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Characterization of xenobiotic metabolizing enzymes in bovine small intestinal mucosa

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The intestinal mucosa plays a capital role in dictating the bioavailability of a large array of orally ingested drugs and toxicants. The activity and the expression of several xenobiotic metabolizing enzymes were measured in subcellular fractions from the duodenal mucosa of male veal calves and beef cattle displaying a functional rumen but differing in both age (about 8 months vs. 18 to 24 months) and dietary regimens (i.e., milk replacer plus hay and straw vs. corn and concentrated meal). Intestinal microsomes showed cytochrome P450 (CYP) 2B, 2C- and 3A-mediated activities and the presence of the corresponding immunorelated proteins, but no proof of CYP1A expression and/or functions could be provided. Intestinal microsomes were also active in performing reactions typically mediated by carboxylesterases (indophenylacetate hydrolysis), flavin-containing monooxygenases (methimazole *S*-oxidation), and uridindiphosphoglucuronyltransferases (1-naphthol glucuronidation), respectively. Cytosolic fractions displayed the glutathione *S*-transferase (GST)-dependent conjugation of 1-chloro-2,4-dinitrobenzene; besides, the GST-mediated conjugation of ethacrinic acid (GSTπ) or cumene hydroperoxide (GSTα) was matched by the presence of the corresponding immunorelated proteins. Conversely, despite the lack of measurable activity with 3,4-dichloronitrobenzene, a protein cross reacting with anti-rat GSTμ antibodies could be clearly detected. Although, as detected by densitometry, CYPs and GST isoenzymes tended to be more expressed in beef cattle than in veal calf preparations, there was a general poor correlation with the rate of the *in vitro* metabolism of the selected diagnostic probes.

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INTRODUCTION

During their production cycles, livestock animals are exposed to a wide array of xenobiotic agents (i.e., veterinary drugs, feed additives, pesticides, pollutants, etc.), the great majority of which are metabolized by different enzyme systems usually yielding less lipophilic, more polar and hence less active metabolites readily eliminated from the body *via* normal excretion routes. In certain instances, however, metabolism may also form more (re)active derivatives through a process called bioactivation. In addition, the metabolic activity of xenobiotic metabolizing enzymes plays a major role in determining the persistence of therapeutically or illegally used compounds in target species, which may additionally impose a risk to the consumers, due to the potential permanence of drug residue levels in edible tissues. This fact is a major concern for public health and consumer's safety.

Although xenobiotic biotransformations take place predominantly in the liver, metabolic activity is apparent in extra-hepatic tissues such as the intestinal mucosa. In addition to its primary role in the absorption of nutrients and water, the intestine represents also a major route of entry into the body for many toxic compounds or orally administered drugs. Consequently, it also has the ability to metabolize a great number of xenobiotics by numerous pathways involving both phase I and phase II reactions (Lin *et al.*, 1999), so that the amount of a drug reaching the systemic circulation – referred to as bioavailability – can be substantially reduced by both intestinal and hepatic

metabolism through a process called first pass metabolism. In this respect, it has been shown that cytochrome P450 (CYP) 3A4 is the major isoenzyme involved in the oxidative biotransformation of xenobiotics in human small intestinal mucosa (Kolars *et al.*, 1994) and largely contributes to the first pass metabolism of many drugs, such as for instance midazolam (Paine *et al.*, 1996; Thummel *et al.*, 1997) and the immunosuppressive drugs cyclosporin and tacrolimus (Benet *et al.*, 1999). As far as ruminant species are concerned, for example, enteric biotransformation of diphenhydramine accounted for approximately a 50% decrease in the bioavailability of such antihistaminic drug in adult sheep (Kumar *et al.*, 1999). In addition, microsomal preparations obtained from cattle small intestinal mucosa were reported to biotransform the worldwide used benzimidazole antiparasitic drugs albendazole and fenbendazole into their respective pharmacologically active sulphoxide derivatives (Virkel *et al.*, 2004).

There is scant information concerning the characterization of xenobiotic metabolizing enzymes in the intestinal mucosa from ruminant species, especially cattle. A number of oxidative and conjugative enzyme activities were measured in the ileal mucosa of sheep and cattle, including benzphetamine- and ethylmorphine *N*-demethylase, as well as uridindiphosphoglucuronyltransferase- (UGT), *N*-acetyltransferase (NAT), and glutathione *S*-transferase (GST) activities toward different substrates; all these reaction rates were lower in the enteric mucosa compared with the liver (Watkins *et al.*, 1987). Both benzphetamine- and erythromycin *N*-demethylases were measured in microsomes obtained from different segments of sheep intestinal mucosa and correlated with the expression of CYP2B- and 3A immunoreacting proteins (Dupuy *et al.*, 2001). More recently, the expression of genes encoding for some of the most important CYPs and conjugative enzymes has been investigated in different parts of the intestine of neonatal calves (Krüger *et al.*, 2005). Notwithstanding this previously published information, the expression and activity of enzymes involved in the biotransformation of xenobiotics in the ruminant's intestinal mucosa need further study.

It is well known that substantial changes in the disposition of xenobiotics may occur in different animal species and human during development. The ontogeny of liver drug metabolizing enzymes may be a major factor associated to age-related changes in drug disposition in ruminants (Kaddouri *et al.*, 1992; Greger *et al.*, 2006), which may contribute to differences in drug clinical efficacy or in the susceptibility to toxic compounds. On the other hand, normal dietary constituents and a large number of drugs and chemical contaminants are long known to modulate the expression and activity of many gut biotransformation enzymes (for a review see Sergent *et al.*, 2008). In the light of the crucial role of the intestine in the disposition of drugs and toxicants, the main goal of this study was to characterize and compare the activity and the expression of several phase I and phase II xenobiotic metabolizing enzymes in subcellular fractions obtained from the duodenal mucosa of bovines belonging to two different commercial categories, i.e., veal calves and beef cattle, differing in both age and dietary regimen.

MATERIALS AND METHODS

Chemicals

Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Roche Applied Science (Monza, Italy). Benzphetamine and ethylmorphine were bought from S.A.L.A.R.S. SpA (Como, Italy), whilst all other chemicals were from Sigma-Aldrich (Milan, Italy).

Animals and preparation of subcellular fractions

Segments of 40–50 cm of the duodenum were obtained from male veal calves (approximately 8 month old) and beef cattle (18–24 month old) slaughtered in an EU-certified abattoir at Sant'Albano (Cuneo, Italy). All animals were inspected prior to slaughter and judged to be healthy by a licensed veterinarian, and their carcasses passed examination by licensed meat inspectors; moreover, a certificate stated that they did not undergo any drug treatment for at least 3 months prior to slaughtering, which in all cases was performed according to the Italian law. Each intestinal segment was opened through a longitudinal incision and the gut content was discarded. The mucosa was then washed with ice-cold KCl 1.15% containing 0.5 mg/mL trypsin inhibitor. Samples were stored in aluminum foils, chilled in ice and brought to the laboratory for subsequent procedures, which started within 2 h from sample collection and were performed between 0 and 4 °C. The intestinal mucosa surface of each segment was blotted dry and thereafter obtained by scraping using a microscope glass slide. The tissue obtained was pooled into a vessel filled with ice-cold homogenization buffer (0.1 M potassium phosphate, pH 7.4, containing 0.15 M Tris acetate, 0.1 M KCl, 1 mM EDTA, 18 μM butylated hydroxytoluene and 1 mg/mL trypsin inhibitor). A total number of 6 pools of either veal calves or beef cattle were processed and each pool was prepared with the mucosal tissue of 8 animals. Pooled samples were filtered through a hydrophilic gauze, weighted and homogenized in a Potter-Elvehjem tool (four to six passes) with two volumes of ice-cold homogenization buffer. The resulting homogenates were filtered again as above, centrifuged at 10 000 ***g*** (Beckman L7-55 ultracentrifuge) for 20 min and the supernatants further spun at 105 000 ***g*** for 60 min. Aliquots of the resulting supernatants (cytosolic fractions) were frozen in liquid nitrogen and stored at −80 °C; pellets (microsomal preparations) were resuspended in a 0.1 M potassium phosphate buffer containing 0.1 mM of EDTA and 20% of glycerol by brief sonication, frozen in liquid nitrogen and stored at −80 °C until used. An aliquot of each subcellular fraction was used to determine protein content using bovine serum albumin as a reference standard (Lowry *et al.*, 1951).

Enzyme assays

All enzyme activities were measured under conditions yielding zero order rates with respect to cofactor and substrate concentrations and ensuring linearity with respect to time and protein

concentrations. Microsomal suspensions were assayed for their ability in metabolizing a number of substrates that in humans, in ruminant or laboratory species (Dupuy *et al.*, 2001; Sivapathasundaram *et al.*, 2001; Ioannides, 2006; Fink-Gremmels, 2008) are thought to be markers for the expression of different CYP isoforms, based also on molecular models of mammalian enzymes (Lewis, 1996). In this respect, it should be noted that even orthologous CYPs may have different substrate specificities (Fink-Gremmels & Van Miert, 1996). In addition, certain substrates may become specific only in individuals pretreated with an inducer of a given isoform and be metabolized by CYPs constitutively expressed to the greatest extent (Nebbia, 2001). Further to the above considerations, the rate of the *in vitro* metabolism of the following substrates was measured: 7-ethoxyresorufin for monitoring CYP1A-mediated activities, 7-ethoxycoumarin (CYP1A and CYP2B), benzphetamine (CYP2B), aminopyrine and 7-methoxy-4-trifluoromethylcoumarin (CYP2C), chlorpheniramine (CYP2C and 3A), and ethylmorphine (CYP3A).

Oxidative CYP-dependent *N*-demethylation activities toward benzphetamine (1 mM), aminopyrine (5 mM), chlorpheniramine (1 mM), or ethylmorphine (6 mM) were assayed using a NADPH-regenerating system ($NADP^+$ 0.32 mM, glucose-6-phosphate 6.4 mM, $MgCl_2$ 5 mM, EDTA 0.8 mM, and 1.25 U of glucose-6-phosphate dehydrogenase in Tris-HCl 0.1 M, pH 7.4) and 1 mg of microsomal protein. After an incubation time ranging from 15 to 20 min according to the nature of the substrate, reactions were quenched with chilled trichloroacetic acid (10%, w/v) and, after centrifugation, the amount of the released formaldehyde was obtained fluorometrically on an aliquot of the clear supernatant with Nash's reagent as detailed by Werringloer (1978). The *O*-deethylation rate of 7-ethoxyresorufin (2 μM) was assayed fluorometrically by measuring the amount of resorufin formed using a continuous method (Burke & Mayer, 1974). The activity of 7-ethoxycoumarin *O*-deethylase was obtained using 0.8 mM substrate concentration as reported by Dent *et al.* (1976). The *O*-demethylation of 7-methoxy-4-trifluoromethylcoumarin (50 μM) was monitored by a continuous fluorimetric method as detailed by Buters *et al.* (1993). Methimazole *S*-oxidation was selected as a flavin monooxygenase (FMO)-dependent pathway and assayed following the method described by Dixit and Roche (1984) using 1 mM substrate concentration and 0.5 mg microsomal protein; in certain experiments, enzyme activities were obtained in the presence of another FMO substrate (thiourea 1 mM) or piperonyl butoxide (1 mM) to ascertain the extent of the CYP contribution to this pathway. Microsomal carboxylesterase activity was assayed using 0.33 mM indophenylacetate (IPA) as substrate and 0.04–0.08 mg of protein (Zemaitis & Greene, 1979).

Microsomal UGT activity was obtained using 1-naphthol (50 μM) as substrate and 0.5 mg of microsomal protein essentially as described by Bock *et al.* (1979). The activities of cytosolic total GST and of GST μ and π isoenzymes were monitored by a continuous spectrophotometric method (Habig *et al.*, 1974) using the following substrates: 1-chloro, 2,4-dinitrobenzene (CDNB) (nonspecific substrate), 3,4-dichloronitrobenzene (DCNB) for GSTμ, and ethacrinic acid for GSTπ. Selenium-independent glutathione peroxidase activity, also referred to as GSTα, was measured using cumene hydroperoxide as substrate following the method by Nebbia *et al.* (1993).

SDS-polyacrylamide gel electrophoresis and Western blotting

Aliquots of each microsomal and cytosolic fraction were diluted in protein sample loading buffer (2% SDS, 10% glycerol, 5% 2-β-mercaptoethanol and 0.25% bromophenol blue) in Tris-HCl 0.0625 M (pH 6.8). SDS-polyacrylamide gel electrophoresis of proteins and Western blotting were carried out following the methodology described by Laemmli (1970) and Towbin *et al.* (1979). One hundred micrograms (100 μg) of microsomal or cytosolic proteins (~25 μL) were loaded in each gel lane. Protein samples of liver microsomes or cytosols, used as positive controls, were also charged. The proteins were then transferred to nitrocellulose membranes, which were firstly incubated with primary antibodies raised against human CYPs 2C (polyclonal rabbit IgGs, 1/1000, Chemicon, Temecula, USA) and 3A4 (polyclonal rabbit IgGs, 1/1000, Oxford Biomedical Research, Oxford, USA), rabbit CYP1A1/1A2 (polyclonal goat IgGs, 1/100, Oxford Biomedical Research), rat CYP2B1 (polyclonal goat IgGs, 1/100, Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan), rat GSTs (polyclonal rabbit IgGs, 1/1000, Alpha Diagnostic International, San Antonio, TX, USA), and α-tubulin (monoclonal mouse IgGs, 1/10000, Sigma-Aldrich). Membranes were then incubated with appropriate secondary peroxidase-labeled antibodies: goat anti-rabbit (1/10000) (CYPs 2C, 3A4 and GSTs), rabbit anti-goat (1/5000) (CYPs 1A1/1A2 and 2B1), or sheep anti-mouse (1/5000) (α-tubulin). Proteins were detected using ECL Western blotting detection reagents (Amersham Biosciences–GE Healthcare, Italy). Integrated optical densities (IODs) were calculated by means of the Bio-Rad software Quantity One (version 4.5.2).

Data and statistical analysis

Metabolic activities (mean ± SD) are expressed as nmoles or pmoles of metabolic products formed/min mg $protein^{-1}$. Statistical comparisons were carried out using the unpaired Student's *t*-test (Instat 3.0, Graph Pad Software, Inc., San Diego, CA, USA). A P value less than 0.05 was considered significant.

RESULTS

Phase I xenobiotic biotransformation enzyme activities

Cytochrome P450-mediated *N*-demethylation activities towards benzphetamine, aminopyrine, chlorpheniramine, or ethylmorphine are shown in Table 1. Compared with those from beef cattle, samples from veal calves displayed somewhat higher values (up to 60%, $P < 0.05$) in the case of benzphetamine and aminopyrine, whereas the reverse held true for chlorpheniramine *N*-demethylation (+40%, $P < 0.05$). Ethylmorphine

Table 1. Rates of N-demethylation of benzphetamine, aminopyrine, chlorpheniramine, and ethylmorphine, and of the hydrolysis of indophenylacetate in duodenal microsomes from veal calves and beef cattle

	CYP isoform	Veal calf	Beef cattle
Benzphetamine N-demethylase	2B	0.23 ± 0.08[a]	0.14 ± 0.01[b]
Aminopyrine N-demethylase	2C	0.02 ± 0.05[a]	0.12 ± 0.02[b]
Chlorpheniramine N-demethylase	2C	0.15 ± 0.03[a]	0.21 ± 0.04[b]
Ethylmorphine N-demethylase	3A	0.14 ± 0.03[a]	0.16 ± 0.04[a]
Indophenylacetate esterase	–	88 ± 17[a]	89 ± 10[a]

Values (nmol/min mg protein–1) are expressed as mean ± SD; six pools for each category were used, each prepared with the mucosal tissue from eight individuals; within a row, values bearing different superscript letters are significantly different ($P < 0.05$ or less, unpaired *t*-test).

N-demethylase activities displayed very similar values in either group and no appreciable microsomal 7-ethoxyresorufin *O*-deethylase, 7-ethoxycoumarin *O*-deethylase, nor 7-methoxy-4-trifluoromethylcoumarin *O*-demethylase activities could be measured in the duodenal mucosa of either cattle category.

There were no substantial differences in the rate of methimazole *S*-oxidation between veal calves and beef cattle preparations. This enzymatic reaction was markedly depressed by thiourea 1 mM to an extent ranging between 43–72% (veal calves) and 45-67% (beef cattle) (Table 2), while piperonyl butoxide was not effective in this respect.

Microsomal fractions obtained from the duodenal mucosa of either veal calves or beef cattle showed almost the same IPA esterase activity (Table 1).

Phase II xenobiotic biotransformation enzyme activities

Microsomal UGT activities measured using 1-naphthol as a substrate displayed very similar values in the duodenal mucosa of veal calves and beef cattle, with mean values of 0.46 ± 0.22 and 0.48 ± 0.28 nmoles/min/mg protein^{-1}, respectively.

The GST accepting CDNB (+47%, $P < 0.05$) or cumene hydroperoxide (+19%, $P < 0.05$) as substrates was slightly higher in the cytosolic fractions of the duodenal mucosa obtained from beef cattle compared with veal calves, while no remarkable differences occurred with ethacrinic acid. Conversely, there was no measurable GST activity toward DCNB in cytosols obtained from the duodenal mucosa of either cattle category (Table 3).

Table 2. Comparative FMO-mediated methimazole *S*-oxidation activities in microsomes determined from the duodenal mucosa of veal calves and beef cattle: inhibitory effect of thiourea (1 mM)

	FMO activity		Percentage inhibition (range)
	−thiourea	+ thiourea	
Veal calf	1180 ± 330[a]	480 ± 100[b]	42.5–72.0
Beef cattle	1050 ± 300[a]	470 ± 120[b]	44.9–67.1

Values (pmol/min mg protein^{-1}) are expressed as mean ±SD; $n = 6$ pools for each category were used, each prepared with the mucosal tissue from eight individuals; within a row, values bearing different superscript letters are significantly different ($P < 0.05$ or less, unpaired *t*-test).

Table 3. Comparative glutathione *S*-transferase activities measured with different substrates in duodenal cytosolic subfractions from veal calves and beef cattle

	Veal calves	Beef cattle
1-chloro-2,4-dinitrobenzene (CDNB)	51.1 ± 10.6[a]	75.1 ± 15.2[b]
3,4-dichloronitrobenzene (DCNB)	n.d.	n.d.
Ethacrinic acid	7.1 ± 0.3[a]	7.8 ± 1[a]
Cumene hydroperoxide	13.6 ± 2.1[a]	16.2 ± 1.1[b]

Values (nmol/min mg protein^{-1}) are expressed as mean ± SD; $n = 6$ pools for each category were used, each prepared with the mucosal tissue from eight individuals; within a row, values bearing different superscript letters are significantly different ($P < 0.05$ or less, unpaired *t*-test); n.d., not detectable.

SDS-polyacrylamide gel electrophoresis and Western blotting

Results of Western blotting analysis of microsomal or cytosolic fractions obtained from the duodenal mucosa of veal calves and beef cattle are depicted in Figs 1 and 2. In all cases, the used antibodies recognized the corresponding immunorelated hepatic proteins in either fraction, resulting in remarkably higher band intensities than those produced by their duodenal counterparts except for GSTπ, in which case the reverse held true (Fig. 2).

Immunoreacting proteins were detected in microsomes probed with antibodies raised to rat CYP2B1, human CYPs 2C8, 2C9, 219, and 3A4 (Fig. 1). By contrast, no immunorelated protein was detectable when antibodies to rabbit CYP1A1/1A2 were used (data not shown).

Immunoblot analysis using antibodies to rat GSTα, GSTμ or GSTπ revealed the presence of the corresponding immunoreacting cytosolic proteins (Fig. 2).

The IOD analysis performed for each protein showed that, compared to veal calves, beef cattle appeared to express intestinal CYPs 2C and 3A, and GSTα to a higher extent.

DISCUSSION

In this study, xenobiotic metabolizing enzyme expression and functions were evaluated in bovine duodenal mucosa by using both biochemical and immunochemical approaches. The former is based on the assumption or the proof that certain molecules are suitable substrates to test the activity of a particular isoenzyme, and the latter relies on the use of antibodies raised in other species, which in previous studies have been reported to cross react with cattle proteins. Although such approaches may be sometimes not conclusive and have been recently subjected to some criticisms (Ioannides, 2006), both have been adopted in the last decades and are particularly useful as the first step in the general characterization of the xenobiotic metabolizing capacity of a given tissue.

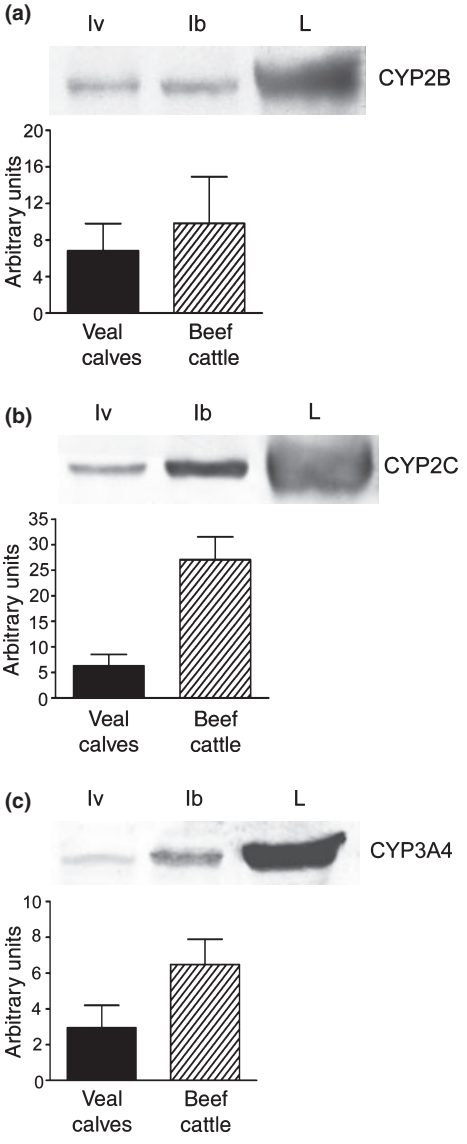

Fig. 1. Representative Western blot analysis of the comparative expression of cytochromes P450 (CYP) 2B (a), 2C (b) and 3A4 (c) in the duodenal mucosa of veal calves and beef cattle. Immunoblotting was performed on microsomal extracts (100 μg per lane), and microsomes from a liver sample were used as a positive control. Iv, intestine from veal calves; Ib, intestine from beef cattle; L, liver.

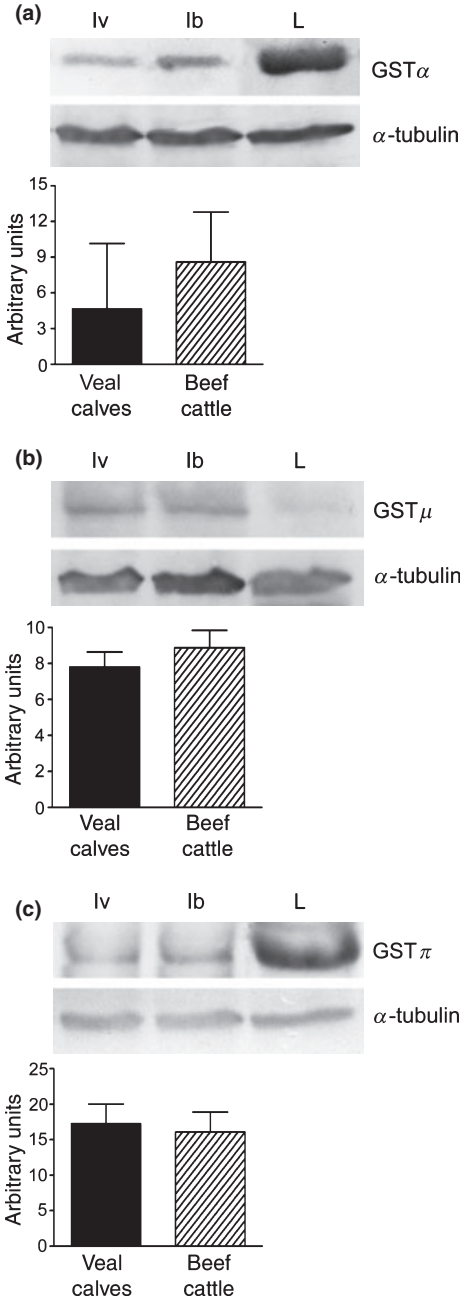

Fig. 2. Representative Western blot analysis of the comparative expression of glutathione *S*-transferases (GST) α (a), μ (b) and π (c) in the duodenal mucosa of veal calves and beef cattle. Immunoblotting was performed on cytosolic extracts (100 μg per lane), and cytosol from a liver sample was used as a positive control. α-tubulin was used as a loading control. Iv, intestine from veal calves; Ib, intestine from beef cattle; L, liver.

Both microsomal and cytosolic fractions obtained from the duodenal mucosa of veal calves and beef cattle were able to metabolize most of the tested substrates. Microsomes of both studied cattle categories showed the ability to perform the CYP-mediated *N*-demethylation of all the tested substrates, but neither *O*-demethylase- nor *O*-deethylase activities apparently occurred (see Table 1).

We were not able to demonstrate any measurable CYP1A-related activities. The CYP1A subfamily, which includes the two members CYP1A1 and CYP1A2, is structurally well conserved and constitutively expressed in vertebrates. Proteins cross reacting with antibodies raised against rat or rabbit CYP1A have been found in cattle liver microsomes from this and from earlier studies, in which the degree of CYP1A expression has been related to the rate of *in vitro* *O*-dealkylation of 7-ethoxy- (EROD) or 7-methoxyresorufin (Sivapathasundaram *et al.*, 2001; Nebbia *et al.*, 2003); incidentally, the ability to *O*-deethylate 7-ethoxyresorufin was remarkably higher in cattle preparations than in those from other food producing species and the rat (Nebbia *et al.*, 2003; Ioannides, 2006). Although the presence of

constitutive CYP1A1 in rat or human small intestine is still controversial (Kaminski & Zhang, 2003), it has been suggested that CYP1A1 is the primary inducible form in enterocytes (Fasco *et al.*, 1993; Zhang *et al.*, 1997). As it has been shown that rat intestinal CYP1A1 may be induced by constituents of commonly used rat chow, this CYP isoenzyme is usually detected in this tissue even in the absence of the administration of known inducers, such as 3-methycholanthrene or β-naphthophlavone (Kaminsky & Fasco, 1991). In our study, the lack of measurable CYP1A-mediated EROD activity was consistent with the absence of a CYP1A immunoreacting protein in the duodenal mucosa of both cattle categories studied. A very low EROD activity (less than 1 pmol/min mg protein^{-1}) was measured in bovine cultured colon epithelial cells (Birkner *et al.*, 2003). In addition, no apparent CYP1A-mediated conversion of either albendazole or fenbendazole sulfoxides to their respective (pharmacologically inactive) sulfone derivatives could be demonstrated in intestinal microsomes from Holstein steers (Virkel *et al.*, 2004). Taken together, these observations may indicate that this subfamily is constitutively poorly expressed in different tracts of cattle intestine but also points to the likely absence of CYP1A inducers in feedstuffs. For instance, certain natural carotenoids like lutein, which are contained in corn based diets, are known to down-regulate CYP1A2 levels in humans (Le Marchand *et al.*, 1997). A poor enteric CYP1A expression could result in a low bioactivation rate of a number of toxins and carcinogens, including aflatoxin B1 (Kuilman *et al.*, 2000).

The CYP2 family is comprised of four subfamilies (2A, 2B, 2C, and 2E) of enzymes, which have been identified in livers from different mammalian species. Immunoreactive proteins to rat anti-CYP2B1/2 have been detected in liver microsomes obtained from cattle (Sivapathasundaram *et al.*, 2001; Nebbia *et al.*, 2003) and reported to increase upon phenobarbital pretreatment (Cantiello *et al.*, 2006). In addition, the presence of a CYP2B6 encoding gene has been documented in 5-day-old calf intestinal mucosa (Krüger *et al.*, 2005); a single band has been recognized in this work probing duodenal microsomes with anti-rat CYP2B1/2 antibodies. It has been shown that benzphetamine *N*-demethylase activity correlated well with the expression of the CYP2B subfamily in ruminants (Kaddouri *et al.*, 1992; Nebbia *et al.*, 2003; Cantiello *et al.*, 2006), even in extrahepatic tissues such as the intestinal tract (Dupuy *et al.*, 2001). The moderately higher CYP2B-mediated activity we observed in the duodenal mucosa of veal calves compared with older animals (see Table 1), however, was not apparently matched by the levels of the CYP2B immunoreacting protein.

Several drugs widely used in veterinary medicine are known CY2C substrates, including sulphonamides, arylpropionic acid nonsteroidal anti-inflammatory derivatives, and the coccidiostat toltrazuril (Fink-Gremmels & Van Miert, 1996). Immunoreactive proteins to antibodies raised against human CYP2C isoforms were identified in cattle liver microsomes (Sivapathasundaram *et al.*, 2001; Cantiello *et al.*, 2006; Giantin *et al.*, 2008). Compared with beef cattle, veal calf duodenal microsomes exhibited a lower ability in performing the *N*-demethylation of chlorpheniramine, but a higher aminopyrine *N*-demethylase activity (see Table 1), thus not mirroring the much less intense band on Western blotting analysis using antibodies raised to human CYP2C detected in such preparations, and stressing again the apparent discrepancy between protein expression and catalytic function toward certain diagnostic substrates.

Like in liver, CYP3A is also well expressed in the human intestine, where it dictates the bioavailability of several drugs, being also involved in many clinically relevant drug-drug interactions (Ioannides, 2006). As far as ruminants are concerned, a triacetyloleandomycin-inducible CYP3A enzyme has been purified from sheep liver, displaying a remarkable activity in the *N*-demethylation of veterinary drugs such as chlorpromazine, chorpheniramine, and bromhexine (Pineau *et al.*, 1990). Proteins cross reacting with anti-rat or anti-rabbit CYP3A antibodies have been identified in cultured hepatocytes or liver microsomes from cattle (van't Klooster *et al.*, 1993; Sivapathasundaram *et al.*, 2001; Nebbia *et al.*, 2003). Using purified antibodies raised against sheep liver CYP3A, the same enzyme was identified in ovine duodenal microsomes (Dupuy *et al.*, 2001). Interestingly, CYP3A mRNA was most abundant in intestinal samples from 5-day-old calves (Krüger *et al.*, 2005). On incubation with antibodies raised to human CYP3A4, duodenal preparations of beef cattle showed the presence of a single band of much stronger intensity than that occurring in veal calves, but, consistent with results concerning the CYP2B and 2C isoforms, a poor correlation was found with the ability to *N*-demethylate chlorpheniramine (modest increase vs. veal calves) or ethylmorphine (no changes).

Microsomal flavin-containing monooxygenases (FMOs) catalyze the oxygenation of nucleophilic nitrogen-, sulfur-, phosphorus-, and selenium-containing xenobiotics to their respective more polar and often less active oxides. Five distinct and functional mammalian FMO isoenzymes (FMO 1 to 5) have been identified, the expression of which being tissue- and species-specific, and related to the ontogenic development: for instance, in humans FMO1 is the major form in fetal liver, and in kidney and intestine, while in the liver of adult individuals FMO3 largely predominates (Ziegler, 2002). Only scant information is available about FMO expression in ruminant species. For example, it has been shown that FMO3 is the predominant flavin-containing isoenzyme in sheep female liver (Longin-Sauvageon *et al.*, 1998), and that the *S*-oxidation rate of the antithyroid compound methimazole was useful to monitor the FMO-mediated metabolic activity in ovine hepatic microsomes (Can Demirdöğen & Adali, 2005) and in camel hepatic or intestinal preparations (Raza *et al.*, 2004). In our study, duodenal microsomal fractions of either cattle category were able to *S*-oxidize methimazole to a similar extent and this metabolic reaction was markedly inhibited by the well known FMO substrate thiourea (Guo *et al.*, 1992), but not by the CYP inhibitor piperonyl butoxide. The above results point to a significant contribution of FMOs to the oxidation of the antithyroid drug and indicate that the intestinal flavin-containing enzyme could play a significant role in presystemic metabolism of the widely used FMO substrates benzimidazole anthelmintics (Virkel *et al.*, 2004).

Mammalian carboxylesterases (CES) hydrolyze a wide variety of xenobiotic esters, being able to activate pro-drugs (e.g., acetylsalicylates) as well as to detoxify several pesticides (e.g., organophosphates, carbamates, and pyrethroids), and five families, called CES 1 to 5, have been identified in the liver and in the intestine of humans and a number of laboratory species (Taketani *et al.*, 2007). As for many other xenobiotic metabolizing enzymes, very little is known concerning such enzymes in cattle, especially as far as their expression and activity in the gut are concerned. It is worth noting that, in this study, IPA esterase activity in the intestinal mucosa of both veal calves and beef cattle (~90 nmol/min mg protein^{-1}) was approximately one fourth of that reported previously in cattle liver microsomes by Gusson *et al.* (2006).

Glucuronidation, a major pathway for the production of easily excretable water-soluble metabolites from endo- or xenobiotics, is carried out by UGTs, a superfamily of membrane bound enzymes catalyzing the conjugation of glucuronic acid to a nucleophilic substrate. Mammalian UGTs have been grouped into two distinct families, UGT1 and UGT2, with several isoforms displaying broad and overlapping substrate specificities; they are expressed mainly in the liver but also at relatively high levels in other tissues, such as the kidney and intestine (Fisher *et al.*, 2001). Proteins belonging to the UGT1A subfamily are the most important UGTs present in the liver and in the intestinal mucosa of humans and other species (Gregory *et al.*, 2004) including domestic ruminants. A cDNA of a bovine phenol UGT isoform was cloned and characterized and designated 'bovUGT1A6' (Iwano *et al.*, 2001). More recently, jejunum and colon cells from neonatal veal calves were reported to express UGT1A1 mRNA, but not UGT1A6, although to a much lower extent in comparison to CYP2B, CYP3A or sulphotransferase genes (Krüger *et al.*, 2005). Interestingly, 1-naphthol is considered a specific probe to evaluate UGT1A activity in humans as well as in cattle preparations (Iwano *et al.*, 2001). In this study, intestinal microsomes obtained from veal calves and beef cattle were able to conjugate 1-naphthol to a similar rate, which was in good agreement with what was earlier reported for ileal cow microsomes (Watkins *et al.*, 1987) and less than one tenth of the activity recorded in beef cattle liver subfractions (Gusson *et al.*, 2006).

The GSTs catalyze nucleophilic attack by reduced glutathione on a wide array of nonpolar compounds that contain an electrophilic carbon, nitrogen or sulfur atom such as plant phenols, mycotoxins (including aflatoxin B1), many chemical carcinogens, insecticides, herbicides, quinones as well as certain reactive oxygen species (Eaton & Bammler, 1999; Hayes *et al.*, 2005). In addition, these transferases are involved in steroid metabolism and in prostaglandin synthesis (Sheehan *et al.*, 2001). Based on physical, catalytical and sequence similarities, four main classes of cytosolic GSTs, and namely α (A), μ (M), π (P) and θ (T) have been identified in humans, rats, and mice, each of them including different isoenzymes (Sherratt & Hayes, 2002). Very limited information is available concerning intestinal GST activity in the bovine species (Watkins *et al.*, 1987; Birkner *et al.*, 2003). In the current investigation, duodenal soluble fractions from beef cattle revealed slight but statistically significant higher activities toward either CDNB (total GST) or cumene hydroperoxide (GSTα), the latter finding being matched by a comparable increase in the immunodetectable corresponding protein (see Fig. 2); conversely, no animal category related differences could be found in the activity and expression of GST accepting ethacrinic acid as substrate (GSTπ). According to the results of the immunochemical assays, it is worth noting that all main GST classes seem to be present in bovine intestine, and, in the case of GSTπ, even more expressed in the duodenal mucosa than in the liver, stressing the importance of the gut in the GSH-mediated detoxification of xenobiotics. An extremely low (Gusson *et al.*, 2006) or even undetectable (Sivapathasundaram *et al.*, 2003) enzyme activity using DCNB has been reported in cattle liver subfractions. In our study, the presence of a clearly visible immunoreacting band in bovine duodenal and hepatic cytosols probed with rat anti-GSTμ antibodies could indicate that, at least in the bovine species, DCNB is not suitable for assessing the GSTμ class.

In this work, which examined a number of phase I or phase II biotransformation pathways in the duodenal mucosa from two meat cattle commercial categories, a slightly higher expression and sometimes the activity of certain oxidative (CYP2C, CYP3A4) and conjugative enzymes (GSTα) was found in beef cattle compared with veal calves. Once again, very limited information is available for the bovine species, in which, to the best of our knowledge, the ontogeny of enzymes involved in xenobiotic metabolism has been addressed only in liver. In this respect, a significant increase in the mRNA abundance was found for CYP2C8 and CYP3A4, but not for UGT1A1, in calves aged 159 days compared with 5-day-old individuals (Greger *et al.*, 2006). A similar behavior was noticed by Kawalek and el Said (1994) when comparing liver phase I and phase II enzyme activities between 35-day-old calves (milk-replacer diet) and 85-day-old calves (conventional diet), i.e., nonruminating animals vs. ruminating ones; interestingly, the latter exhibited small differences with 105-day-old ruminating calves. In fact, although certain enzyme activities like GSTs are reported to increase as a function of age in rat small intestine (Kaminski & Zhang, 2003), it is conceivable that the nature of diet is a major factor regulating the enteric biotransformation pattern. Indeed, veterinary drugs, pesticides, environmental pollutants as well as secondary mold (mycotoxins) or plant metabolites (polyphenols) (Sergent *et al.*, 2008), or even crop constituents (e.g., lutein) (Le Marchand *et al.*, 1997) are reported to modulate actively most phase I and phase II xenobiotic metabolizing enzymes in the gut. Consequently, it is difficult to decide whether the slightly higher expression and/or activity of certain duodenal enzymes we observed in beef cattle preparations with respect to veal calves could be ascribed to age (16 to 24 months vs. approximately 8 months) or to the dietary regimen (the so called unifeed diet based on corn and concentrated meal vs. milk replacer plus hay and straw) or to one or more of the remaining factors mentioned above.

Another feature of this work was the apparent poor correlation between enzyme expression, as detected by immunoblotting,

and the rate of the *in vitro* metabolism of diagnostic probes. Aside from the problems already outlined in previous papers (Ioannides, 2006) arising from the use of nonspecific antibodies, the possible contribution provided by other enzyme systems to the metabolism of certain substrates remains to be established. For instance, microsomal prostaglandin H synthase (PGHS), which is able to co-oxidize a broad spectrum of chemicals such as phenolic compounds, aromatic amines, and polycyclic aromatic hydrocarbons (Strolin Benedetti *et al.*, 2006), was found to be highly expressed in bovine colon epithelial cells (Birkner *et al.*, 2003).

In conclusion, this study has demonstrated that a number of CYPs and of GST isoenzymes are expressed and active in the bovine duodenal mucosa along with some biotransformative pathways pointing to the presence of FMO, microsomal carboxylesterases, and UGT enzymes. Further studies at a molecular level are warranted to investigate factors regulating the xenobiotic metabolizing enzyme expression in the small intestine of cattle as well as to characterize the role not only of PGHS but also of drug transporters (for a review see Martinez *et al.*, 2008) in that tissue.

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