differences in NR1I2/NR1I3 transactivation and consequent CYP3A induction have been observed in the presence of different CYP3A inducers such as dexamethasone, rifampicin and pregnenolone-16alpha-carbonitrile (Burk and Wojnowski, 2004; Luo et al., 2004; Kojima et al., 2011). Interestingly, a similar behavior (no effect upon NR1I2, 1I3, 2A1 and 2B1 genes) was noticed in veal calves administered with growth promoting dosages of dexamethasone (Cantiello et al., 2009). Collectively, previous and present results suggest that basic regulatory mechanisms by which NR ligands transactivate cattle NR1I3/NR1I2/NR2A1/NR2B1 genes network and increase the transcription of target genes need further basic and applied clarifying molecular studies.

Phenobarbital induces hepatic CYP genes in human and rodents (Joannard et al., 2000; Wagner et al., 2005; Pelkonen et al., 2008) as well as in rabbit (Chirulli et al., 2005), goat (van't Klooster et al., 1993), pig (Puccinelli et al., 2010), chicken (Goriya et al., 2005) and dog (Makino et al., 2009). As regards cattle, just preliminary post-translational data have

been published (Cantiello et al., 2006). In the current study, PB significantly up-regulated hepatic CYP2B22 (about 90-fold the CTRL value), 2C31, 2C87 and 3A, likewise to humans and rodents (Waxman and Azaroff, 1992; Handschin and Meyer, 2003). The transcriptional effect upon target CYPs was reflected at the protein level, as demonstrated by the significant augmentation of CYP2B, 2C and 3A apoprotein amounts. Moreover, catalytic activity assays confirmed present gene/protein results (Cantiello et al., 2006).

Conjugative DMEs may be induced by PB, and UGT1A1 is perhaps the most important one. Phenobarbital up-regulates the hepatic UGT1A1 gene in humans and rodents (Smith et al., 2005; Olinga et al., 2008; Buckley and Klaassen 2009), and such an effect occurs through the same transcriptional pathway (NR1I3 activation: Sugatani et al., 2001). Likewise, PB significantly increased hepatic UGT1A1-*like* mRNA and apoprotein amount. A similar behavior was noticed for GSTA1-*like*, for which a significant mRNA increase (two-fold the CTRL value) was noticed and confirmed, albeit not significantly, at the protein level. Overall, these findings agree with preceding rodent data (Langouet et al., 1996; Bulera et al., 2001; Ejiri et al., 2005), even though it should be here mentioned that present UGT1A1-*like* and GSTA1-*like* data did not find confirmation at the catalytic activity level (Cantiello et al., 2006). On the other hand, hepatic SULTs were unaffected by PB. In general, murine SULT1A1 and 2A1/2 mRNA expression is not affected by PB or PB-*like* compounds (Alnouti and Klaassen, 2008), while diverse PB dosages (35, 80 and 100 mg/kg for 3 days) elicit a dose-dependent inhibitory effect upon SULT1/2 transcription in the rat (Runge-Morris et al., 1998). Thus, present transcriptional results and the post-translational inhibitory trend noticed by using an anti-rat SULT1A1 antibody (data not shown) would be consistent with abovementioned rodent data.

Above and beyond liver, PB increases DMEs gene transcription in the intestine and, though to a lower extent, in other extra-hepatic tissues (Denison and Whitlock, 1995;

Kakizaki et al., 2003; Goriya et al., 2005; van de Kerkhof et al., 2008). In cattle duodenum, a general inhibitory trend was noticed, reaching the level of statistical significance merely for SULT1A1-*like*; moreover, such a finding was confirmed at the protein level. Therefore, duodenal SULT1A1-*like* results match with those obtained in the liver. As far as the other target genes is concerned, most of studies about the *in vivo/in vitro* transcriptional effects of PB upon the gastrointestinal tract describe a common CYP and UGT genes up-regulation, although some variations/exceptions attributable either to the species or the barbiturate dosage regimen have been reported (Zhang et al., 2003; Martignoni et al., 2004; Shelby and Klaassen, 2006; Martin et al., 2008; van de Kerkhof et al., 2007; van de Kerkhof et al., 2008). To the best of our knowledge, few data about PB transcriptional effects on GSTs and SULTs have been published, but human GSTA1 gene expression is down-regulated following the *in vitro* exposure (24 hours) to 5mM PB (Romero et al., 2006).

Phenobarbital did not elicit transcriptional effects in the other extra-hepatic tissues subject of investigation. Few comparative data about PB effects on target gene mRNA levels in kidney and lung have been published, while no information are actually available for testis, adrenal and skeletal muscle. What's more, these information usually refer to CYPs. As a whole, present results are consistent with published data. Phenobarbital did not affect CYP2B gene expression in human and mouse kidney (Jarukamjorn et al., 2001; Kakizaki et al., 2003). Concerning the lung, rat and rabbit pulmonary CYP2Bs did not respond to PB, even though NR1I2 and NR1I3 were expressed in both species (Lee et al., 1998; Skarin et al., 1999; Chirulli et al., 2005); likewise, mouse pulmonary CYP2B gene and protein were not modulated by PB, but measurable amounts of NR1I3 mRNA were never detected in this species (Pustylnyak et al., 2009).

Collectively, present data suggest that PB, orally administered in cattle by using a prototypical induction protocol, increases the transcription of six out of nine PB-responsive

genes coding for DMEs (CYP2B22, 2C31, 2C87 and 3A, UGT1A1-*like* and GSTA1-*like*) in the liver; what's more, such an effect was confirmed at the post-translational level. On the other hand, duodenum was the only extra-hepatic tissue in which PB elicited an effect, just consisting in a down-regulation of SULT1A1-*like* mRNA and protein amounts. Finally, no transcriptional effects of PB on NRs gene expression were ever noticed. In the majority of cases, present transcriptional results agree with previously published comparative ones, but some contradictory results need further molecular investigations.

Conclusions

In the present study, the constitutive expression and tissue distribution of a number of PB and PB-*like* compounds responsive genes was investigated in cattle liver and extra-hepatic tissues. Likewise to humans and rodents, these genes were shown to be expressed to a lower extent compared to the liver with few exceptions, i.e. NR2A1 in kidney, NR2B1, CYP2B22 and SULT1A1-*like* in lung and NR2B1 in skeletal muscle. Phenobarbital is a prototypical DME inducer, whose transcriptional and post-translational effects in humans and rodents occur through NRs transactivation. Following cattle exposure to an oral PB inducing protocol, hepatic CYP2B22, 2C31, 2C87, 3A as well as UGT1A1-, GSTA1- and SULT1A1-*like* mRNAs and protein amounts were significantly modulated; among target extra-hepatic tissues, only the duodenum showed a significant down-regulation of both SULT1A1-*like* mRNA and corresponding apoprotein. Collectively, present data are suggestive of possible species-differences in DMEs expression, regulation and function between cattle, humans and laboratory species, whose relevance needs to be better understood by using basic molecular investigations, particularly addressed to define the molecular nature of PB-induction response and its tissue-specific control in this important species of veterinary interest. Looking at the bigger picture, this study improves knowledge about drug metabolism in veterinary species, a

subject of investigation always considered of lesser importance compared with that of humans and rodents. This will be useful for veterinary pharmaco-toxicologists, cattle practitioners and, ultimately, for consumers. In fact, data here obtained might be useful for the extrapolation of pharmaco-toxicological data among the different veterinary species or the extension from veterinary drug licenses from major to minor or exotic species; the development of new drugs tailored to reach a target tissue and exert their action; the characterization of potentially clinically relevant drug-drug interactions; and, finally, the evaluation of cattle and consumers' risk caused by the formation and consumption of harmful xenobiotic residues.

Acknowledgements

This work was funded by grants from Università degli Studi di Padova ("Studi biomolecolari sull'espressione degli enzimi biotrasformativi epatici in bovini trattati con fenobarbitale", 60A08-8818/08 and 60A08-5793/09) and Ministero della Salute ("Genesi del boldenone in urina di vitello. Sviluppi e applicazioni inerenti la ricerca del residuo", IZSPLV009/01) to M.D. and C.N., respectively, as well as a PhD fellowship from Università degli Studi di Padova to V.Z.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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Figure captions

Figure 1. Hepatic CYP2B (A), 2C (B) and 3A (C) qPCR and immunoblotting. Data (mean arbitrary units, a.u. ± SD) from PHEN group were normalized to the $\Delta\Delta C_t$ (qPCR) and the integrated density (immunoblotting) mean values of CTRL, to whom an arbitrary value of 1.00 was assigned.

**: significant differences ($p<0.01$) *vs* CTRL (Student's *t*-test).

***: significant differences ($p<0.001$) *vs* CTRL (Student's *t*-test).

CYP, cytochrome P450; CTRL, control group; PHEN, animals orally administered with PB.

Figure 2. Hepatic GSTA1-*like* (A), UGT1A1-*like* (B) and small intestinal SULT1A1-*like* (C) qPCR and immunoblotting. Data (mean arbitrary units, a.u. ± SD) from PHEN group were normalized to the $\Delta\Delta C_t$ (qPCR) and the integrated density (immunoblotting) mean values of CTRL, to whom an arbitrary value of 1.00 was assigned.

*: significant differences ($p<0.05$) *vs* CTRL (Student's *t*-test).

**: significant differences ($p<0.01$) *vs* CTRL (Student's *t*-test).

GSTA1, glutathione *S*-transferase alpha 1; UGT1A1, UDP glucuronosyltransferase 1A1; SULT1A1, sulfotransferase 1A1. Lv, liver; I, small intestine; CTRL, control group; PHEN, animals orally administered with PB.

Table 1. GenBank accession numbers, oligonucleotide sequences or references of primers used for qPCR, and amplicon size.

gene acronym	GenBank ID	primer reference or sequence (5' → 3')	amplicon size (bp)
NR1I2	NM_001103226	Cantiello et al. 2009	68
NR1I3	NM_001079768	Cantiello et al. 2009	63
NR2A1	NM_001015557	*forward*: 5'-TTGACGATGGGCAATGACACA-3' *reverse*: 5'-GCACTGACACCCAGGCTGTT-3'	74
NR2B1	XM_881943	Cantiello et al. 2009	120
CYP2B22	NM_001075173	Giantin et al. 2008	80
CYP2C31	XM_600421	Giantin et al. 2008	56
CYP2C87	XM_612374	Giantin et al. 2008	71

CYP2C88	NM_001076051	Giantin et al. 2008	51
CYP3A	NM_174531	Cantiello et al. 2009	77
UGT1A1-*like*	NM_001105636	Giantin et al. 2008	71
GSTA1-*like*	NM_001078149	Giantin et al. 2008	84
SULT1A1-*like*	NM_177521	Lopparelli et al. 2010	84
SULT2A1-*like*	NM 001046353	Lopparelli et al. 2011	81
ACTB	NM_173979	Toffolatti et al. 2006	84
RPLP0	NM_001012682	Robinson et al. 2007	227
B2M	NM_173893	*forward*: 5'-TCGTGGCCTTGGTCCTTCT-3'	71

reverse: 5’-AATCTTTGGAGGACGCTGGAT-3’

Table 2. Main features, namely slope, efficiency (E_x), linear regression coefficients (R^2) and linear dynamic range (cycle threshold, C_t), of each qPCR assays, measured by using two different tissue pools (liver, intestine, adrenal gland and testis; kidney, lung and skeletal muscle). Tissues in which target genes were not constitutively expressed or showed C_t values over 30 (or close to thermal cycler limit of quantification) were not taken into consideration.

Lv, liver; I, small intestine; K, kidney; L, lung; T, testis; A, adrenal; M, muscle.

assay	cDNA pool	slope	E_x	R^2	linearity range (C_t)
NR1I2	Lv. I. T. A.	-3.25	2.031	0.998	24.35 – 34.29
	Ln. K. M.	-3.12	2.092	0.972	29.29 – 33.04
NR1I3	Lv. I.	-3.30	2.009	0.996	25.43 – 33.31
	K.	-3.27	2.022	0.998	27.68 – 33.66
NR2B1	Lv. I. T. A.	-3.30	2.009	0.994	23.90 – 36.00
	Ln. K. M.	-3.29	2.013	0.986	24.61 – 30.47

NR2A1	Lv. I. T. A.	-3.33	1.997	0.988	23.97 – 30.1
	Ln. K. M.	-3.48	1.938	0.990	25.84 – 34.15
CYP2B22	Lv. I. T. A.	-3.40	1.968	0.999	19.98 – 32.21
	Ln. K. M.	-3.35	1.988	0.996	23.50 – 33.30
CYP2C31	Lv. I. T. A.	-3.25	2.031	0.992	23.26 – 31.04
	Ln.	-3.32	2.001	0.995	28.53 – 32.52
CYP2C87	Lv. I. T.	-3.35	1.988	0.982	23.36 – 31.68
CYP2C88	Lv. I. A.	-3.42	1.961	0.995	23.85 – 32.17
	Ln.	-3.16	2.072	0.987	28.36 – 36.19
CYP3A	Lv. I. T.	-3.50	1.931	0.988	20.08 – 30.35

UGT1A1-*like*	Lv. I. T. A.	-3.30	2.009	0.990	24.10 – 34.11
GSTA1-*like*	Lv. I. T. A.	-3.33	1.997	0.993	19.70 – 29.82
	Ln. K. M.	-3.28	2.018	0.996	24.09 – 32.04
SULT1A1-*like*	Lv. I. T. A.	-3.35	1.988	0.998	21.45 – 33.68
	Ln. K. M.	-3.21	2.049	0.993	24.42 – 30.16
SULT2A1-*like*	Lv. I. T. A.	-3.21	2.049	0.998	23.34 – 32.96
	Ln. K. M.	-3.15	2.077	0.993	28.15 – 31.95
ACTB	Lv. I. T. A.	-3.39	1.972	0.993	18.2 – 26.17
	Ln. K. M.	-3.51	92.7	0.995	23.24 – 28.45
RPLP0	Lv. I. T. A.	-3.35	98.8	0.990	19.61 – 26.76
	Ln. K. M.	-3.28	101.8	0.987	21.53 – 28.46

B2M	Lv. I. T. A.	-3.32	100.1	0.993	18.61 – 25.07
	Ln. K. M.	-3.32	100.1	0.985	23.36 – 29.43

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Table 3. Nuclear receptors, CYPs and conjugative DMEs mRNA relative abundances (arbitrary units, a.u.) in liver and small intestine of cattle exposed to PB induction protocol. Data (arithmetic means ± S.D.) are expressed as n–fold change (normalized to $\Delta\Delta C_t$ mean value of the respective control group to whom an arbitrary value of 1 was assigned). D.N.Q., detected but not quantifiable; D.N., not detected.

*,**,***: significant differences (p<0.05, 0.01 and 0.001) *vs* CTRL (Student's *t*-test)

	n-fold change (a.u.) ± S.D.			
Target gene	Liver		Small intestine	
	CTRL	PHEN	CTRL	PHEN
NR1I2	1.00 ± 0.46	0.99 ± 0.09	1.00 ± 0.24	0.73 ± 0.29
NR1I3	1.00 ± 0.44	0.90 ± 0.14	1.00 ± 1.05	0.37 ± 0.26
NR2A1	1.00 ± 0.28	1.19 ± 0.11	1.00 ± 0.03	0.84 ± 0.35
NR2B1	1.00 ± 0.17	1.02 ± 0.16	1.00 ± 0.10	0.76 ± 0.20

CYP2B22	1.00 ± 0.78	90.51 ± 19.66***	1.00 ± 0.51	0.53 ± 0.36
CYP2C31	1.00 ± 0.58	6.03 ± 0.99***	D.N.Q.	
CYP2C87	1.00 ± 0.57	3.73 ± 0.59**	1.00 ± 0.51	0.60 ± 0.20
CYP2C88	1.00 ± 0.43	1.29 ± 0.40	1.00 ± 0.43	1.17 ± 0.82
CYP3A	1.00 ± 0.25	2.67 ± 0.29***	1.00 ± 0.68	0.29 ± 0.25
UGT1A1-*like*	1.00 ± 0.22	1.62 ± 0.16**	1.00 ± 0.27	1.12 ± 0.71
SULT1A1-*like*	1.00 ± 0.25	0.71 ± 0.21	1.00 ± 0.17	0.65 ± 0.12*
SULT2A1-*like*	1.00 ± 0.34	1.59 ± 0.55	1.00 ± 1.10	0.61 ± 0.34
GSTA1-*like*	1.00 ± 0.42	2.75 ± 0.94*	1.00 ± 0.83	1.05 ± 1.24

Figure 1

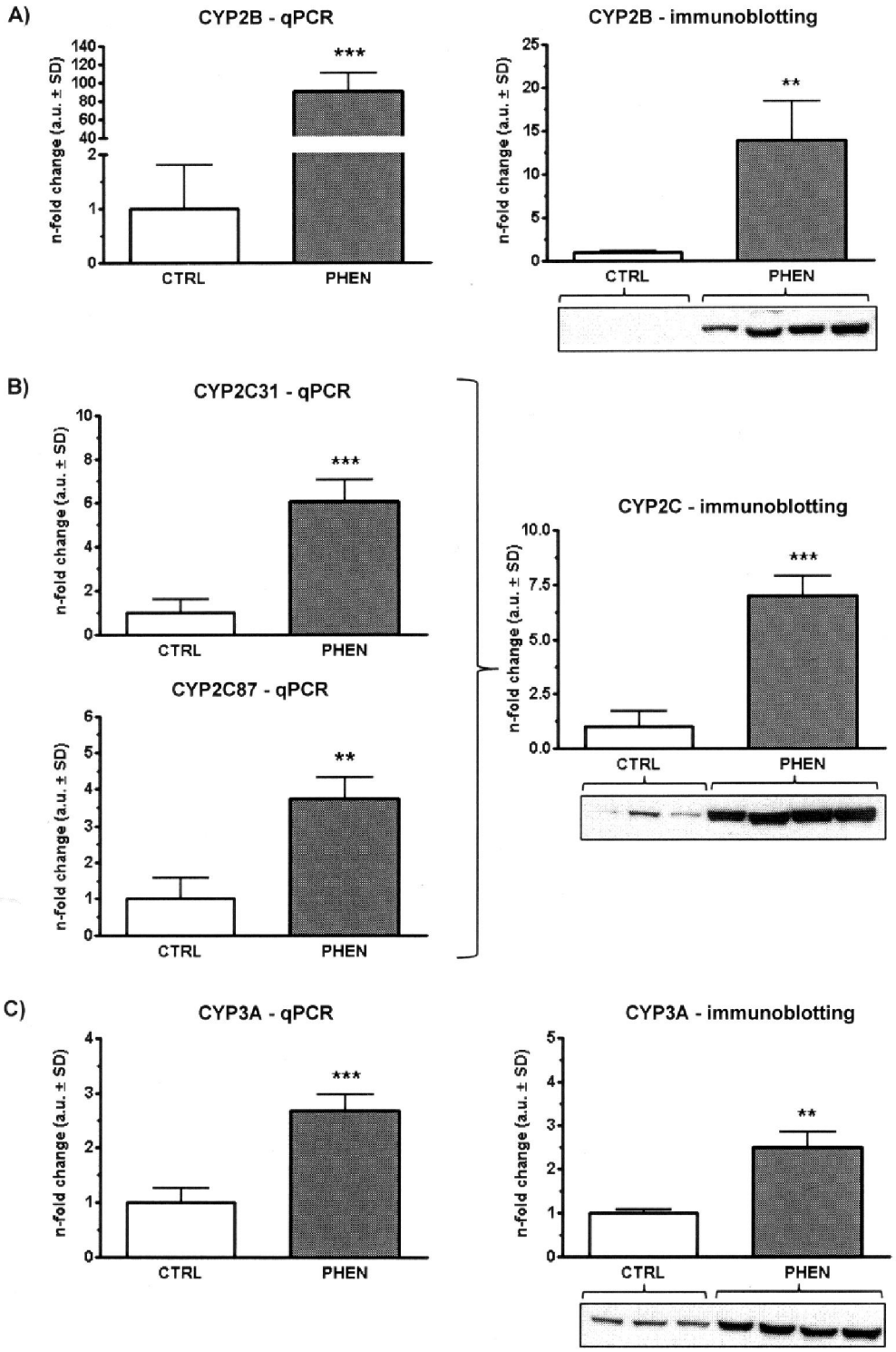
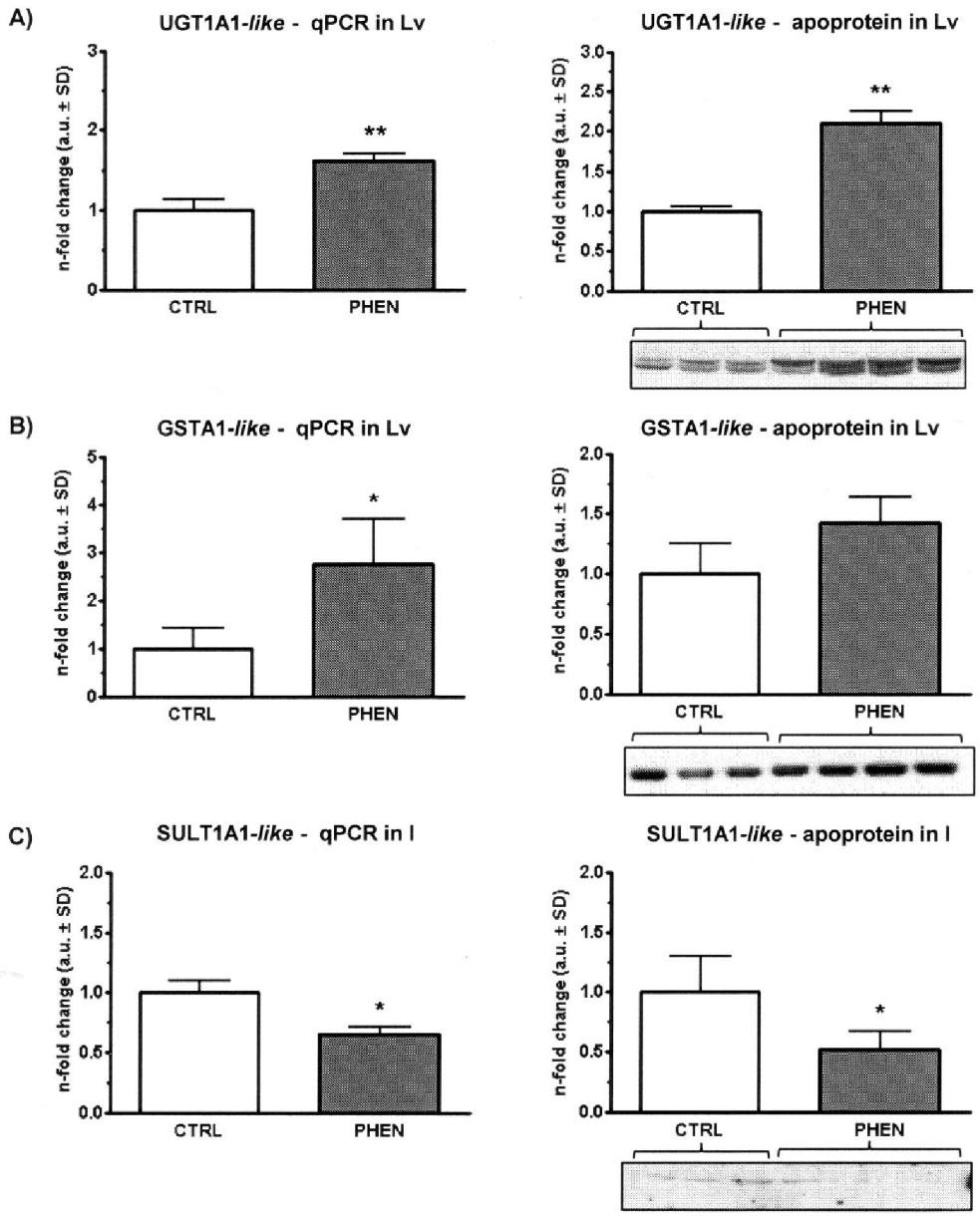

A)
CYP2B - qPCR
n-fold change (a.u. ± SD)
***
CTRL
PHEN
CYP2B - immunoblotting
n-fold change (a.u. ± SD)
**
CTRL
PHEN
B)
CYP2C31 - qPCR
n-fold change (a.u. ± SD)
***
CTRL
PHEN
CYP2C87 - qPCR
n-fold change (a.u. ± SD)
**
CTRL
PHEN
CYP2C - immunoblotting
n-fold change (a.u. ± SD)
***
CTRL
PHEN
C)
CYP3A - qPCR
n-fold change (a.u. ± SD)
***
CTRL
PHEN
CYP3A - immunoblotting
n-fold change (a.u. ± SD)
**
CTRL
PHEN

Figure 2


A)
UGT1A1-*like* - qPCR in Lv
n-fold change (a.u. ± SD)
CTRL
PHEN
UGT1A1-*like* - apoprotein in Lv
B)
GSTA1-*like* - qPCR in Lv
GSTA1-*like* - apoprotein in Lv
C)
SULT1A1-*like* - qPCR in I
SULT1A1-*like* - apoprotein in I