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Absolute quantification and modulation of cytochrome P450 3A isoforms in cattle liver

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ABSTRACT

In humans and laboratory animals, knowledge about cytochrome P450 (CYP) regulation and function is detailed and very extensive. However, CYPs have still been incompletely characterized in veterinary species so far. In this study, mRNA levels of three CYP3A enzymes (CYP3A28, CYP3A38 and CYP3A48) were measured in cattle liver by using quantitative real-time RT-PCR (qPCR) assays and an absolute quantification approach. In particular, the possible presence of breed-differences in CYP3A expression was investigated in five different meat cattle breeds (Charolais, CH; Piedmontese, PM; Blonde d'Aquitaine, BA; Marchigiana, MA; Valdostana, VALD) and the potential transcriptional effect of the prototypical inducer phenobarbital (PB) upon the CYP3A isoforms was evaluated.

Cytochrome P450 3A38 showed the highest amounts of gene copy numbers, followed by CYP3A48 and CYP3A28. Significant breed-differences in CYP3A gene abundances were found, and PB significantly up-regulated all the CYP3A isoforms. The data provide new information about CYP3A expression in cattle, particularly the heterogeneity in the pattern of expression of distinct hepatic CYP3As (CYP3A38 > 3A48 >> 3A28), the significant effect of breed, and their common up-regulation following the exposure to PB, although with different orders of magnitude.

Introduction

Cytochrome P450 (CYP) comprises a very large group of heme drug metabolizing enzymes (DMEs), mostly expressed in the liver, gastrointestinal tract, lung, and kidney. In humans, CYP 1–4 and 7 gene families play a major role in xenobiotic metabolism as well as in the oxidation of endogenous compounds (Zhou, 2008). The human CYP3A subfamily includes CYP3A4, 3A5 and two minor isoforms (CYP3A7 and 3A43) of which CYP3A4 is the most abundantly expressed hepatic CYP, representing on average ~14–30% of the microsomal CYP pool. Owing to their broad substrate selectivity, human CYP3A enzymes metabolize ~30–40% of all clinically used drugs (Klein and Zanger, 2013; Zanger and Schwab, 2013).

In contrast to human and rodent model species, data about CYP expression, regulation and function in veterinary species are far from being complete. This is rather peculiar for farm animals (and particularly for cattle) as, during their lifetime, this globally important food-producing species is exposed to xenobiotics of either natural (i.e. mycotoxins, plant secondary metabolites) or anthropogenic (i.e. drugs, pesticides and environmental pollutants) origin, and both the occurrence of potentially harmful drug–drug interactions and the accumulation of residues in edible tissues may represent a risk not only for the animal itself but also for consumers (Darwish et al., 2010; Fink-Gremmels, 2010).

Four cattle CYP3A genes are known and a new nomenclature, reflecting the true evolutionary relationships among bovine CYP3A isoforms, has been recently proposed: CYP3A28 (corresponding to GenBank acronym CYP3A4), CYP3A38 (CYP3A5) and CYP3A48 (CYP3A4 nifedipine oxidase; Zancanella et al., 2010). The fourth gene, annotated as CYP3A24, still refers to a predicted coding sequence. In cattle, CYP3A is involved in the metabolism of drugs pharmacologically relevant in bovine medicine, such as the macrocyclic lactone moxidectin (Dupuy et al., 2001), tiamulin and macrolide antibiotics (Zweers-Zeilmaker et al., 1999), as well as the ionophore monensin (Nebbia et al., 2001).

Some physiological factors, such as age, gender and breed, affect cattle CYP3A expression and/or biological activity (Dacasto et al., 2005; Greger et al., 2006; Giantin et al., 2008; Ashwell et al., 2011). Among xenobiotics, the non-specific and pleiotropic CYP inducer phenobarbital (PB) has been shown to increase hepatic CYP3A mRNA levels and apoprotein amounts in cattle (Zancanella et al., 2012), and similarly in humans, rodent model species, dogs and pigs (Madan et al., 2003; Anakk et al., 2004; Graham et al., 2006; Magnusson et al., 2006; Puccinelli et al., 2010). In contrast, unexpected results have been observed following the exposure to a known human CYP3A

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Table 1
Primer oligonucleotide sequences used for quantitative real-time RT-PCR, GenBank accession number and amplicon size.

Gene acronym	Primer	5′ → 3′ sequences or primer reference	GenBank ID	Amplicon size (bp)
CYP3A28	Forward	CCCCTTGAAAATAAGCAGTCA	NM_001099367	159
	Reverse	ATCAAGCCCCCTGAAATTCT		
CYP3A38	Forward	GTGGCTATGAGACCACTAGCACTT	NM_001075888	286
	Reverse	ACAGAGATTGGCACCGTCAACC		
CYP3A48	Forward	CCAGAGACGTGGTCTACTTTGA	NM_174531	76
	Reverse	CCCTACTCACCAGCAAGTACAGT		
RPLP0*	Forward	CAACCCTGAAGTGCTTGACAT	NM_001012682	227
	Reverse	AGGCAGATGGATCAGCCA		

bp, base pairs; CYP, cytochrome P450; ID, identity.
* From Robinson et al. (2007).

inducer like dexamethasone (Greger and Blum, 2007; Cantiello et al., 2009).

In humans, CYP3A4 and minor CYP3A isoforms contribute to xenobiotic metabolism, owing to overlap in substrate specificity, inducers and inhibitors; moreover, accumulating evidence has revealed that CYP3As exhibit marked ethnic and individual variability, with obvious consequences (Daly, 2006; Liu et al., 2007; Okubo et al., 2013). So far, no data about the effects of physiological factors or prototypical CYP inducers on the expression of cattle hepatic CYP3A individual isoforms have been published. The analysis of gene expression represents a first important step in the characterization of a gene and the corresponding coded protein biological activity.

The aim of this study was to assess, using an absolute quantitative real time RT-PCR (qPCR) approach, the constitutive expression of the chief CYP3A isoforms in cattle liver, their likely modulation in different meat cattle breeds, and their responsiveness to a prototypical CYP inducer such as PB.

Materials and methods

Samples

A total of forty-four liver samples, obtained from previous studies, were used. The experiments were run according to the European Community Directive 86/609, recognized and adopted by the Italian Government (DLgs 116/92). Experimental plans were approved by the Italian Ministry of Health. As a general rule, beef cattle were allotted into pens on a weight-basis and fed with Unifeed.

Possible breed-differences in mRNA abundance of CYP3A28, 3A38 and 3A48 were assayed in 37 male beef cattle of five different breeds, namely, Charolais (CH; $n = 10$; ~18–20 months old; ~700 kg body weight [bw]), Piedmontese (PM; $n = 7$; ~18–20 months old; ~650 kg bw); Blonde d'Aquitaine (BA; $n = 7$; ~18–20 months old; ~600 kg bw) (Giantin et al., 2008); Marchigiana (MA; $n = 8$; ~14–16 months old; ~490 kg bw) (Giantin et al., 2010); Valdostana (VALD; $n = 5$; ~13–18 months old; 490 kg bw) (Lopparelli et al., 2011).

The transcriptional effects of PB on individual specific CYP3A isoforms were measured on seven male Friesian cattle (10 months old, ~300 kg bw). Three animals served as controls (CTRL), while the other individuals (PB group) were given PB orally for 7 days at a dose rate of 18 mg/kg/day (Zancanella et al., 2012).

Liver samples were collected in an abattoir about 45 min after slaughter. Liver tissue aliquots (~100 mg) were obtained from the right side of the liver (same position for all animals), snap frozen in liquid nitrogen or alternatively placed in RNAlater (Life Technologies) and then stored at –80 °C until use.

Total RNA isolation and cDNA synthesis

For total RNA isolation, approximately 100 mg of frozen hepatic tissue was used, and the nucleic acid was extracted by using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The nucleic acid integrity was confirmed by denaturing agarose gel electrophoresis, while RNA quantity and purity were determined by using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). The RNA integrity number (RIN) and the absence of co-extracted genomic DNA were checked in all available samples using a 2100 Bionalyzer platform and a RNA 6000 Nano kit (Agilent Technologies). The reverse transcription of 1 μg of RNA was made using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) and random primers according to the purchaser's procedure (the final assay volume was 40 μL).

Preparation of CYP3A28, 3A38, 3A48 and ribosomal protein, large, P0 (RPLP0) standard curves

Primers for *Bos taurus* CYP3A28, 3A38 and 3A48 genes were designed ex novo, while for RPLP0 the primers previously published by Robinson et al. (2007) were used. The primer pairs design was performed by using the free Primer3Plus[1] software (Untergasser et al., 2007). Primer specificity was checked in silico using the NCBI Nucleotide Basic Local Alignment Search (BLAST) Tool, Primer-BLAST[2] (Ye et al., 2012); an OligoAnalyzer 3.1[3] was used to confirm the absence of primer dimer and hairpin formation.

Oligonucleotide primers were synthesized by Integrated DNA Technologies[4] and sequences are listed in Table 1. PCR amplification was performed using the TaKaRa LA Taq Hot Start DNA polymerase (TaKaRa Biotechnology Co.), on a TPersonal thermocycler (Biometra). Unpurified fragments were inserted into the TOPO TA pCR2.1 vector using the TOPO TA Cloning Kit (Life Technologies). Colonies were randomly picked up and the plasmid DNA (pDNA) was isolated using a QIAprep Spin Miniprep Kit (QIAGEN). At least five clones from three independent PCR reactions and cloning procedures were verified by sequencing (Macrogen). Subsequently, a larger amount of verified plasmids was produced using the Plasmid Midi Kit (QIAGEN). Plasmid concentrations were determined with a Qubit fluorometer and Qubit dsDNA BR Assay Kit (Life Technologies).

As circular pDNA is unsuitable as a standard in absolute qPCR (Hou et al., 2010), plasmids were linearized with FastDigest KpnI restriction enzyme (Thermo Scientific) and purified using the High Pure PCR Cleanup Micro Kit (Roche Applied Science). The concentration of eluted pDNA was measured with the Qubit fluorometer and aliquots were stored at –80 °C. The number of copies per volume was calculated using the average product molecular weight and Avogadro's constant. Eight order of magnitude serial dilutions of plasmids, ranging from 1×10^8 to 10 copies/2.5 μL (the sample volume loaded in the qPCR reaction) were used as standards for calibration curves.

Quantitative real time RT-PCR

The fluorescent dye SYBR Green I was initially used to carefully validate primer pairs. The reaction was performed, in a final volume of 20 μL using 12.5 ng of pooled liver cDNA, the Power SYBR Green PCR Master Mix (Life Technologies) and a Stratagene Mx3000P thermal cycler (Agilent Technologies), under standard qPCR conditions. The presence of specific amplification products was confirmed by dissociation curve analysis.

For each qPCR assay, negative controls (with total RNA or water as template) were run to exclude, respectively, genomic DNA and cDNA contamination during sample loading. Each primer pair was optimized in the 300–900 nM range to identify the primer concentration providing the highest sensitivity. Then, the web-based software ProbeFinder[5] was used to select a suitable Universal ProbeLibrary (UPL) probe (Roche Applied Science) to be used in combination with the target-specific primer pairs. Standard curves and samples analysis were made in a LightCycler 480 Instrument (Roche Applied Science) using standard qPCR conditions. The reaction consisted of 1X LightCycler 480 Probe Master (Roche Applied Science), 300 nM

[1] See: http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/ (accessed 29 July 2014).
[2] See: http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome (accessed 29 July 2014).
[3] See: http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx (accessed 29 July 2014).
[4] See: http://www.idtdna.com/site (accessed 29 July 2014).
[5] See: http://lifescience.roche.com/webapp/wcs/stores/servlet/CategoryDisplay?catalogId=10001&tab=&identifier=Universal+Probe+Library&langId=-1#tab-3 (accessed 29 July 2014).