CLONING AND SEQUENCING OF SOME RELEVANT DRUG METABOLISM GENES IN CATTLE: THE PROGRAMMA GALILEO 2003 SUMMARY REPORT

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Short title: cloning and sequencing of cattle liver genes

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ABSTRACT

Objective. Liver biotransformations play a critical role in the individual response to harmful substances and increasing importance has been recently given to drug metabolising enzymes (DMEs) gene expression analysis. In cattle, little is known about the expression of DMEs and their transcription factors (i.e. nuclear receptors, NRs). Therefore, some relevant bovine liver DMEs and NRs genes have been cloned and sequenced.

Method. Liver samples from control or phenobarbital-induced cattle were snap-frozen in liquid nitrogen and stored at –80°C. Total mRNA was extracted, purified and quantified. Specific primer pairs were designed from highly conserved regions among different mammalian species. Single band PCR products were cloned and plasmids containing the expected PCR fragment submitted to confirmatory enzyme restriction analysis and resolution on agarose gels. Therefore, they were amplified, purified and sequenced.

Results. Eleven cattle genes (CAR, $CYP1A_1$, CYP2B, CYP2C, CYP4A, GRα, HNF4α, PgP, PXR, RXRα and UDPGT) were submitted to GenBank. Some of them were then successfully used for liver gene expression analysis (northern blotting) in cattle treated, *in vivo*, with corticosteroids alone or in combination with steroids and β-agonists.

Discussion. Cattle are economically important worldwide, but little is known about the expression of liver DMEs and NRs. Their molecular characterization is important for understanding mechanisms of detoxification, or bioactivation, in basic, pharmacological and environmental research. Particularly, these genes might be used in the future to define some biomarkers of effect or dose response in cattle administered with illicit drugs.

Key words: cattle, drug metabolising enzymes, nuclear receptors, liver, cloning.

INTRODUCTION

In the living organism biotransformations, conjugation and transport of exogenous substances as well as of endogenous metabolic end-products (steroids, vitamins and fatty acids) are critical processes, which occur mainly in the liver. Drug-metabolising enzymes (DMEs) exert a key role in such a context and are usually classified, depending on their activity, in phase I (biotransformations), phase II (conjugation) or phase III (transport) enzymes (1,2).

The cytochrome P450 (CYP) superfamily comprises a large family of haem-containing phase I enzymes, which act mainly as monooxygenases. Among CYPs, those included in gene families 1-4 are mainly involved in drug metabolism, whereas other ones exert a prominent role in biosynthetic pathways (i.e., thromboxane and prostacyclin synthesis or steroidogenesis: 3). Given their importance, molecular mechanisms of CYP regulation have been intensively studied in humans and laboratory animals; nowadays, many characterised liver-enriched transcription factors [i.e. hepatocyte nuclear factors (HNF)1α and HNF4α] and nuclear receptors [NRs, i.e. pregnane X receptor (PXR), constitutive androstan receptor (CAR), retinoid X receptor (RXR)] appear to be involved in the control of CYP genes (4).

In veterinary species, there is little information concerning the liver DMEs expression and regulation compared to man, rat and mouse. Cattle represent an economically important food-producing species, which significantly impact on the worldwide animal meat market. In the European Union, the use of growth promoting agents in cattle is prohibited (Council Directive 96/22/EC and 96/23/EC); in spite of this, the use of anabolic steroids, β-agonists and corticosteroids, administered either alone or in combination, is still practiced. These drugs were proven to affect the catalytical activity of cattle liver DMEs (5-7).

Recently, increasing importance has been given to gene expression profiling, particularly in toxicogenomics studies (8). In the present preliminary study, which is part of projects aiming to ascertain the effects of illicit drugs upon cattle DMEs gene expression, some relevant bovine liver DMEs, NRs and transcription factors genes were cloned and sequenced.

MATERIALS AND METHODS

Four male, 10 months old Friesian calves, were orally given phenobarbital (PB) at a dose level of 18 mg/kg bw/day for 7 days. Other three ones remained untreated and processed as controls (K). At slaughtering, liver samples (about 100 mg) were collected in sterility, immediately snap-frozen in liquid nitrogen and stored at -80°C.

TRIzol® reagent (Invitrogen) was used for cell lysis and subsequent total RNA isolation. The concentration and purity of this latter was determined at the spectrophotometer (260 nm and 280 nm readings).

Selected genes were amplified by a RT-PCR technique. Gene specific primers pairs were designed after the alignment and identification of highly conserved regions among different mammalian species (*Homo sapiens, Mus musculus, Rattus norvegicus, Canis familiaris, Sus scrofa, Capra hircus*). In brief, 2,5 µg total RNA were reverse-transcribed in a final volume of 25 µl containing 0,4 mM of each dNTP, 200 units of MMLV reverse transcriptase, 10 mM dithiothreitol, 40 units of RNasin® ribonuclease inhibitor (all from Promega), 500 ng of oligo dT_{12-18} (Invitrogen), 50 mM Tris-HCl (pH 8,3), 75 mM KCl and 3 mM $MgCl_2$. Reverse transcriptase reactions from K and PB-induced calves were carried out in a T-Personal Biometra termocycler as follows: RNA denaturation (68°C for 5 min and, then, at 4°C for 5 min), RNA elongation at 40°C for 50 min and reverse transcriptase inactivation at 70°C for 10 min. Samples were therefore stored at -20°C until used.

Complementary DNA (cDNA) was amplified in a final reaction volume of 50 µl containing 20mM Tris-HCl, 50 mM KCl, 0,2 mM of each dNTP, 1,5 mM $MgCl_2$, 0.6 µM of each primer and 0,6 units of DyNAzyme EXT DNA polymerase (Finnzymes). Polymerase chain reactions (PCR) were carried out by using the following conditions: hot start at 95°C for 3 min; 35 cycles of PCR amplification at 95°C for 45 sec., 60°C for 1 min and 72°C for 1 min; then, a final extension step at 72°C for 7 min. This program was run for all genes, because specific primers pairs were designed *ad hoc* with a melting temperature next to 60°C.

Aliquots (10 µl) of the PCR amplification product were analyzed by agarose gel electrophoresis (1,2%) and visualized as a single bright band after ethidium bromide staining. These products were then cloned with TOPO TA cloning kit (Invitrogen); briefly, they were ligated into a pCR®2.1-TOPO® vector and the recombinant plasmid transformed into One Shot® TOP10F' chemically competent cells. Therefore, competent cells were incubated overnight at 37°C and plasmids, obtained from bacteria lysis, were finally purified with Midiprep kit (Qiagen, Milano, Italy).

The cDNA inserts were then excised from plasmid DNA with EcoRI (Fermentas) and cut with specific restriction enzymes. Fragments obtained were visualized as bright bands on a 1,2%-1,5% agarose gel stained with ethidium bromide; if fragments' length corresponded to the expected ones, the insert was sequenced after enzyme purification with ExoSAP (Celbio).

Finally, the bovine partial sequence was analyzed with Chromas Windows application program and then submitted to GenBank (http://www.ncbi.nlm.nih.gov/).

RESULTS

Four phase I ($CYP1A_1$, CYP2B, CYP2C, CYP4A) and a conjugative (UDP glucuronyltransferase) DMEs, *p*-glycoprotein (PgP), four NRs or transcription factors (CAR, HNF4α, PXR, RXRα,) and a cytosolic receptor (GRα) were successfully cloned, sequenced and submitted to GenBank. In Fig. 1, an example of PCR products amplified from cattle liver cDNA is reported. In Table 1, GenBank accession number, size and primers for PCR amplification for each *Bos taurus* gene are reported. In particular, for eight of these genes (*) bovine sequences were not yet available in GenBank.

The possible effect of illicit drugs upon the liver $CYP1A_1$ and CYP2C, CYP3A and $CYP2E_1$ gene expression level was investigated by using the northern blotting technique and specific probes obtained in the present study (for $CYP1A_1$ and CYP2C). A significant decrease in CYP3A gene expression, compared to controls, was observed in animals administered orally and intramuscularly with dexamethasone (DEX: -30%, $P<0.05$ and -34%, $P<0.01$, respectively). On the contrary, no significant differences *vs* controls were ever noticed in the liver of veal calves administered with a cocktail consisting of 17β-oestradiol, clenbuterol and DEX.

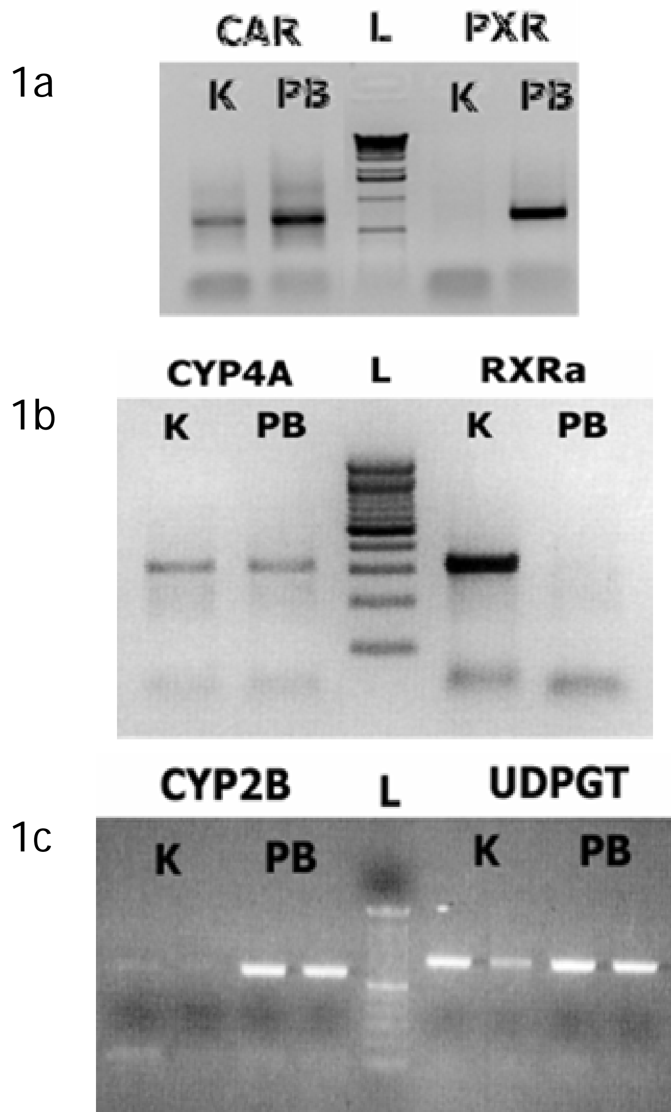

Figure 1a-c. Agarose gel electrophoresis (1.2%) of PCR products (cDNA) amplified starting from the liver of control and phenobarbital-induced cattle.

Legend: K: control; PB, Phenobarbital-induced; L, 1 kb ladder marker in Fig. 1a, 200 bp in Fig.1b and 100 bp in Fig.1c; CAR, constitutive androstan receptor; PXR, pregnane X receptor; CYP4A, cytochrome P450 4A; RXRa, retinoid X receptor alpha; CYP2B, cytochrome P450 2B; UDPGT, uridindiphosphoglucuronyl transferase.

Table 1: List of *Bos taurus* cloned and sequenced liver genes, GenBank accession number, size (expressed in base pairs, bp) and their 5'-3' reverse and forward primers.

*: cattle genes whose sequence was not available in GenBank.

GENE	GenBank Accession Number	SIZE (bp)	PRIMERS for PCR amplification
*CAR	AY789649	641	5'-GCAGACGTTCCTTGTTTTCC-3' 5'-CCTTGAGAAGGGAGATCTGGT-3'
*CYP1A$_1$	AY265991	946	5'-GCCTGAAGAGTCCACCAGAG-3' 5'-CTGCCAATCACTGTGTCCAG-3'
*CYP2B	DQ087595	676	5'-GCTCTACTCCGCCTTCTTGA-3' 5'-AGTTCTGGAGGATGGTGGTG-3'
*CYP2C	AY265992	704	5'-ACCAAGAATCCCTGGACCT-3' 5'-CATCTCACAGAAGGGTGGAAT-3'
*CYP4A	DQ100360	560	5'-AGAATGGAGAATGGGAGCAG-3' 5'-GGATCTCAGCACCATGATTG-3'
GRα	DQ192585	850	5'-GGAAGCTCGAATGAGGACTG-3' 5'-AGCAGTGACACCAGTGTTGG-3'
HNF4α	DQ192586	638	5'- CGTGGTGGACAAAGACAAGA-3' 5'- TACTGGCGGTCGTTGATGTA-3'
*PgP	AY789648	655	5'-GCTGTGGGACAGGTCAGTTC-3' 5'-CCGAGGTAGCTTCATCCAGA-3'
*PXR	AY789647	673	5'-TTTTCAGGAGGGCCATGAAG-3' 5'-AATCTCAGCTGGCACAACTC-3'
*RXRα	DQ100361	573	5'-ACGAGTCGTGTGGAAAACG-3' 5'-GGAACGAGCTGCTCATCG-3'
UGT1A$_4$	DQ192584	837	5'-AAGTCAACCTGCTCCTCCAA-3' 5'-AAGTCAACCTGCTCCTCCAA-3'

DISCUSSION

In recent years, an increasing interest toward the food safety has been noticed: as a direct consequence, the request of labelling regulations, useful to certify the quality of animal food-products, increased noteworthy. All of this is particularly true in the bovine meat products "from farm to fork"; in fact, the use of illicit drugs to increase animal performances has been clearly demonstrated in this food-producing species.

In toxicology, relevant importance is nowadays given to the risk assessment evaluation; such an objective is usually pursued by using biomarkers (BMs: 9). Drug metabolizing enzymes may represent either BMs of susceptibility (genetic polymorphism) or response/effect (induction/inhibition phenomena: 9-11). Recent advances in biotechnology provided an array of molecular technologies, including those allowing global gene expression analysis (genomics, transcriptomics: 12); these were successfully applied in drug metabolism studies, too (13-15).

As far as illicit treatments and DMEs are concerned, expression profiling of candidate genes, executed using either the low-density DNA microarray technology or the quantitative real-time PCR technique (or both) seem to be very helpful (16). In this respect, these data represent a preliminary step; future perspectives will essentially focus on the improvement of cattle DMEs genes database and the definition of an –omics technique for the screening of cattle treated with forbidden drugs.

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