Effect of Breed upon Cytochromes P450 and Phase II Enzyme Expression in Cattle Liver

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ABSTRACT:

Cattle represent an important source of animal-derived food-products; nonetheless, our knowledge about the expression of drug-metabolizing enzymes (DMEs) in present and other food-producing animals still remains superficial, despite the obvious toxicological consequences. Breed represents an internal factor that modulates DME expression and catalytic activity. In the present work, the effect of breed upon relevant phase I and phase II DMEs was investigated at the pretranscriptional and post-translational levels in male Charolais (CH), Piedmontese (PM) and Blonde d’Aquitaine (BA) cattle. Because specific substrates for cattle have not yet been identified, the breed effect upon specific cytochrome P450 (P450), UDP-glucuronosyltransferase (UGT), or glutathione S-transferase (GST) DMEs, in terms of catalytic activity, was determined by using human marker substrates. Among P450s, benzphetamine N-demethylase, 16β-, 6β-, and 2β-testosterone hydroxylase, aniline and p-nitrophenol hydroxylase, and α-naphthol and p-nitrophenol UGT activities were significantly higher in CH; in contrast, lower levels of CYP1A1-, CYP1A2-, CYP2B6-, CYP2C9-, CYP2C18-, CYP3A4-, and UGT1A1-like mRNAs were noticed, with CH < PM ≤ BA as a trend. CYP2B and CYP3A mRNA results were confirmed with immunoblotting, too. As regards conjugative DMEs, UGT1A6-like mRNA levels were consistent with respective catalytic activities. Both 1-chloro-2,4-dinitrobenzene and 3,4-dichloronitrobenzene GST activities were higher in BA, and these results agreed with GSTA1-, GSTM1-, and GSTP1-like mRNA amounts. Correlation analysis between catalytic activities and mRNAs showed either significant or uneven results, depending on the substrate. These findings confirm previous data obtained in laboratory species; however, further studies are required to ascribe this behavior to pretranscriptional or post-translational phenomena.

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ABBREVIATIONS: DME, drug-metabolizing enzyme; P450, cytochrome P450; PM, Piedmontese; CH, Charolais; BA, Blonde d’Aquitaine; Q RT-PCR, quantitative real-time polymerase chain reaction; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; TST, testosterone; 2βOH-T, 2β-testosterone hydroxylase; 6βOH-T, 6β-testosterone hydroxylase; 16βOH-T, 16β-testosterone hydroxylase; EROD, ethoxyresorufin O-deethylase; ETR, ethoxyresorufin; UGT, UDP-glucuronosyltransferase; GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 3,4-dichloronitrobenzene; GSH, glutathione; ANOVA, analysis of variance; BENZDEM, benzphetamine N-demethylase; TBT4-OH, tolbutamide 4-hydroxylase; Aniline-OH, aniline hydroxylase; pNP-OH, p-nitrophenol hydroxylase; ETDEM, ethylmorphine N-demethylase; ERDEM, erythromycin N-demethylase; TAODEM, triacetyloleandomycin N-demethylase; a.u., arbitrary unit(s).
Cattle represent one of the major economically important veterinary species worldwide. A few studies, mostly addressed to investigate the post-translational effects of species, age, and gender (some of the constitutional factors influencing the overall biotransformation capability; Nebbia, 2001), upon the cattle liver DMEs have been published in the past decade (Machala et al., 2003; Nebbia et al., 2003; Sivapathasundaram et al., 2003b; Sztóková et al., 2004; Dacasto et al., 2005; Gusson et al., 2006). In contrast, only recently, DME expression and regulation have begun to be studied at the gene expression level (Gereger et al., 2006).

In one of aforementioned studies, breed differences in the hepatic CYP3A apoprotein and CYP3A-dependent catalytic activities have been reported among Limousine and Piedmontese (PM) cattle (Dacasto et al., 2005). In the present one, the effect of breed upon the most relevant phase I and phase II DMEs has been investigated both at the pretranscriptional and/or post-transcriptional level, in two French [Charolais (CH) and Blonde d’Aquitaine (BA)] and one Italian (PM) meat cattle breeds.

Since in cattle, as well as in most veterinary species, the definition of substrates that are specifically metabolized by a P450 isoform or a conjugative DME is still debatable, catalytic activities were measured by using probes whose selectivity toward each DME had been previously established in rat and human species. Likewise, commercially available antibodies, raised toward human and rat antigens, were used for CYP1A, 2B, 2C, 2E, and 3A immunoblotting; in fact, to the best of the authors’ knowledge, mono- or polyclonal antibodies directed to cattle antigens have not yet produced so far. In effect, such an approach has been already adopted, at least partly, in prior studies where cattle liver drug metabolism was compared with that of other ruminants, other farm animals, or reference species (i.e., the rat) as well (Sivapathasundaram et al., 2001, 2003a,b; Machala et al., 2003; Nebbia et al., 2003, 2004; Sztóková et al., 2004; Dacasto et al., 2005; Gusson et al., 2006; Ioannides, 2006). On the contrary, bovine-specific primer pairs were designed for P450s and phase II enzyme isoform mRNA to be used in the relative quantification by means of quantitative real-time polymerase chain reaction (Q RT-PCR). In fact, the entire bovine genome has been recently sequenced but a definitive nomenclature for bovine P450s as well as phase II DMEs, is still actually lacking. For this reason, in the present study, bovine DME sequences were identified with the name of the human sequence sharing the highest percentage of identity, based on protein sequence alignments, followed by the suffix -like. Finally, a correlation analysis between each gene expression profile and the relative supposed catalytic activity was performed in an attempt to define the bovine P450 or phase II enzyme mostly involved in the metabolism of chosen model substrates.

**Materials and Methods**

**Chemicals and Reagents.** Bovine serum albumin, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP+, and 4-aminophenol were from Boehringer Ingelheim GmbH (Ingelheim, Germany). All other reagents used for the estimation of catalytic activities were obtained from Sigma-Aldrich (St. Louis, MO), except for methanol, acetoneitrile, NADPH, and glacial [high-performance liquid chromatography (HPLC) grade] acetic acid (from Mallinkrodt-Baker, Milan, Italy) and ammonium acetate and hydrogen chloride (from Fluka, Buchs, Switzerland). The MilliQ Synthesis water purification system (Millipore Corporation, Billerica, MA) provided purified deionized water. The goat anti-rabbit CYP1A1/2 polyclonal antibody was from Oxford Biomedical Research (Oxford, MI), the goat anti-rat CYP2B1/2 polyclonal antibody was from Daiichi Pure Chemicals Co. (Tokyo, Japan), the anti-rat CYP3A1/2 monoclonal antibody was a kind gift of H.V. Gelboin (National Institutes of Health and Cancer, Bethesda, MD), and rabbit anti-human CYP2C8/9/19 and rat CYP2C12 as well as sheep anti-human and rat CYP2E1 polyclonal antibodies were from Millipore Bioscience Research Reagents (Temecula, CA). Rabbit anti-goat and donkey anti-sheep peroxidase-conjugated IgGs were purchased from Sigma-Aldrich; donkey anti-rabbit and sheep anti-mouse peroxidase-conjugated IgGs were from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). All the other immunoblotting reagents were of molecular biology grade. Chloroform, isopropyl alcohol, and ethyl alcohol were obtained from Thermo Electron Corporation (Waltham, MA), whereas TRIZol reagent and agarose were from Invitrogen (Carlsbad, CA). High Capacity cDNA Archive Kit and Power SYBR Green PCR Master Mix were from Applied Biosystems (Foster City, CA). Oligonucleotide primers were synthesized by Invitrogen.

**Animals.** Certified male CH (n = 10, approximately 700 kg b.wt.), PM (n = 8, approximately 650 kg b.wt.), and BA (n = 7, approximately 600 kg b.w.t.) beef cattle from private breeding farms located in the Piedmonte Region (Italy) were used. All animals were about 18 to 20 months old and were selected in the course of a research project whose major role was to define basic immunological, histological, zootechnical, and pharmacotoxicological parameters to be used in the screening of illicit drug treatments in meat cattle farming. At the slaughterhouse, after the exsanguination step, the liver lobe was removed, and small aliquots for the total RNA extraction (about 100 mg each) were collected in sterile, immediately snap-frozen in liquid nitrogen, and stored at −80°C. The remaining part of the lobe was cut in small pieces, washed in chilled isotonic 1.15% KCl, brought to the laboratory within 2 h after the animal death, wrapped in aluminum foil, and kept in ice.

**Preparation of Subcellular Fractions.** Once in the laboratory, 20- to 30-g aliquots free from pathological lesions were blotted dry, chopped, and homogenized in a Potter homogenizer. Therefore, cytosolic and microsomal subcellular fractions were isolated by differential ultracentrifugation as reported elsewhere (Nebbia et al., 2003). Aliquots of supernatants (cytosolic fraction) were frozen in liquid nitrogen and stored at −80°C until use. Pellets (microsomal fraction) were resuspended with 0.1 M phosphate-buffered saline (PBS) containing 0.1 M EDTA and glycerol 20% (v/v) and stored as above. Protein concentration was determined according to Lowry et al. (1951), using bovine serum albumin as standard.

**Enzyme Assays.** With regards to phase I DMEs catalytic activities, the rate of N-demethylation of benzphetamine (1 mM), erythromycin (1 mM), ethylmorphine (6 mM), and triacetyloleandomycin (0.3 mM) as well as the hydroxylation of aniline (5 mM), 4-nitrophenol (0.2 mM), and benzol[a]pyrene (0.08 mM) were measured with an Uvikon 941 spectrophotometer (Kontron Instruments, Watford, Herts, UK) or, alternatively, an LS30 fluorescence spectrometer (PerkinElmer, Monza, Italy), after an aerobic incubation step at 37°C for 0.5 h, under the conditions previously reported by Nebbia et al. (2003). The testosterone (TST; 250 µM) hydroxylations in the 2β-, 6α-, and 16β positions (2βOH-T, 6βOH-T, and 16βOH-T, respectively) was measured by an HPLC method (Capolongo et al., 2003). The ethoxyresorufin-O-deethylase (EROD) activity was measured by a modified HPLC method with fluorimetric detection, according to Hanioka et al. (2000). Briefly, 0.2 mg of microsomal protein were diluted in PBS (50 mM, pH 7.4) and incubated (37°C, 3 min) in the presence of 0.1 M MgCl2, 10 mM NADPH, and 0.5 µM ethoxyresorufin (ETR) as substrate. The final assay volume was 400 µl. The reaction was stopped by adding 400 µl of ice-cold methanol. Samples were vortexed, cooled on ice for 15 min, and centrifuged (3500g, for 20 min at +4°C). Supernatant (10 µl) was finally injected onto a Luna C18 column (100 Å, 150 × 4.6 mm, 5 µm; Phenomenex, Torrance, CA). The produced analyte (resorufin) was eluted isocratically with a mobile phase consisting of PBS (25 mM, pH 7.0)-methanol [58:42 (v/v)] at a 0.8 ml/min flow rate and detected fluorometrically at 530-nm (excitation) and 580-nm (emission) wavelengths. The HPLC analysis was run by using a Jasco HPLC apparatus consisting of a PU 980 pump, an AS-1555 Autoinjector, a DG1580 –53 degasser, and an FP-920 fluorescence detector (Jasco Europe, Cremella, Lecco, Italy). The rate of tolbutamide hydroxylation was measured by HPLC as reported by Palamanda et al. (2000), with minor modifications. In short, 1.25 mg/ml microsomal protein diluted in PBS (0.1 M, pH 7.4) was incubated with 1.5 mM tolbutamide.
Among phase II DMEs, UDP-glucuronosyltransferase (UGT) and glutathione S-transferase (GST) activities were measured. The activity of the former toward substrates 1-naphthol and p-nitrophenol was measured using microsomal proteins, according to Gusson et al. (2006). With regards to cytosolic GSTs, the conjugation rate of either the substrate was measured on microsomal proteins, according to Gusson et al. (2006). With the densitometric analysis of results as well as a molecular marker, respectively.

### Total RNA Isolation from Bovine Liver.

Total RNA was isolated from frozen samples using the TRIzol reagent according to the manufacturer’s instructions. Briefly, 1 ml of TRIzol was added to a small aliquot (80 mg) of liver tissue in a Qbiogene Lysing Matrice Tube (MP Biomedicals, Irvine, CA) and immediately homogenized using a glass Fast-Prep FP120 (MP Biomedicals) twice for 20 s. Then, samples were put on ice and purified with a classical phenol-chloroform extraction step. Total RNA concentration and quality were checked by using a Nanodrop ND-1000 spectrophotometer (Labtech France, Paris, France). The RNA quality was estimated by the 260-/280- and 260-/230-nm absorbance ratios and confirmed by denaturing agarose gel electrophoresis.

### Reverse Transcription.

The reverse transcription was performed using the High Capacity cDNA Archive Kit, following the manufacturer’s procedure. Two micrograms of total RNA was added to the reaction mixture to reach a final volume of 20 μl. The reaction was performed in a 96-Well GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: 10 min at 25°C and 2 h at 37°C. Complementary DNA was then stored at –20°C until use.

### Quantitative Real-Time Polymerase Chain Reaction.

**Bos taurus** mRNA sequences of target genes were obtained from the GenBank and Ensembl Genome Browser web sites (http://ncbi.nlm.nih.gov and http://www.ensembl.org, respectively). Primers sequences for Q RT-PCR were designed using Primer Express Software (version 2.0; Applied Biosystems) and were reported in Table 1. Primers concentrations were optimized in the 300 to 900 nM range. Melting curve analysis and agarose gel electrophoresis confirmed

### TABLE 1

<table>
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<tr>
<th>DMEs</th>
<th>GenBank Accession No.</th>
<th>5′ → 3′ Primer Sequence</th>
<th>Primer Length</th>
<th>Amplicon Size</th>
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<td>β-Actin</td>
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<td>R: AAGCAGGCAGCAAGCAGTT</td>
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<td>85</td>
</tr>
</tbody>
</table>

F: forward; R, reverse.

(30 μM; Phenomenex), at a flow rate of 0.5 ml/min with 10 mM ammonium acetate, pH 4.3 (A), and acetonitrile (B) as mobile phase. The hydroxylated metabolite was separated by using a linear gradient, consisting of 20% B for 14 min, which increased up to 50% in 2 min, remained constant for 7 min, and immediately decreased to 20% in 2 min, and then kept constant until the end of runtime (30 min). The HPLC analysis was performed using a Jasco HPLC system consisting of a PU-2089 PLUS pump, a PU 2089 PLUS degasser, a AS 2055 PLUS degasser, a AS 2055 PLUS degasser, and a AS 2055 PLUS degasser (Bologna, Italy), as previously reported by Laemmli (1970) and Towbin et al. (1979). Among phase II DMEs, UDP-glucuronosyltransferase (UGT) and glutathione S-transferase (GST) catalytic activities were measured. The activity of the former toward substrates 1-naphthol and p-nitrophenol was measured on microsomal proteins, according to Gusson et al. (2006). With regards to cytosolic GSTs, the conjugation rate of either the substrate was measured on microsomal proteins, according to Gusson et al. (2006). With the densitometric analysis of results as well as a molecular marker, respectively.
Nanomoles per milligram of protein (P450 content); micromoles of GSH per gram liver weight (GSH content); nanomoles per minute per milligram of protein (NADPH-R and NADH-R).

+ Statistically significant differences between CH and PM (ANOVA + Tukey’s post-test; p < 0.01).

Micrograms of quinine sulfate per minute per milligram of protein (BPOH); picomoles per minute per milligram of protein (EROD); nanomoles per minute per milligram of protein (BENZDEM, 16OH-T, TBT4-OH, Aniline-OH, pNP-OH, 6OH-T, 2OH-T, ERDEM, ETDEM, TAODEM).

+ Statistically significant differences between CH and PM, CH and BA, and PM and BA, respectively (ANOVA + Tukey’s post-test; a, b, and c, p < 0.05; aa and bb, p < 0.01; aaa, p < 0.001).

Micrograms of quinine sulfate per minute per milligram of protein (BPOH); picomoles per minute per milligram of protein (EROD); nanomoles per minute per milligram of protein (BENZDEM, 16OH-T, TBT4-OH, Aniline-OH, pNP-OH, 6OH-T, 2OH-T, ERDEM, ETDEM, TAODEM).

+Statistically significant differences between CH and PM, CH and BA, and PM and BA, respectively (ANOVA + Tukey’s post-test; a, b, and c, p < 0.05; aa and bb, p < 0.01; aaa, p < 0.001).

Micrograms of quinine sulfate per minute per milligram of protein (BPOH); picomoles per minute per milligram of protein (EROD); nanomoles per minute per milligram of protein (BENZDEM, 16OH-T, TBT4-OH, Aniline-OH, pNP-OH, 6OH-T, 2OH-T, ERDEM, ETDEM, TAODEM).

+Statistically significant differences between CH and PM, CH and BA, and PM and BA, respectively (ANOVA + Tukey’s post-test; a, b, and c, p < 0.05; aa and bb, p < 0.01; aaa, p < 0.001).

Micrograms of quinine sulfate per minute per milligram of protein (BPOH); picomoles per minute per milligram of protein (EROD); nanomoles per minute per milligram of protein (BENZDEM, 16OH-T, TBT4-OH, Aniline-OH, pNP-OH, 6OH-T, 2OH-T, ERDEM, ETDEM, TAODEM).

+Statistically significant differences between CH and PM, CH and BA, and PM and BA, respectively (ANOVA + Tukey’s post-test; a, b, and c, p < 0.05; aa and bb, p < 0.01; aaa, p < 0.001).

Micrograms of quinine sulfate per minute per milligram of protein (BPOH); picomoles per minute per milligram of protein (EROD); nanomoles per minute per milligram of protein (BENZDEM, 16OH-T, TBT4-OH, Aniline-OH, pNP-OH, 6OH-T, 2OH-T, ERDEM, ETDEM, TAODEM).

+Statistically significant differences between CH and PM, CH and BA, and PM and BA, respectively (ANOVA + Tukey’s post-test; a, b, and c, p < 0.05; aa and bb, p < 0.01; aaa, p < 0.001).
TABLE 6
Liver P450 and phase II DMES mRNA relative abundance (a.u.) in CH, PM, and BA cattle breeds

<table>
<thead>
<tr>
<th>Genes</th>
<th>-Fold Change</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CH</td>
</tr>
<tr>
<td>CYP1A1-like</td>
<td>1.00 ± 0.35a,b,b</td>
</tr>
<tr>
<td>CYP1A2-like</td>
<td>1.00 ± 0.54a</td>
</tr>
<tr>
<td>CYP2B6-like</td>
<td>1.00 ± 2.34a</td>
</tr>
<tr>
<td>CYP2C8-like</td>
<td>1.00 ± 1.89</td>
</tr>
<tr>
<td>CYP2C9-like</td>
<td>1.00 ± 0.52a,b,b</td>
</tr>
<tr>
<td>CYP2C18-like</td>
<td>1.00 ± 0.29b</td>
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<tr>
<td>CYP2C19-like</td>
<td>1.00 ± 0.84</td>
</tr>
<tr>
<td>CYP2E1-like</td>
<td>1.00 ± 1.52</td>
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<tr>
<td>CYP3A4-like</td>
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<tr>
<td>UGT1A6-like</td>
<td>1.00 ± 0.68a</td>
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<td>GSTM1-like</td>
<td>1.00 ± 0.60b</td>
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<td>GSTP1-like</td>
<td>1.00 ± 0.23b</td>
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</table>

CYP2C Activity, Protein, and mRNA. Differences in tolbutamide 4-hydroxylase (TBT4-OH) and CYP2C protein amount were never observed among cattle breeds. Likewise, no statistically significant differences were noticed in CYP2C8- and CYP2C19-like mRNAs (despite the higher amounts found in PM). In contrast, significant lower amounts of CYP2C9- and CYP2C18-like mRNAs were found in CH (p < 0.01 versus PM and BA and p < 0.001 versus BA, respectively); in the case of CYP2C18-like transcript, significant differences were noticed between BA and PM, too (p < 0.05). The TBT4-OH catalytic activity was significantly correled to liver CYP2C19-like mRNA expression in the CH group (rs = 0.82, p < 0.05).

CYP3A Activity, Protein, and mRNA. Aniline and p-nitrophenol were chosen as CYP2E1 model substrates. Liver CH microsomes showed the greater catalytic activity toward the aforementioned substrates. In particular, the aniline hydroxylase (Aniline-OH) activity was about 2- and 1.5-fold higher than that reported in PM and BA microsomes (p < 0.001 and p < 0.05, respectively). With regards to p-nitrophenol hydroxylase (pNP-OH), a significantly higher catalytic activity was pointed out in CH, if compared with the BA one (p < 0.05). Immunoblotting was performed by using a human and rat anti-CYP2E1 polyclonal antibody raised in sheep; faint cross-reacting bands, which hampered their consequent densitometric analysis, were noticed (data not shown). No statistically significant differences were ever observed in CYP2E1-like mRNA, even if, and quite unexpected, BA showed a higher CYP2E1-like mRNA abundance (BA > CH > PM). As a whole, these results were partially confirmed by the correlation analysis; in fact, in the BA group, the Aniline-OH catalytic activity was negatively correlated to CYP2E1-like gene expression (rs = −0.94, p < 0.05).

CYP3A Activity, Protein, and mRNA. No differences were found among CH, PM, and BA in the N-demethylation of CYP3A3-dependent substrates ethylmorphine (ETDDEM), erythromycin (ERDEM), or triacetyloleandomycin (TAODEM). On the contrary, another CYP3A3 substrate, was metabolized to a greater extent in CH; the rate of TST hydroxylations in 6β and 2β positions was about 2-fold higher in PM and BA (p < 0.01). In contrast, CYP3A protein was expressed to a greater extent in the BA group (p < 0.05 versus CH). The Q RT-PCR results were in line with those obtained with immunoblotting because BA was the cattle breed gifted of the higher CYP3A4-like transcript (p < 0.05, BA versus CH). No significant correlations were noticed between used substrates and the CYP3A4-like gene expression profile.

UGT Activity and mRNA. In our experimental conditions, CH was proved as the cattle breed that metabolized to a greater extent either α-naphthol or p-nitrophenol, two UGT1-specific substrates. As regards the former, the catalytic activity was about 1.5- and 2-fold higher than in PM (p < 0.05) and BA (p < 0.01), respectively. At the mRNA level, UGT1A6-like expression was perfectly in line with the highest CYP2B apoprotein content (p < 0.05 versus CH). In Fig. 1, a representative CYP2B immunoblotting is reported. Such a result was confirmed also at the CYP2B6-like gene expression level; in both cases, the trend was BA > PM > CH. The linear correlation analysis between 16βOH-T and CYP2B6-like gene expression pointed out a positive correlation for PM and BA (p < 0.05) and a negative one for CH (rs = 0.71, 0.79 and −0.38, respectively). In addition, a good correlation coefficient was noticed in PM if we took into account benzphetamine N-demethylation (BENZDEM, rs = 0.74, p < 0.05). In Fig. 2, the overall effect of breed upon CYP2B catalytic activity, apoprotein content, and mRNA together with the linear correlation analysis between 16βOH-T and CYP2B6-like mRNA is reported.
-nitrophenol UGT activity results; in fact, CH showed a mRNA relative abundance 3- and 8-fold greater than that measured in PM (p < 0.01) and BA (p < 0.01), respectively. In contrast, UGT1A1-like transcript was found highly expressed in BA (p < 0.01), with an opposite trend: BA/PM > CH. No significant correlation was ever observed between the two UGT1-dependent conjugation activities and UGT1A1- and UGT1A6-like gene expression profiles.

GST Activity and mRNA. With regards to GST-dependent conjugation reactions, in Fig. 3 the activity of GSTs accepting two different substrates, namely CDNB for total GST and DCNB for GST μ1, are reported. The pattern of CDNB metabolism was lowest in CH (p < 0.01 versus PM and p < 0.001 versus BA), whereas the DCNB one was of the same order of magnitude in CH and PM, but more elevated in BA (p < 0.001). At the pretranscriptional level, likewise to enzymatic activities, CH showed an overall lesser amount of considered GSTs transcripts, reaching in certain instances the level of statistical significance (p < 0.05 versus PM for GSTA1-like and p < 0.05 versus BA for GSTP1-like). As far as GSTM1-like mRNA is concerned, CH showed about 3- and 6-fold lower amounts than PM and BA (p < 0.05 and p < 0.001, respectively; see Fig. 3). The correlation analysis between the two selected DMEs activities, and GSTM1-like gene expression was statistically significant (p < 0.05) in PM cattle, with rS values of 0.82 and 0.89 for CDNB and DCNB, respectively.

Discussion

In the present study, the effect of breed upon most DMEs was investigated in cattle liver both at pretranscriptional and post-translational levels. In fact, previously published studies were mostly addressed to the evaluation of catalytic activities and apoprotein content, and only recently, DME gene expression has gained increasing interest in this veterinary species (Cantiello et al., 2005; Greger et al., 2006).

Substrates mostly used to monitor CYP1A activity are benzo[a]pyrene and ETR, the latter metabolizing to a greater extent in cattle than rats (Nebbia et al., 2003; Ioannides, 2006). In the present study, both catalytic activities and apoprotein levels were in line with previously published ones (Sivapathasundaram et al., 2001; Nebbia et al., 2003; Sztáková et al., 2004). In the rat, EROD reflects CYP1A1 activity, but it has been hypothesized that CYP1A2 might participate, albeit much less efficiently than CYP1A1, to ETR metabolism. In the cattle liver, the higher EROD activity might be due to a more relevant
expression of CYP1A1, an extrahepatic isoform only inducible in the liver (Sivapathasundaram et al., 2001); in this respect, the presence of liver constitutive CYP1A1-like mRNA was confirmed by the Q RT-PCR technique, but correlation indexes between catalytic activities and CYP1A1- or CYP1A2-like mRNA were higher for this latter. The observed significant breed differences in CYP1A1/2 expression were consistent with previous data reported in mouse strains (Casley et al., 1997).

Benzphetamine and 16α-hydroxylated TST are considered CYP2B-dependent substrates in man and rat, respectively (Ioannides, 2006; Wang et al., 2006). In cattle, this isoform was believed to be poorly involved in TST hydroxylation (Sivapathasundaram et al., 2001; Ioannides, 2006), but recently, veal calf liver microsomes were proved able to produce, out of 6β- and 2β-OH TST, detectable amounts of 16β-OH TST, too (Capolongo et al., 2003); besides, a 3-fold increase in 16β-OH TST production was noticed in liver microsomes from beef cattle induced with phenobarbital (Cantiello et al., 2006), which up-regulates CYP2B10 expression in the mouse (Pefeller et al., 2007). In our study, CYP2B-dependent DME activities were clearly and significantly higher in CH. The immunoblotting, where we used the same anti-rat CYP2B1/2 polyclonal antibody reported by Nebbia et al. (2003) and Sivapathasundaram et al. (2001), gave significant results that agreed with CYP2B6-like expression profile, but not with catalytic activities. A good correlation was obtained between 16β-OHT and CYP2B6-like gene expression (especially in BA), thereby confirming data reported in the mouse by Pefeller et al. (2007).

Tolbutamide hydroxylation was chosen as a CYP2E-dependent DME because it reflects human 2C9, and it has been already measured in cattle, too (Ioannides, 2006). Breed did not affect either TBT4-OH or CYP2C apoprotein level, which were not correlated each other (data not shown); similar results were obtained by Löfgren et al. (2004) in the mouse, where the same polyclonal antibody that recognizes several CYP2C isoforms as a single immunoreacting band was successfully used. On the contrary, significant breed differences were noticed at the mRNA level except for CYP2C19-like, whose expression was significantly correlated with TBT4-OH in CH. In veterinary species, P450 substrates are usually classified according to their specificity toward human or rat isoforms; unfortunately, a substrate specifically metabolized by a certain P450 in aforementioned species might undergo different biotransformation pathways in other ones, with consequent changes in P450 selectivity (Sztókóvá et al., 2004). Therefore, further studies are required to identify bovine CYP2C isoform sequences and clarify their involvement in xenobiotic metabolism.

Several rodent CYP2E-dependent DME activities, including pNP-OH and Aniline-OH, have been measured in cattle (Ioannides, 2006). Present results are consistent with those reported by Nebbia et al. (2003) and suggestive of differences in catalytic activities among cattle breeds. A poor cross-reactivity was noticed at the immunoblotting level, which did not allow a correlation between enzymatic activity and CYP2E1 protein; on the other hand, contrasting results in terms of cross-reactivity, number of cross-reacting bands, as well as accordance with corresponding catalytic activities have already been reported in veterinary species, following the use of anti-human or anti-rat CYP2E1 antibodies (Machala et al., 2003; Nebbia et al., 2003; Sztókóvá et al., 2004; Ioannides, 2006). No differences were noticed at the mRNA level, consequent to the high interindividual variability. Noteworthy was the correlation coefficient found for Aniline-OH (significant for BA), which would indirectly confirm this latter as a CYP2E substrate in cattle, too; this isoform represents...
one of the mostly conserved subfamilies, and corresponding orthologous proteins often show the same substrate specificity (ioannides, 2006).

Concerning CYP3A, both 6βOH-T and 2βOH-T activities pointed out significant differences among breeds, whereas ERDEM, ETDEM, and TAODEM ones did not. These contrasting results partially disagreed with those reported by Dacasto et al. (2005), where significant differences, between PM and LIM cattle, were found for ERDEM, ETDEM, and 2βOH-T. Out of ERDEM, whose protocol and the resulting catalytic activity in PM (the only used in both studies) were similar, methodological differences might be probably offered as a justification for such a discrepancy; for ETDEM, in terms of substrate concentration or instrument sensitivity; about 2βOH-T, of more sensitivity and precision of the used method (HPLC). In this respect, the correlation analysis among catalytic activities and CYP3A4-like mRNA gave better coefficients with HPLC results, which indirectly suggest TST as a more specific and useful substrate to study CYP3A expression in cattle. The immunoblotting, where the same anti-rat CYP3A1/2 cited by Nebbia et al. (2003) was used, gave contradictory results compared with catalytic activities (according to Dacasto et al., 2005) but consistent with CYP3A4-like mRNA.

With regards to phase II DMEs, we choose human microsomal UGT1A substrates α-naphthol and p-nitrophenol, which had already been used in cattle (Gusson et al., 2006). Both were metabolized to a greater extent in CH. These data were in line with UGT1A6-like mRNA levels but disagreed with UGT1A1-like ones. Moreover, no correlation was found between catalytic activities and gene expression. Interestingly a UGT2B17-like isoenzyme, involved in human steroid conjugation (Xu et al., 2005), was highly expressed in BA, likewise to UGT1A1-like (data not shown). Present results might be justified by the fact that many UGTs (including those here selected and whose cattle sequences are available in databases) show broad overlapping substrate specificities (Nagar and Remmel, 2006), even in the mouse animal model (Katoh et al., 2005).

Today, seven GSTs classes are recognized in mammals (Frova, 2006); among their substrates, CDNβ is considered a general one, whereas DCNB is specific for the 1 μ isof orm in rat, mouse, man, and rabbit (Gusson et al., 2006). In our study, BA elicited the most efficient pattern of biotransformation, in contrast with P450s and UGTs data; besides, such a behavior was confirmed pretranscriptionally, too. Furthermore, in PM, the GSTM1-like mRNA was significantly correlated with both substrates. On a knowledge basis, this is the first report concerning GSTs mRNA expression in cattle, suggestive of breed differences as previously noticed in rats (Jang et al., 2001).

As a whole, our data are suggestive of breed differences in DME expression in cattle, likewise to laboratory species (Casley et al., 1997; Jang et al., 2001; Löfgren et al., 2004; Saito et al., 2004; Stott et al., 2004). Differences in DME expression are likely to influence both the bioavailability and clinical efficacy of xenobiotics (Sallovitz et al., 2002), and these same might be of particular concern in farm animals, for the hypothetical presence of potentially harmful residues in foodstuffs of animal origin. Moreover, the presence of uneven results suggests that such an effect cannot be clearly set at the level of enzyme translation rather than transcription a priori.

Microsomal and cytosolic proteins as well as P450 and GSH contents were entirely similar among breeds; nonetheless, CH, comparatively showed, for the great majority of considered DMEs, the lower protein and gene expression levels but the more elevated catalytic activity. A first possible explanation of such a behavior might be a more efficient catalytic cycle, a hypothesis that might be justified by the higher NADPH cytochrome c reductase activity measured in CH itself; in this respect, a similar behavior has been recently recorded, for CYP3A, in the horse (Tydén et al., 2007). A second one might be traced back in the presence either of post-translational phenomena (like phosphorylation) or, alternatively, post-transcriptional effects (i.e., micro-RNA); in fact, the former ones have been proved able to modulate P450 activity and substrate specificity as well (Oesch-Bartlomowicz and Oesch, 2005), whereas the second ones, instead, might play a role in the regulation of DME expression (as recently reported in the case of CYP1B1; Tsuchiya et al., 2006). Finally, the hypothetical presence of single nucleotide polymorphisms in DMEs sequence cannot be altogether excluded; recently, an increased metabolic activity found in CH compared with German Holstein cattle has been attributed to single nucleotide polymorphisms in some functional liver and intestine genes (Schwerin et al., 2006). Ongoing studies aiming to sequence genes coding for major DMEs in these cattle breeds are envisaged in our laboratory.

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