Effects of dexamethasone, administered for growth-promoting purposes, upon the hepatic cytochrome P4503A expression in the veal calf

Effects of dexamethasone, administered for growth promoting purposes, upon the hepatic cytochrome P450 3A expression in the veal calf

Biochemical Pharmacology 77(2009):451-463
http://dx.doi.org/10.1016/j.bcp.2008.10.025

Effects of dexamethasone, administered for growth promoting purposes, upon the hepatic cytochrome P450 3A expression in the veal calf

Michela Cantiello[b], *Mery Giantin*[a], Monica *Carletti*[b], Rosa M. *Lopparelli*[a], *Francesca Capolongo* [a], *Frederic Lasserre* [d], *Enrico* Bollo[c], Carlo Nebbia [b], Pascal G.P. Martin [d], Thierry Pineau[d], Mauro Dacasto[a,*]

[a] *Dipartimento* di Sanita Pubblica, Patologia *Comparata* ed Igiene *Veterinaria, area* di *Farmacologia e* Tossicologia, Universita di Padova,
viale dell'Universita 16,I-35020 *Agripolis Legnaro* (Padova), Italy

[b] Dipartimento di Patologia Animale, sezione di Farmacologia e Tossicologia, Universita di Torino, via Leonardo da Vinci 44,
I-10095 Grugliasco (Torino), Italy

[c] Dipartimento di Patologia Animale, sezione di Anatomia Patologica, Universita di Torino, via Leonardo da Vinci 44,
I-10095 Grugliasco (Torino), Italy

[d]Laboratoire de Pharmacologie et Toxicologie, Equipe de Pharmacologie Moleculaire, Institut National de la Recherche Agronomique,
180 chemin de Tournefeuille, F-31931 Toulouse, France

Keywords:

Cattle
Dexamethasone
Liver
CYP3A
Illicit protocol
Nuclear receptors

A B S T R A C T

Dexamethasone (DEX) exerts its known anti-inflammatory and immunosuppressant activities through the interaction with the glucocorticoid receptor (GR). In human liver, DEX is metabolized by cytochrome P450 3A (CYP3A); moreover, it is among those xenobiotics which induce CYP3A itself. The transcriptional regulation of CYP3A involves GR and nuclear receptors (NRs). In cattle, DEX is used at low dosages as a growth promoter; besides, CYP3A is expressed in the liver. In the present study, the effects of two illicit DEX protocols upon liver CYP3A were investigated in the veal calf. Dexamethasone, administered *per* os (DOS) or injected intramuscularly (DIM) at growth promoting purposes, increased GR mRNA (+25.62% and +73.02% of CTRL for DOS and DIM, respectively), while tyrosine aminotransferase (TAT) and NRs gene expression profiles were unaffected; decreased CYP3A mRNA (-20.64% and -16.07% with QRT-PCR; -30.55% and -34.31% with Northern blotting); at the post-transla-tional level, decreased TAT activity (-19.84% and 44.34%), CYP3A apoprotein (-27.65% and —42.85%) and CYP3A-dependent enzyme activities (erythromycin N-demethylase, —78.89% and -23.87%; ethylmorphine N-demethylase, -44.26% and -28.37%; testosterone 6b-hydro-xylase, -44.60% and -18.07%; testosterone 2b-hydroxylase, -43.95% and -11.69%); by contrast, an increase (about 2-fold) of the urinary 6b-hydroxycortisol:cortisol ratio was observed in vivo. In summary, DEX modulates cattle liver CYP3A at pre- and post-translational level. Species-differences in GR-NRs-CYP3A regulation and in their response to differing DEX dosages might justify present results. Furthermore, the urinary 6b-hydroxycortisol:cortisol ratio is not useful to monitor in vivo CYP3A activity in DEX-treated individuals.

1. Introduction

Glucocorticoids (GCs) are steroid hormones including both natural and synthetic derivatives. In humans, cortisol (CTS) and cortisone represent main natural GCs, playing an outstanding role in the regulation of many homeostatic functions; dexamethasone (DEX) and prednisolone, instead, are well-known synthetic derivatives, whose major therapeutic usage derive from their anti-inflammatory and immunosuppressant properties [1]. Glucocorticoids exert their actions through the interaction with the glucocorticoid receptor (GR), a member of the nuclear hormone receptor superfamily which comprise also other steroid, retinoid and thyroid hormone receptors; once activated by GCs, GR translocates from the cytosol of target cells to the nucleus, where it binds to specific glucocorticoid response elements within the regulatory DNA sequences and, finally, transcriptionally regulates the expression of GC-responsive genes [2-4].

In humans, CTS and DEX undergo hepatic biotransformations mostly catalysed by the cytochrome P450 3A (CYP3A) [5,6]; this enzyme belongs to the cytochrome P450 superfamily of haem-containing drug metabolising enzymes, which are known to play a key role in the oxidative drug metabolism; cytochromes P450 are ubiquitous, but show their higher concentration in the liver, which is therefore considered the hinge of drug metabolism. The cytochrome P450 3A is the most abundant P450 subfamily in the human liver, in charge of the metabolism of most drugs and many endogenous compounds as well (i.e., steroids, bile acids). Many structurally diverse compounds, which are likely to be or not its substrates, are known to induce CYP3A expression [4,7-9]; among these ones there are also some GCs, such as CTS, corticosterone, DEX, prednisone and prednisolone [7]. In the past decade, many efforts were done to better understand the transcriptional regulation of CYP3A induction: at present, it is believed that GR and some members of the class II of nuclear receptors (NRs) such as the pregnane X receptor (PXR), the constitutive androstan receptor (CAR), the retinoid X receptor alpha (RXRa) and, to a lower extent, the hepatocyte nuclear factor 4-alpha (HNF4a) and the vitamin D receptor (VDR), are responsible of such a phenomenon; clearly, the regulation of CYP3A transcription is considered complex and multifactorial, occurring both at pre- or post-translational level, and involving many DNA and ligand-binding factors [4,7-14].

In veterinary medicine DEX is widely used, even coadministered with other drugs, for the treatment of inflammation, shock, stress and metabolic diseases of ruminants (i.e., ketosis); likewise to humans, it is usually preferred among available GCs because it lacks a sodium and water retention effect, has a high potency and long half-life [15,16]. Dexamethasone and other GCs are also frequently used illicitly as growth promoters (GPs), to improve quality and quantity of meat in cattle [15,17]; at first, they were used in cocktails with b-agonists and/or anabolic steroids, essentially for their interactions at target receptors level [18,19]. Nowadays, they are often used alone and at low dosages; in fact, lower GC concentrations improve feed intake, increase the body weight gain, reduce feed conversion and nitrogen retention and, finally, increase water retention and fat content [17,19]. Altogether, such a practice allows the achievement of the desired effect but a reduction of GP concentrations in biological fluids, thereby

representing a major problem for the European Community, that in 1996 forbid (Directives 96/22/EC and 96/23/EC) the use of anabolic compound and re-partitioning agents in cattle [15,17,20].

With regards to CYP3A expression, large inter-individual variations probably attributable to both environmental and genetic factors, have been reported in humans; moreover, species- and dose-dependent differences in its pattern of induction, following DEX exposure, have been noticed; finally, very few studies on the effect of DEX, administered at clinically relevant dosages, upon its expression and regulation have been published. All these evidences, together with the fact that most drugs are metabolized by CYP3A, justify the substantial interest on the relationship between DEX and CYP3A transcription, also for the risk of potential drug-drug interactions [4,7,8,16,21].

In cattle liver, CYP3A is expressed in terms of catalytic activity, protein and mRNA [22-29]; moreover, the basic molecular knowledge about CYP3A regulation in this food-producing species has been recently extended to most relevant NRs, and particularly PXR, CAR and RXRa [30,31]. Most veterinary pharmacologists and toxicologists affirm that more comparative studies focused on the expression and regulation of drug metabolising enzymes are needed [22,23,28]. Such an objective is of great particular concern when DEX is illicitly administered in cattle for growth promoting purposes; in fact, there is a lack of knowledge about its metabolic fate and distribution, its possible modulation of CYP3A expression and regulation as well as the potential consumers' risk due to the presence of uncontrolled drug residues in edible tissues. Therefore, in the present study the effect of DEX misuse upon CYP3A expression and regulation was investigated in the veal calf liver; the glucocorticoid was administered either intramuscularly (im) or *per* os and according to two protocols often used in farm practice.

2. Materials and methods

2.1. Chemicals

Dexamethasone sodium phosphate (Desashock1) and DEX isonicotinate (Voren1) were from Fort Dodge Animal Health (Bologna, Italy) and Boehringer Ingelheim Italia S.p.A. (Milano, Italy), respectively. Bovine serum albumin, glucose 6-phos-phate, glucose 6-phosphate dehydrogenase, $NADP^+$ and 4-aminophenol were from Boehringer Ingelheim (Ingelheim, Germany). Methanol and acetonitrile (high performance liquid chromatography, HPLC, grade) were from Mallinkrodt-Baker (Milano, Italy), whereas ammonium acetate and hydrochloric acid from Fluka (Milano, Italy). All other reagents used for the estimation of tyrosine aminotransferase (TAT) and CYP3A-dependent catalytic activities were obtained from Sigma-Aldrich (Milano, Italy). Anti-rat CYP3A1/2 monoclonal antibody was a kind gift of Dr. H.V. Gelboin, from the National Institute of Health and Cancer (Bethesda, Maryland, USA), whereas the sheep anti-mouse peroxidase-conjugated IgG was from GE Healthcare (Milano, Italy). Chloroform, isopropyl alcohol and ethyl alcohol were purchased from Carlo Erba Reagents (Val de Reuil, France); both TRIzol1 reagent and

agarose from Invitrogen (Paris, France), whereas [a^{32}P] deoxycytidine-5-triphosphate (dCTP, specific activity 3000 Ci mmor^{l}) was from ICN Biomedicals (Illkirch, France). Oligonucleotide primers for Real Time PCR were synthesised by Invitrogen (Paris, France).

2.2. Animals **and** experimental design

Fifteen male cross-bred Italian Friesian veal calves (15 days old, about 40 kg) were bought by local breeders, allowed to acclimatize for 2 months and fed, twice a day, with increasing amounts (from 13 up to 16 L day^{-1}) of a liquid milk replacer (Finition 60, Covel Italia, Marene, Italy); after 1 month, 0.5 kg day^{-1} of straw was included into the diet according to the European Commission rules (97/182/EC). When calves were about 130 days old, they were divided on a weight basis into three experimental groups, each consisting of five animals. The first one (176 ± 18 kg bw) was used as control (CTRL); the second one (166 ± 12 kgbw) was given, *per* os and for the 23 days, 0.4 mg day^{-1} DEX sodium phosphate (DOS); the last one (170 ± 16 kg bw) was intramuscularly injected, 14 and 21 days from the beginning of the oral administration, with 2 mg DEX isonicotinate (DIM). At 10 days from the end of oral DEX administration, calves were deprived of food overnight and then slaughtered with a captive bolt pistol. The treatment phase lasted for a total of 35 days (see Fig. 1). All experiments were carried out according to the European Economic Community Council Directive 86/609, recognised and adopted by the Italian Government (D.L. 27/01/1992 no. 116).

2.3. Sampling

2.3.1. Urine

Fifty milliliters urine were collected from each animal, with a kettle, early in the morning (7.30 ± 0.30 a.m.) when spontaneous urination occurred. Scheduled sampling times were: just before the beginning of the experiment (T_0); 15 days after

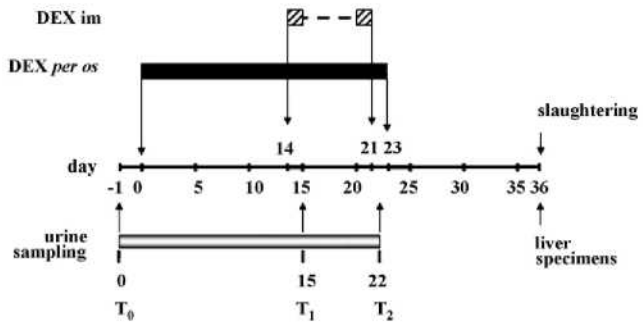

Fig. 1 - Treatment protocol and samples/specimens collection. Calves were divided into three experimental groups and either administered 0.4 mg day^{S1} dexamethasone (DEX) sodium phosphate *per* os and for 23 days (DEX *per* os, DOS group), or intramuscularly injected with 2 mg DEX isonicotinate 14 and 21 days far away from the onset of the oral administration (DEX im, DIM group); the third group was used as control (CTRL). Urine samples (50 mL) were withdrawn just before the onset of the experiment (T_0), 15 (T1) and 22 (T2) days after the beginning of the oral DEX administration (corresponding to the day after the first and the second DEX im injection, respectively). Liver specimens for both total RNA extraction and the preparation of microsomal subcellular fractions were collected at the slaughterhouse, after the bleeding step.

the onset of oral DEX administration and 1 day after the first im DEX injection (T1); 22 days after the onset of the oral DEX administration and 1 day after the second im DEX injection (T_2: see Fig. 1). Each sample, without preservative, was divided in 2 mL aliquots and stored at -20 8C until used.

2.3.2. *Liver specimens and* subcellular *fractions* At the slaughterhouse and after the bleeding step, the liver caudate lobe was removed from each animal. Small aliquots (about 100 mg each), for the total RNA extraction, were collected in sterility, immediately snap-frozen in liquid nitrogen and stored at -80 8C until used. The remaining part of the lobe was cut in small pieces, washed in chilled isotonic 1.15% KCl and brought to the laboratory within 2 h, wrapped in aluminium foils and kept in ice. Once in the laboratory, about 20-30 g of tissue free of pathological lesions were blotted dry, chopped and homogenised in a Potter homogeniser. Hepatic subcellular fractions were isolated by differential ultracen-trifugation as reported elsewhere [27]. Microsomal pellets were resuspended with a 0.1 M phosphate buffer solution containing 0.1 M EDTA and glycerol 20% (v/v); therefore, both cytosolic and microsomal fractions were frozen in liquid nitrogen and stored at -80 8C. Protein concentration was determined [32] by using bovine serum albumin as standard.

2.4. Cytochrome P450 3A **and** NRs quantitative real time polymerase *chain* reaction (Q RT-PCR)

2.4.1. Total RNA isolation

Total RNA was isolated from frozen bovine liver samples by

using the TRIzol1 reagent, according to the manufacturer's instructions. Briefly, 1 mL TRIzol1 was added to about 80 mgof liver tissue in a Lysing Matrice Tube (Qbiogene, MP Biomedicals, Illkirch, France), and immediately homogenised twice for 20 s using Fast-Prep1 FP120 (Qbiogene, MP Biomedicals, Illkirch, France); therefore, samples were purified, at 4 8C, with a standard phenol-chloroform extraction. Total RNA concentration and quality (assessed by the 260/280 and 260/ 230 nm absorbance ratios) were determined by using the Nanodrop ND-1000 spectrophotometer (Labtech France, Paris, France); the nucleic acid quality was confirmed by denaturing gel electrophoresis.

2.4.2. *Reverse transcription*

Total RNA was reverse transcribed by means of the Two High Capacity cDNA Archive Kit (Applied Biosystems, Courtaboeuf, France); the reaction mixture (20 mL as final volume) was prepared following the purchaser's procedure and by adding 2 mg of total RNA. The reaction was performed in a 96-Well GeneAmp1 PCR System 9700 (Applied Biosystems, Courtaboeuf, France), 10 min at 25 8C and, then, 2 h at 37 8C.

2.4.3. *Quantitative real time polymerase chain* reaction

Bos taurus mRNA sequences of candidate genes were obtained from GenBank and Ensembl Genome Browser web sites (http:// ncbi.nlm.nih.gov/ and http://www.ensembl.org/, respectively). Primers sequences for the Q RT-PCR were designed by using the Primer Express^{M} Software (version 2.0, Applied Biosystems, Courtaboeuf, France), with the following settings: the melting temperature for primers was set between 58 and 60 8C, and the G/C content was kept in the 20-80% range. Oligonucleotides were designed at the exon-exon junctions, to avoid genomic DNA amplification, and primers sets were subjected to primer test analysis (Primer Test Document application in Primer Express^{M} Software) to exclude dimers synthesis. Primers and reference sequences accession numbers are shown in Table 1. Primers sequence specificity, for each candidate gene, was checked against the NCBI BLAST database, by using the agarose gel electrophoresis as well as the analysis of melting curves. Each set of primers was optimised in the 300-900 nM range. This optimisation step identified the primers concentration providing highest sensitivity and specificity for each target sequence. Calibration curves were obtained after the amplification of decreasing amounts of a cDNA pool, diluted at 10-fold intervals, to evaluate the Q RT-PCR performances. The quality of each Q RT-PCR assay could be gathered from standard curve slopes and correlation coefficients. The PCR efficiency (E_x) was determined using the equation $E_x = io\sim^{1/slope}$. Bovine b-actin was chosen as the reference gene, as its amplification efficiency was proved to be approximately equal to target genes; besides, slight differences noticed in its expression profile were never statistically significant. Quantitative real time RT-PCR was performed by using the ABI Prism 7000 Sequence Detector System (Applied Biosystems, Courtaboeuf, France). The reaction was performed in a 96-wells microtiter plate. A reaction mixture (20 mL), consisting of 1x Power SYBR1 Green PCR Master Mix (Applied Biosystems, Courtaboeuf, France) and an optimised concentration of each primers set, was added to each well together

with 5 mL of cDNA diluted 1:20, in order to obtain a final volume of 25 mL. The thermal cycler conditions were 95 8C for 10 min to activate Amplitaq Gold DNA Polymerase, denaturation at 95 8C for 15 s and anneal/extension at 60 8C for 1 min (40 cycles). Messenger RNA relative quantification was performed by using the DDCt method [33] and data were expressed as fold-change compared to CTRL values.

I Table 1 - Reference sequences and primers for Q RT-PCR.

Gene	GenBank accession no.	5′ → 3′ primer sequence	Primer length (bp)	Amplicon size (bp)
CYP3A4	NM_174531	F: GCC AGA GCC CGA GGA GTT R: GCA GGT AGA CGT AAG GAT TTA TGC T	18 25	77
CAR	NM_001079768	F: GAA GGA CAT GAT CCT ATC GAC AGA R: CGT CGC TGG GCC TGT CT	24 17	63
PXR	XM_582516	F: TGA AGG CCT ACA TCG AGT TCA AC R: GGC CAT GAT CTT CAG GAA CAA	23 21	68
RXRα	XM_881943	F: GCC TCA ATG GTG TCC TCA AAG R: AGC TGT ACA CCC CGT AGT GCT T	21 22	120
HNF4α	NM_001015557	F: CGA CAA CGA GTA CGC CTG C R: CCC CTT GGC ATC TGG GTC	19 18	58
GR	ENSBTAT00000025941	F: AGC AGT GGA AGG ACA GCA CAA R: TTC TTC GAA TTT TAT CAA TGA TAC AAT CAT	21 30	71
TAT	NM_001034590	F: CTG AAG TTA CCC AAG CAA TGA AAG R: CCT CCC GAC TGG ATA AGT AGC C	24 22	90
ACTB	NM_173979	F: GTC GAC ACC GCA ACC AGT T R: AAG CCG GCC TTG CAC AT	19 17	85

ACTB: bovine β-actin; bp: base pairs; CAR: constitutive androstan receptor; CYP3A4: cytochrome P450 3A4; F: forward; GR: glucocorticoid receptor; HNF4α: hepatocyte nuclear factor 4-alpha; PXR: pregnane X receptor; R: reverse; RXRα: retinoid X receptor alpha; and TAT: tyrosine aminotransferase.

2.5. Cytochrome P450 3A *Northern* blotting

2.5.1. Northern blotting probe synthesis

The reverse transcription of total RNA (2 mg) from cattle liver was performed in a final volume of 25 mL, containing 50 mM Tris-HCl pH 8.3, 75 mM KCl,3 mM $MgCl_2$,10 mM dithiothreitol, 0.25 mM deoxynucleotide triphosphates (dNTPs, Invitrogen, Paris, France), 40 U ribonuclease inhibitor (RNasin1, Promega, Madison, WI), 500 ng oligodT_{12}-18 (Invitrogen, Paris, France) and 200 U of a murine Moloney leukaemia virus reverse transcriptase (Promega, Madison, WI). The reaction was carried out at present conditions: 68 8C for 5 min and, then, 5 min at 4 8C (RNA denaturation), 50 min at 40 8C (RNA elongation), and reverse transcriptase inactivation at 70 8C. Cytochrome P450 3A and 36b4 forward (F) and reverse (R) primers (CYP3A F: 5^0-ATAGAACTCGTGGCCCAAAG-3^0; CYP3A R: 5^0-TAACTGGGGTGGATGGAGAG-3^0; 36b4 F: TCGTTGGAGT-GACATCGTCT; 36b4 R: GACCGAATCCCATATCCTCA) were then designed with Primer3 (http://frodo.wi.mit.edu/cgi-bin/ primer3/primer3_www.cgi), by using available bovine sequences. Then, a PCR amplification was performed in a total volume of 50 mL, containing 5 mL of cDNA template, lx buffer (Promega, Madison, WI), 200 mM of each dNTP (Invitrogen, Paris, France), 600 nM of each F/R primers (Eurobio, Courtaboeuf, France), 1.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Promega, Madison, WI), by using the following basic program: hot start at 95 8C for 3 min; 35 cycles at 95 8C for 45 s, 60 8C for 1 min, 72 8C for 1 min; then, a final step at 72 8C for 10 min. Finally, PCR products (903 and 1000 bp for CYP3A and 36b4, respectively), were cloned with the TOPO TA cloning kit (Invitrogen, Paris, France); briefly, they were inserted into a pCR12.1-TOPO1 vector and recombinant plasmids transformed into One Shot1 TOP10F' chemically competent cells. Recombinant plasmids were then extracted from bacteria and submitted to enzymatic digestion to obtain the cDNA insert. This latter was purified by agarose gel electrophoresis and spectrophotometrically quantified.

2.5.2. Northern blot analysis

Total RNA was extracted from bovine liver samples as previously reported, except for the use of an Ultra-Turrax1 T8 (IKA1Werke, Staufen, Germany) homogeniser. Twenty micrograms of total RNA were loaded on 1.2% agarose denaturating gel. After the electrophoresis, RNA samples were submitted to a capillary transfer onto a nylon membrane (Nytran1 SuPerCharge, Schleicher & Schuell, Ecquevilly, France). Complementary DNA probes were radio-labelled with [a^{32}P]dCTP using Multi random Priming System Ready-to-go^{T}M DNA Labelling Beads (Amersham Biosciences, Piscataway, NJ) according to the suppliers' protocol and, finally, purified using ProbeQuant G-50 micro Columns (Amersham Biosciences, Piscataway, NJ). Blots were then pre-hybridised and hybridised with the labelled cDNA overnight at 42 8C in a Hybridization Mini-Oven (Qbiogene, MP Biomedicals, Illkirch, France), then washed and exposed to a phosphor screen (Storage Phosphor

Screen Kodak - Amersham Biosciences, Piscataway, NJ). After the autoradiography, the hybridization intensity was measured by using a Storm1 Phosphorimager (Molecular Dynamics, GE Healthcare, Munich, Germany), and spots quantified by the means of Image Quant Software (Molecular Dynamics, GE Healthcare, Munich, Germany). For Northern blot data normalization, the amount of 36b4 mRNA level was measured by using a specific probe. Results were expressed as a ratio between CYP3A and the respective 36b4 values.

2.6. Cytochrome P450 3A immunoblotting

Microsomal proteins (30 mg) were firstly separated by a 10% sodium dodecyl sulphate polyacrilamide gel electrophoresis in a Bio-Rad Miniprotean cell (Bio-Rad, Milano, Italy) and, then, transferred to Protran1 nitrocellulose membranes (Whatman Schleicher & Schuell, Dassel, Germany) as previously reported [34,35]. Therefore, membranes were incubated with an anti-rat $CYP3A_{1/2}$ monoclonal antibody (final dilution of 1:500) and, then, with a peroxidase-conjugated sheep anti-mouse IgG (final dilution of 1:5000). The band immunodetection was performed by using a chemiluminescence kit (SuperSignal1 West Pico Chemiluminescent Substrate, Pierce, Milano, Italy), according to manufacturer's instructions. Immunopositive bands were captured by the Agfa ScanWise^{T}M2.0. software for the Agfa Snapscan 1212U scanner and their optical density analysed by the ImageJ 1.34 s image analysis software. On each minigel, two further samples were blotted, consisting of microsomal proteins obtained from the liver of DEX-induced or control (untreated) rats: the former was used as a molecular marker; the second one, to allow the densitometric analysis as well as the normalization of cattle results (particularly for any developing and fixing discrepancies among different blots). Results were expressed as arbitrary units.

2.7. Tyrosine aminotransferase and CYP3A enzyme activities

2.7.1. Tyrosine aminotransferase

Tyrosine aminotransferase activity was determined in cyto-sols by using 10 mM a-chetoglutaric acid and 50 mM pyridoxal-phosphate (final concentrations) as a substrate and a cofactor, respectively [36,37]. Results were expressed as $nmolmin^{-1}$ $mgprotein^{-1}$.

2.7.2. Erythromycin and ethylmorphine N-demethylation Microsomal erythromycin and ethylmorphine N-demethylase catalytic activities (ERDEM and ETDEM, respectively) were measured by an Uvikon 941 spectrophotometer (Kontron, Milano, Italy) after an aerobic incubation step at 37 8C at previously published conditions [27], with 1 mM erythromycin or 6 mM ethylmorphine as final substrate concentrations. For both catalytic activities, results were expressed as nmol min^{-1} mg $protein^{-1}$.

2.7.3. Testosterone (TST) hydroxylation

The cytochrome P450 3A-dependent TST hydroxylations at the 2b- and 6b-position (2b-OHTST and 6b-OHTST, respectively), were measured (as nmol min^{-1} $mgprotein^{-1}$) by HPLC [38], by using 250 mM TST as the final substrate concentration.

2.7.4. *Urinary* 6b-hydroxycortisol:cortisol (6b-OHCTS/CTS) ratio

Free urinary CTS ($ngmL^{-1}$) was quantitatively measured by the Urinary Cortisol direct immunoenzymatic kit (DIA.METRA, Segrate, Milano, Italy), according to the manufacturer's instructions. A competitive enzyme immunoassay kit (Stabili-gen, Nancy, France) was used to measure urinary 6b-hydroxycortisol (6b-OHCTS) concentrations ($ngmir^{1}$). The 6b-hydroxycortisol:cortisol ratio was obtained dividing, for each animal and at every sampling time, the 6b-OHCTS concentration by the respective CTS one.

2.8. Other *parameters*

2.8.1. Liver absolute and *relative* weights

At the slaughterhouse, just before to proceed specimens collection, each calf liver was weighed (kg). The organ relative weight was calculated by using the following formula: liver weight (kg)/animal body weight (kg) x 100. Results were expressed either as kilograms (absolute organ weight) or percent (relative organ weight).

2.8.2. Total cytochrome P450 (P450) content

The amount of total P450 (nmol mg $protein^{-1}$) was determined as the carbon monoxide difference spectrum of sodium dithionite-reduced microsomal suspensions [39].

2.9. Statistics

Data were expressed as mean values ± standard error. Statistical analysis (Graph Pad Instat 2.01, San Diego, CA, USA) was performed by the nonparametric Mann-Whitney Utest, with the level of significance set at $p < 0.05$.

3. Results

3.1. Effects ***of*** illicit **DEX upon** GR, **TAT,** NRs **and** CYP3A *gene* expression *profiles*

Glucocorticoids exert their actions through their binding with GR [2]. The effect of DEX, if used as a GP, upon GR gene is reported in Fig. 2. Increasing GR mRNA levels were noticed in both DEX-treated groups (+25.62% and +73.02% of CTRL value for DOS and DIM, respectively), reaching the significance level ($p < 0.05$) in DIM one.

Several studies demonstrate that the tyrosine aminotransferase gene, coding for a key metabolic enzyme, is regulated by GR [40,41]; consequently, TAT gene expression profile is usually measured whenever there is a need to monitor, in vivo and in the whole organism, changes in GR-mediated gene expression [42]. In the present study, DEX prompted on a slight (not significant) decrease of TAT mRNA (see Fig. 2).

The glucocorticoid receptor, besides glucocorticoid response elements, is likely to interact with other transcription factors such as the nuclear factor-kB and NRs *[2-4]*. The pregnane X receptor, PXR, CAR and RXRa represent those NRs mostly involved in CYP3A regulation either in man and laboratory species [7,8]; moreover, these same transcription factors are expressed in cattle liver, too [30,31]. Unlike GR, DEX administration did not affect, independently from the chosen protocol, PXR, CAR, and RXRa gene expression profiles; neither that of HNF4a, another NR known to contribute, with PXR and/ or CAR, to CYP3A transcriptional regulation [7] (see Table 2).

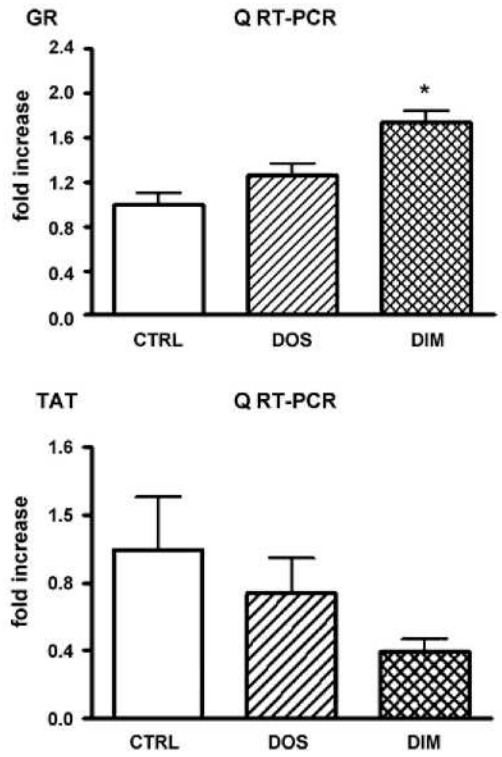

Fig. 2 - Effect of the illicit dexamethasone (DEX) administration upon glucocorticoid receptor (GR) and tyrosine aminotransferase (TAT) gene expression profiles in the liver of veal calves. Total RNA was isolated from liver specimens obtained from control (CTRL) or DEX-treated cattle, in which the corticosteroid was administered orally (0.4 mg day^{S1} DEX sodium phosphate for the 23 days: DOS) or injected intramuscularly (2 mg DEX isonicotinate, 14 and 21 days from the beginning of the oral administration: DIM). For each animal, the amount of both GR and TAT mRNAs were measured by Q RT-PCR by using 25 ng cDNA, and then normalised to that of b-actin, a housekeeping gene. Data (fold-changes of CTRL values) are expressed as means W standard error. *: $p < 0.05$ vs CTRL (nonparametric Mann-Whitney U-test).

Likewise to human and rodent model species [7,8], CYP3A gene is expressed in cattle liver [30,31]; in former species, CYP3A expression might be induced by several compounds including some GCs and, notably, DEX [7]. The DEX effect upon liver CYP3A gene expression, measured either by Q RT-PCR or Northern blotting, is shown in Fig. 3. Quite unexpected both DEX illicit protocols substantially reduced CYP3A gene expression, although a significant reduction ($p < 0.05$) was noticed only with the Northern blotting. In DOS and DIM groups the decrease accounted for -20.64% and -16.07% (Q RT-PCR) and -30.55% and -34.31% (Northern blotting) of CTRL values, respectively.

3.2. Effects ofillicit **DEX upon** CYP3A apoprotein

Previously published studies demonstrated the presence of a single immunoreactive cross-reacting band, in immunoblots of cattle liver microsomes, by using several mono- or

Table 2 - Effect of the illicit dexamethasone (DEX) administration upon nuclear

NR gene	CTRL	DOS	DIM
CAR	1.00 ± 0.11	1.09 ± 0.10	1.04 ± 0.16
PXR	1.00 ± 0.18	1.12 ± 0.19	1.18 ± 0.29
RXRα	1.00 ± 0.13	1.05 ± 0.14	0.99 ± 0.06
HNF4α	1.00 ± 0.19	1.01 ± 0.13	1.28 ± 0.20

Total RNA was isolated from liver specimens from control (CTRL) or DEX-treated cattle, in which the corticosteroid was administered orally (0.4 mg day^{-1} DEX sodium phosphate for the 23 days: DOS) or intramuscularly injected (2 mg DEX isonicotinate, 14 and 21 days from the beginning of the oral administration: DIM). For each animal, the mRNA amount of every gene was measured by Q RT-PCR, by using 25 ng cDNA, and normalised to that of the housekeeping gene β-actin. Data (fold-changes of CTRL values) are expressed as means ± standard error S.E. CAR: constitutive androstan receptor; PXR: pregnane X receptor; RXRα: retinoid X receptor alpha; and HNF4α: hepatocyte nuclear factor 4-alpha.

polyclonal antibodies [22-24,27]; likewise, in the present investigation an unique band, corresponding to CYP3A protein, was clearly detected (see Fig. 4). Interestingly, the pre-transcriptional inhibitory effect of DEX upon CYP3A mRNA was reflected at the protein level; in fact, an overall significant ($p < 0.05$) reduction of CYP3A apoprotein amount was noticed in DEX-treated animals (-27.65% and -42.85% of CTRL value for DOS and DIM groups, respectively).

3.3. Effects *of* illicit **DEX upon TAT and CYP3A-dependent** catalytic activities

Tyrosine aminotransferase activity is considered a suitable assay to investigate effects of xenobiotics (including DEX) upon GR in vivo [42]; therefore, TAT catalytic activity was measured in cytosols from control and DEX-treated calves to confirm GR, PXR and TAT transcriptional data. The enzyme activity was decreased by DEX in DOS (-19.84%) and DIM (-44.34%, $p < 0.05$) group (see Table 3).

Post-translational effects of DEX upon CYP3A-dependent catalytic activities were investigated in liver microsomes by using substrates which have been shown to be either N-dealkylated (erythromycin and ethylmorphine) or hydroxy-lated (TST) by this relevant P450 subfamily in cattle, too [22-24,27,28,38]. All considered enzyme activities (ERDEM, ETDEM, 6b-OHTST and 2b-OHTST) were decreased by DEX, independently from the illicit protocol used (see Table 3). These reductions were more relevant and significant in DOS group ($p < 0.01$ for ERDEM; $p < 0.05$ in the case of ETDEM, 6b-OHTST and 2b-OHTST); in particular, they accounted for a -78.89%, -44.26%, -44.60% and -43.95% of CTRL value, respectively. As far as DIM is concerned, the reduction was less than -30.00% (-23.87%, -28.37%, -18.07% and -11.69% for ERDEM, ETDEM, 6b-OHTST and 2b-OHTST, respectively).

In recent years, urinary 6b-OHCTS/CTS ratio has been proposed as a non-invasive indicator of hepatic CYP3A4 activity [5,43,44]. In light of this, such a ratio was measured in urine samples

withdrawn from each animal at the beginning of the in vivo animal phase and at scheduled time points (see Fig. 1). The glucocorticoid, independently from the chosen

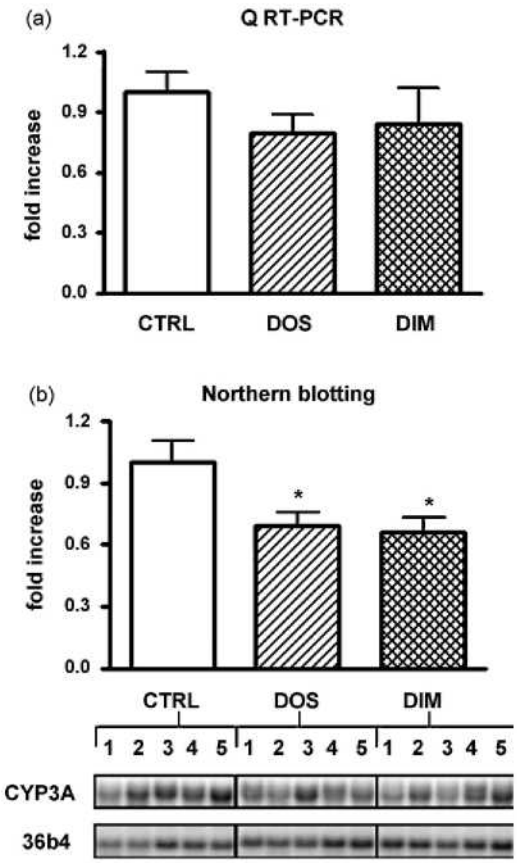

Fig. 3 - Effect of the illicit dexamethasone (DEX) administration upon the liver cytochrome P450 3A (CYP3A) gene expression in the veal calf. Total RNA was isolated from liver specimens obtained from control (CTRL) or DEX-treated cattle, in which the corticosteroid was administered orally (0.4 mg day^{S1} DEX sodium phosphate for the 23 days: DOS) or injected intramuscularly (2 mg DEX isonicotinate, 14 and 21 days from the beginning of the oral administration: DIM). For each animal, CYP3A mRNA was measured either by a Q RT-PCR approach (a) or Northern blotting (b). In the former, 2 mg of total RNA were reverse transcribed and 25 ng cDNA were used; for Northern blotting, 20 mg of total RNA, electrophoresed and transferred onto a nylon membrane, were hybridised with [a^{32}P]dCTP-labelled cDNA probes for CYP3A and then exposed to a phosphor screen. In both cases, CYP3A mRNA levels were respectively normalised to those of b-actin and 36b4, two housekeeping genes. Data (fold-changes of CTRL values) are expressed as means W standard error.*: $p < 0.05$ vs CTRL (nonparametric Mann-Whitney U-test).

protocol, caused a significant reduction of both 6b-OHCTS and CTS urinary concentrations ($p < 0.01$ vs CTRL, except for the DOS group at T1, where a $p < 0.05$ value was measured; see Table 4). As a direct consequence, an overall significant ($p < 0.05$) increase of 6b-OHCTS/CTS ratio was

observed in DOS, whereas for DIM it was recorded only at T_2. Whenever a significant increase of 6b-OHCTS/CTS ratio occurred, it was around 2-fold the CTRL vale.

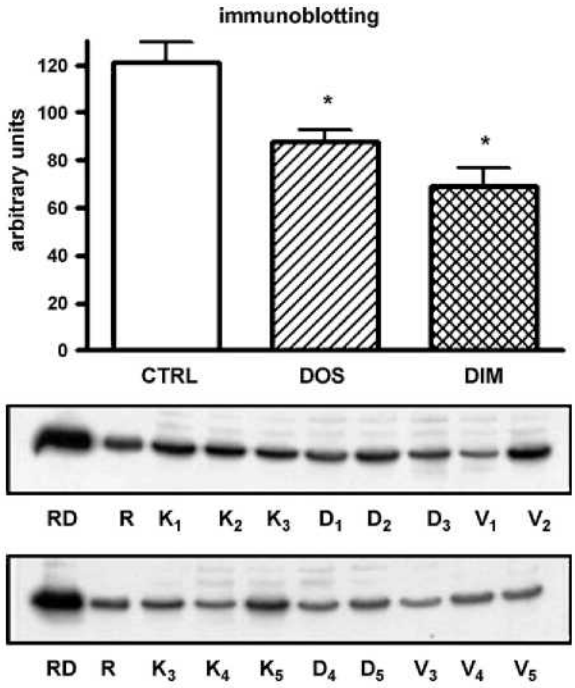

Fig. 4 - Effect of the illicit dexamethasone (DEX) administration upon the liver cytochrome P450 3A (CYP3A) apoprotein in the veal calf. Microsomal subcellular fractions were obtained from the liver of control (CTRL) or DEX-treated cattle, in which the corticosteroid was administered orally (0.4 mg day[S1] DEX sodium phosphate for the 23 days: DOS) or injected intramuscularly (2 mg DEX isonicotinate, 14 and 21 days from the beginning of the oral administration: DIM). Microsomal proteins (30 mg) were firstly separated by a 10% SDS-PAGE and transferred to nitrocellulose membranes; these ones were firstly incubated with an anti-rat $CYP3A_{1/2}$ monoclonal antibody (1:500 final dilution) and, then, with a peroxidase-conjugated sheep anti-mouse IgG (1:5000 final dilution). The band immunodetection and optical density analysis were performed by using a chemiluminescence kit and the ImageJ 1.34 s image analysis software, respectively. Two samples, consisting of microsomal proteins from the liver of untreated or DEX-induced rats, were run on each blot, to allow the densitometric analysis and the normalization of cattle results and as a molecular marker, respectively. Results are expressed as mean arbitrary units W standard error. *: $p < 0.05$ vs CTRL (nonparametric Mann-Whitney *U*-test). RD: liver microsomes obtained from DEX-induced rats; R: rat liver microsomes; K_{1-5}: liver microsomes from CTRL cattle; *D1-5:* liver microsomes from cattle administered DEX per os (DOS group); and *V1-5:* liver microsomes from cattle intramuscularly injected with DEX (DIM group).

3.4. *Effects of dexamethasone upon the* absolute and relative liver weight, and total cytochrome P450 content

The effect of DEX on absolute and relative liver weights as well as on total P450 content is reported in Table 5.

No differences were ever found between CTRL and DEX-treated animals.

Table 3 - Effect of the illicit dexamethasone (DEX) administration upon TAT and CYP3A catalytic activities in the liver of veal calves.

Catalytic activity	CTRL	DOS	DIM
TAT	8.57 ± 0.35	6.87 ± 0.21	4.77 ± 0.08*
ERDEM	0.80 ± 0.08	0.17 ± 0.08**	0.61 ± 0.19
ETDEM	1.99 ± 0.32	1.11 ± 0.13*	1.42 ± 0.36
6β-OHTST	0.85 ± 0.14	0.47 ± 0.03*	0.70 ± 0.15
2β-OHTST	0.50 ± 0.09	0.28 ± 0.02*	0.44 ± 0.13

Cytosolic and microsomal subcellular fractions were obtained from the liver of control (CTRL) or DEX-treated cattle, in which the corticosteroid was administered orally (0.4 mg day^{-1} DEX sodium phosphate for the 23 days: DOS) or injected intramuscularly (2 mg DEX isonicotinate, 14 and 21 days from the beginning of the oral administration: DIM). The tyrosine aminotransferase activity, CYP3A-dependent rate of N-dealkylation of erythromycin and ethylmorphine (ERDEM and ETDEM), as well as the hydroxylation of testosterone at the 6β- and 2β-positions (6β-OHTST and 2β-OHTST), were measured as reported in Section 2.7. Results ($nmol^{-1}$ min mg $protein^{-1}$) are expressed as mean ± standard error. 2β-OHTST: 2β-hydroxytestosterone hydroxylase; 6β-OHTST: 6β-hydroxytestosterone hydroxylase; ERDEM: erythromycin N-demethylase; ETDEM: ethilmorphine N-demethylase; and TAT: tyrosine aminotransferase.

* $p < 0.05$ *vs* CTRL (nonparametric Mann–Whitney *U*-test).

** $p < 0.01$ *vs* CTRL (nonparametric Mann–Whitney *U*-test).

4. Discussion

In humans, several clinical studies suggest how the oxidation of drugs and steroids might be perturbed by synthetic and

Table 4 - Effect of the illicit dexamethasone (DEX) upon urinary 6b-hydroxycortisol (6b-OHCTS), cortisol (CTS), and 6b-hydroxycortisol to cortisol (6b-OHCTS/CTS) ratio in the liver of veal calves.

Time	Groups	6β-OHCTS	CTS	6β-OHCTS/ CTS ratio
T_0	CTRL	60.98 ± 6.20	20.76 ± 1.59	2.94 ± 0.17
	DOS	60.59 ± 11.51	27.78 ± 8.85	2.23 ± 0.36
	DIM	92.75 ± 23.10	23.00 ± 8.48	4.24 ± 0.56
T_1	CTRL	71.00 ± 8.17	27.01 ± 4.40	2.82 ± 0.43
	DOS	$27.67 \pm 5.52^{*}$	$4.93 \pm 0.71^{**}$	$5.59 \pm 0.75^{*}$
	DIM	$20.26 \pm 3.22^{**}$	$5.69 \pm 1.54^{**}$	3.22 ± 0.30
T_2	CTRL	55.21 ± 6.63	33.41 ± 5.18	1.72 ± 0.11
	DOS	$10.58 \pm 2.99^{**}$	$4.41 \pm 1.19^{**}$	$3.46 \pm 0.79^{*}$
	DIM	$9.45 \pm 1.26^{**}$	$3.51 \pm 0.91^{**}$	$3.19 \pm 0.54^{*}$

Fifty milliliters urine were collected, early in the morning when spontaneous urination occurred, from control (CTRL) or DEX-treated cattle, in which the corticosteroid was administered orally (0.4 mg day^{-1} DEX sodium phosphate for the 23 days: DOS) or intramuscularly injected (2 mg DEX isonicotinate, 14 and 21 days from the beginning of the oral administration: DIM). Scheduled sampling times were: just before the beginning of the experiment (T_0); 15 days after the onset of the oral DEX administration and 1 day after the first im DEX injection (T_1); then, 22 days after the onset of the oral DEX administration and 1 day after the second im DEX injection (T_2). Data (ng mL^{-1}) are expressed as means ± stan-standard error.

* $p < 0.05$ *vs* CTRL (nonparametric Mann–Whitney *U*-test).

** $p < 0.01$ *vs* CTRL (nonparametric Mann–Whitney *U*-test).

Table 5 - Effect of the illicit dexamethasone (DEX) upon absolute and relative liver weights

Parameter	CTRL	DOS	DIM
Absolute liver weight	4.40 ± 0.15	3.92 ± 0.13	4.32 ± 0.24
Relative liver weight	2.00 ± 0.07	2.06 ± 0.12	2.02 ± 0.10
P450 content	0.18 ± 0.02	0.20 ± 0.02	0.17 ± 0.04

Veal calves were illicitly treated with DEX either orally (0.4 mg day^{-1} DEX sodium phosphate for the 23 days: DOS) or intramuscularly (2 mg DEX isonicotinate, 14 and 21 days from the beginning of the oral administration: DIM). At the slaughterhouse, the liver of each calf was weighed (kg). The organ relative weight was calculated according to the following formula: liver weight (kg)/animal body weight (kg) × 100. Results were expressed either as kilograms (absolute organ weight) or percent (relative organ weight) ± standard error. As regards the total cytochrome P450 content, liver microsomal subcellular fractions were isolated by differential ultracentrifugation. The total cytochrome P450 content was determined as the carbon monoxide difference spectrum of sodium dithionite-reduced microsomal suspensions. Results ($nmol^{-1}$ min mg $protein^{-1}$) were expressed as means ± standard error.

natural GCs, including DEX [45,46]. These effects might be substantial if referred to CYP3A. This P450 subfamily accounts for about 30% of total P450 proteins in human liver; it is involved in the metabolism of more than 50% of common drugs as well as of many endogenous compounds; finally, it might be induced by many xenobiotics [8]. In this respect, CTS and DEX themselves are metabolized by CYP3A and are likely to induce CYP3A expression [5-7].

Likewise to humans, CYP3A is expressed in cattle liver [22-29]; such an enzyme is involved in the metabolism of relevant veterinary drugs, such as the antiparasitic moxidectin [47], macrolide antibiotics [48] and the ionophore monensin [49]. As mentioned above, in men CTS and DEX are metabolized by CYP3A to 6b-hydroxy derivatives (just as TST), to yield inactive and readily eliminable metabolites [6,8,50]. Interestingly, cattle physiologic corticosteroids are similar to human ones [51]; moreover, DEX is frequently in common veterinary clinical practice, often co-administered with other drugs [16]. Therefore, even in cattle CYP3A might play a role in potential and therapeutically significant drug-drug interactions. This would be a matter of particular concern for those GCs illicitly used in cattle breeding as GPs [17], essentially for the absence of data concerning the possible modulation of CYP3A expression and regulation phenomena. In the present study, the effects of two common illicit DEX protocols upon the veal calf liver CYP3A and related transcription factors were investigated, by using a common tier-approach consisting of: (a) the evaluation of gene expression profiles (by Northern blot and/or Q RT-PCR); (b) the protein amount quantification (by immunoblotting); and (c) the determination of enzyme activities on subcellular fractions by using putative specific substrates [52].

Basically, GCs interact with GR that, in turn, regulates the expression of corticosteroid-responsive genes, thus modulating amounts and functions of proteins synthesised by target tissues [53]. As far as the molecular bases of CYP3A regulation, in the past decade GR has been subject of extensive debate,

due to the different behaviour disclosed by some GCs (including DEX and hydrocortisone) upon CYP3A expression. In essential, three possible mechanisms have been identified: (a) a direct interaction of GR with CYP3A; (b) an activation of CYP3A through PXR; and (c) an interaction of GCs with GR, which in turn stimulates other transcription factors interacting with CYP3A promoter [54-56]. In the case of DEX, its effects upon CYP3A mRNA are biphasic; at nanomolar concentrations, the induction (usually of low amplitude, 3^-fold) is under GR control, which in turn up-regulates PXR, RXR and CAR; by contrast, in presence of supramicromolar concentrations, the inductive process (of higher amplitude, 15-30-fold) is under PXR control [4,8,57]. A GR-[PXR/CAR]-CYP3A signal transmission cascade has been proposed, whose relevance relies on the fact that any process altering GR transcriptional activity should consequently involve PXR, CAR and RXR and target P450s expression, with a reduction of drug metabolism and excretion processes. Nevertheless, it is actually believed that GR-[PXR/CAR]-CYP3A represents rather a complex regulatory loop showing sequential, synergic or ''in *tandem*'' interrelationships [4,7,57-61]. As an example, GCs transcriptionally regulate CAR gene expression in HepG2 hepatoma cells [62]; dexamethasone is a ligand/activator of human PXR [7,63] and promotes the CAR nuclear translocation in human hepatocytes [59]; functional glucocorticoid responsive elements have been found in the human CAR promoter and in the rat CYP3A23 gene [11,64]. In the present study, low DEX dosages increased liver GR mRNA levels (being such a finding more relevant in DIM), while no effect was ever observed, regardless of the adopted protocol, upon NRs gene expression profiles; by contrast, this same GC down-regulated liver CYP3A mRNA (significantly when measured by Northern blotting). An explanation of present results might lie in the very low DEX concentrations here used: in fact, CYP3A induction and PXR activation require GC concentrations higher than those needed to activate GR [4,7,8,57]; besides, DEX is a ligand for human PXR, but only at concentrations higher than 10 mM. Presumably, DEX induces CYP3A4 *via* GR rather than the GR-[PXR] pathway depending on the concentration (nanomolar or micromolar, respectively) [4]. Probably, in the current study the needed high DEX concentrations were never reached and, consequently, only GR mRNA was modulated by the GC. This interpretation would confirm the hypothesis that GCs, at physiological levels, maintain GR in the activate state and control NRs basal levels (particularly PXR and CAR) which in turn can, albeit to a lower extent, function as CYP3A transcriptional regulators [4,57,64]. The glucocorticoid receptor is involved also in the regulation of several key metabolic enzymes, such as TAT [65,66], which represents a good model to monitor changes in GR gene expression in vivo [42]; moreover, DEX concentrations able to induce GR mRNA increased also TAT mRNA (even if these concentrations were about 100-fold lower than those required for CYP3A up-regulation) [4]. Thus, it is worldwide accepted that any effect on GR should be confirmed at the TAT mRNA level, too [67]. In this respect and just to confirm GR and PXR data, in the present study TAT gene expression was measured, but GR mRNA up-regulation was not similarly reflected at the TAT mRNA level. However, some contradictory results have already been published; as an example, in human hepatocytes the azole derivative ketoconazole and colchicine as well down-regulated TAT mRNA,

without or slightly affecting GR and PXR gene expression profiles, respectively [11,67]. It has been reported that DEX induces rat TAT activity, but the magnitude of the effect depends on animal's age [42]. In the present study, DEX reduced TAT catalytic activity, confirming QRT-PCR data; besides, similar pre- and post-translational results were confirmed in another experiment, where DEX was administered *per* os to beef cattle (Giantin et al., unpublished results). Altogether, present and cited data suggest that in cattle definitive conclusions on DEX effects upon GR cannot be drawn, at least at the pre-transcriptional level.

Despite the fore-mentioned close relationship between DEX and human GR, NRs, and CYP3A, in cattle these relevant liver transcription factors were not affected by the GC. In a previously published study, where SC^gkgbw^{-1} DEX were given twice a day and for 5 days to veal calves fed with colostrum or milk-based formula, a down-regulation of both PXR and CAR mRNA with respect to untreated animals was observed, whereas CYP3A mRNA was unaffected [68]. Beyond DEX dosage, definitely higher than those estimated to be adopted at growth promoting purposes, it should be hypothesised that the GR-[PXR/CAR]-CYP3A network, in DEX-treated cattle, might show different regulation phenomena if compared to humans. Such a hypothesis would further confirm marked species-differences found in CYP3A pattern of induction [8]. Among hypotheses offered as a justification of such a behaviour, there is the possible difference in the affinity between the inducer and PXR protein [7]; in PXR, the DNA or the ligand-binding domain regions shared more than 90% or 75-90% amino acid residue identity among considered species, respectively. An extensive variation of this latter should provide a rational basis to explain fore-mentioned species-differences in CYP3A induction [8,64]. Thus, a PXR protein sequence alignment was made and a phylogenetic tree designed taking into account man, model laboratory species and cattle; three sets, showing similar features, were obtained: a first one with rat, mouse and, partly, the rabbit; a second one, with man and non-human primates; and a last one, with dog and cattle (Giantin et al., unpublished results). These results agree, at least under a general point of view, with recent published data [69]. It is of interest that cattle and dog were grouped together: in fact DEX, administered in the dog at clinically relevant doses, elicits the same post-transcriptional effect (inhibition of CY3A-dependent enzyme activities) [16] here observed. Therefore, a possible difference in DEX affinity toward PXR, which might in turn result in a different CYP3A expression, cannot be at least hypothetically excluded.

In the neonate, the rapid development of detoxifying enzymes represents always a critical step: in cattle, both NRs and NR target genes showed specific and marked ontogenic changes in pre-term, full-term, 5 and 159 days old animals (being these latter more or less of the same age than those here used) [30]. Diet is another constitutional non pharmacogenetic factors (together with gender) which modulates the xenobiotic drug metabolism. In this respect, liver NRs mRNA abundance, in full-term calves administered either colostrum or a milk-based formula containing or not vitamin A, did not show significant changes, except for the RXRb [31]. However, a comparison of liver NRs gene expression profiles between

present CTRL calves and untreated beef cattle, fed respectively with a milk replacer or unifeed *ad* libitum, never showed differences due to aging or diet as well (Giantin et al., unpublished results). Experimental evidences about a possible effect of gender upon cattle liver NRs are still lacking; however, in control or DEX-treated mice, sex affected at the post-transcriptional level, both PXR- and CAR-mediated regulation of CYP3A44 [70]. Taken together, present and above-mentioned results suggest how DEX effect upon liver NRs in cattle needs further basic and applied clarifying studies.

About CYP3A, there are experimental evidences showing that CYP3A mRNA is expressed in cattle liver [23,30,31]. In the present study DEX down-regulated liver CYP3A mRNA, and such a decrease was more evident when measured by Northern blotting. In a previous study an illicit cocktail consisting of 17b-oestradiol, DEX and clenbuterol, administered to veal calves, slightly reduced the amount of liver CYP3A mRNA [71]. There is a consistent literature indicating DEX as a representative CYP3A inducer [7]. The inducibility of this relevant P450 isoform by different xenobiotics (i.e., rifampicin, DEX itself) has been subject of comparative investigations and marked species-differences have been observed [8]; in fact, DEX induces CYP3A in rat hepatocyte primary cultures, but not in human, rabbit, minipig and dog ones [8]. If we exclude above-mentioned individual factors (i.e., gender, age, strain), the different CYP3A gene responsiveness to prototypical inducers (and, particularly, to DEX), might be a consequence of other molecular mechanisms: as an example, the number of nucleotide mismatches within DEX responsive elements (DRE-1 and DRE-2) are responsible for differences in the pattern of induction as well as of basal catalytic activity among rat CYP3A2 and CYP3A23 [72]. In cattle hepatocytes, only a light increase in CYP3A apoprotein was noticed following the incubation with DEX [73]. Moreover, the administration of a high DEX dose (30 mg kg^{-1} DEX, twice a day and for 5 days) did not positively affect the liver CYP3A gene expression in veal calves [68]. Therefore, it looks like that DEX might not be a CYP3A inducer in cattle, likewise to the dog [8] and confirming once more how data extrapolation from rodent model species and humans to veterinary species and vice *versa* might sometimes be problematic, if not a hazardous risk.

At the post-translational level, DEX decreased both CYP3A apoprotein and CYP3A-dependent enzyme activities (ERDEM, ETDEM, 6b-OHTST and 2b-OHTST). Similar results were obtained either in calves administered with an illicit cocktail containing DEX (where a less intense CYP3A protein band was noticed at the immunoblotting, too) [71] as well as in the liver of dogs orally given 2.5 mg or 7.5 mg DEX (considered as clinically relevant doses), where the P450 content and CYP3A-dependent enzyme activities were inhibited [16]; moreover, in this same study DEX administration (0.75 or 48mgkg~^{1}) to Wistar rats showed a dual effect: the lower GC dose inhibited DMEs, whereas the higher one up-regulated them [16]. These results would confirm a possible repressive effect of DEX when used at low dosages, the presence of species-differences in CYP3A expression and regulation and a possible similitude in the way by which the dog and cattle respond to DEX.

In the past decade, several assays have been used to measure CYP3A activity in vivo: among these ones, 6b-OHCTS/CTS ratio is considered an useful non-invasive assay for CYP3A activity [5,8,43].

Nonetheless, some doubts on 6b-OHCTS/CTS effectiveness have been raised [43,44,50,74]. Since CTS, likewise TST and DEX, is mainly hydroxylated in the 6b-position by CYP3A [50], its circadian variation mirrors CYP3A activity [75] and, finally, cattle physiologic corticosteroids are similar to human ones [51], 6b-OHCTS/CTS ratio was measured. Both CTS and 6b-OHCTS levels were lowered by DEX; by contrast, 6b-OHCTS/CTS ratio was increased. This result follows the reduction of urinary CTS concentrations, which is due to the DEX-dependent down-regulation of adrenocortical cytochrome P450 11 and 17 mRNAs [76]. In man, hydrocortisone serum levels fluctuate between 0.1 and 0.45 mM, a range of concentrations believed to permit GR full activation and, perhaps, the regulation of both NRs and basal CYP3A expression [4,57]; likewise, it has been hypothesised that illicit DEX, administered *per* os and at low dosages, might reach steady state concentrations which might interfere with CTS synthesis and release [15]. Therefore, an indirect modulating effect of CTS upon CYP3A and related transcription factors could not be excluded *a priori*. In light of these results, 6b-OHCTS/CTS ratio should not be used to monitor CYP3A activity in individuals treated with DEX; rather, it has been proposed as a promising indirect biomarker to detect GC misuse in cattle [15,77].

In conclusion, results obtained in the present study show that DEX, when used at growth promoting purpose in the veal calf: (a) up-regulates liver GR mRNA, whereas gene expression profiles of TAT or those NRs which contribute with GR to CYP3A expression and regulation were unaffected; (b) down-regulates liver CYP3A mRNA; (c) decreases liver CYP3A protein and CYP3A-dependent and TAT catalytic activities; and (d) increases the urinary 6b-OHCTS/CTS. Therefore, DEX exerts its effects upon CYP3A both at the pre- and post-translational level. Probably cattle show different regulatory phenomena of GR-[PXR/CAR]-CYP3A signal transmission cascade if compared to humans and rodentmodel species; all of this would confirm the relevance of species-differences in the CYP3A regulation. However, it looks likely that DEX dosage regimens (i.e., growth promoting ones, clinically relevant, high) might play a key role in the regulation of fore-mentioned transcription factors and CYP3A, just as reported for humans and laboratory species (i.e., the well-known biphasic effect). Clearly further studies, which should firstly clarify if DEX induce or not CYP3A in cattle and, therefore, go deeper inside the molecular mechanisms of CYP3A regulation, are required and envisaged in this same laboratory. Moreover, the usefulness of 6b-OHCTS/CTS ratio as a tool to be used in the screening of illicit use of DEX in cattle is currently under investigation.

Acknowledgements

This study was supported by grants from Universita degli Studi di Padova (CPDA054599: messa a punto di una tecnica -omics per la valutazione dei profili di espressione genica di biomarcatori del metabolismo in bovini trattati con sostanze illecite) to M.D.; from Ministero Universita e Ricerca Scientifica e Tecnologica and Universita Italo-Francese (Programma Galileo 2004: definizione di possibili biomarcatori per l'evi-

denziazione di trattamenti con sostanze illecite nel bovino; and Programma Galileo 2006: valutazione degli effetti dei trattamenti illeciti sugli enzimi farmaco-metabolizzanti del bovino mediante tecniche di PCR quantitativa) to M.D. and T.P.; from Direzione di Sanita Pubblica, Assessorato alla Sanita, Regione Piemonte (Ricerca Sanitaria finalizzata in materia di sicurezza alimentare: valutazione delle modifica-zioni indotte sul sistema immunitario dalla somministrazione di corticosteroidi in vitelli a carne bianca) to E.B.; and finally, PhD fellowships (to M.C. and M.G.) from Universita degli Studi di Torino and Padova, respectively.

REFERENCES

[1] Schimmer BP, Parker KL. Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. In: Harman JG, Limbird LE, Goodman Gilman A, editors. Goodman & Gilman's the pharmacological basis of therapeutics, 1649. New York: McGraw-Hill; 2001. p. 77.

[2] Yudt MR, Cidlowski JA. The glucocorticoid receptor: coding a diversity of proteins and responses through a single gene. Mol Endocrinol 2002;16:1719-26.

[3] Sonneveld E, Jonas A, Meijer OC, Brouwer A, van der Burg B. Glucocorticoid-enhanced expression of dioxin target genes through regulation of the rat aryl hydrocarbon receptor. Toxicol Sci 2007;99:455-69.

[4] Pascussi J-M, Gerbal-Chaloin S, Drocourt L, Maurel P, Vilarem M-J. The expression of CYP2B6, CYP2C9 and CYP3A4 genes: a tangle of networks of nuclear and steroid receptors. Biochim Biophys Acta 2003;1619:243-53.

[5] Galteau MM, Shamsa F. Urinary 6b-hydroxycortisol: a validated test for evaluating drug induction or drug inhibition mediated through CYP3A in humans and in animals. Eur J Clin Pharmacol 2003;59:713-33.

[6] Gentile DM, Tomlinson ES, Maggs JL, Kevin Park B, Back DJ. Dexamethasone metabolism by human liver in vitro, metabolite identification and inhibition of 6-hydroxylation. J Pharmacol Exp Ther 1996;277:105-12.

[7] Burk O, Wojnowski L. Cytochrome P450 3A and their regulation. Arch Pharmacol 2004;369:105-24.

[8] Luo G, Guenthner T, Gan L-S, Griffith Humphreys W. CYP3A induction by xenobiotics: biochemistry, experimental methods and impact on drug discovery and development. Curr Drug Metab 2004;5:483-505.

[9] Xu C, Li CY, Kong AN. Induction of phase I, II and III drug metabolism/transport by xenobiotics. Arch Pharm Res 2005;28:249-68. [10] Down MJ, Arkle S, Mills JJ. Regulation and induction of CYP3A11, CYP3A13 and CYP3A13 and CYP3A25 in C57BL/6J mouse liver. Arch Biochem Biophys

2007;457:105-10. [11] Duret C, Daujat-Chavanieu M, Pascussi J-M, Pichard-Garcia L, Balaguer P, Fabre J-M, et al. Ketoconazole and miconazole are antagonists of the human glucocorticoid receptor: consequences on the expression and function of the constitutive androstane receptor and the pregnane X receptor. Mol Pharmacol 2006;70:329-39.
[12] Gu X, Ke S, Liu D, Sheng T, Thomas PE, Rabson AB, et al. Role of NF-κB in regulation of gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. J Biol Chem 2006;281:17882-9. [13] Timsit YE, Negishi M. CAR and PXR: the xenobiotic-sensing receptors. Steroids 2007;72:231^16.
[14] Zhou C, Tabb MM, Nelson EL, Griin F, Verma F, Sadatrafiei A, et al. Mutual repression between steroid and xenobiotic receptor and NF-κB signaling pathways links xenobiotic metabolism and inflammation. J Clin Invest 2006;116: 2280-9.
[15] Capolongo F, Tapparo M, Merlanti R, Ravarotto L, Tealdo E, Gallina G, et al. Illicit treatments in cattle and urinary 6beta-hydroxycortisol/cortisol ratio. Anal Chim Acta 2006;586:228-32.
[16] Zhang K, Kuroha M, Shibata Y, Kokue E, Shimoda M. Effect of oral administration of clinically relevant doses of dexamethasone on regulation of cytochrome P450 subfamilies in hepatic microsomes from dogs and rats. Am J Vet Res 2006;67:329-34.
[17] Courtheyn D, Le Bizec B, Brambilla G, De Brabander HF, Cobbaert E, Van de Wiel M, et al. Recent development in the use and abuse of growth promoters. Anal Chim Acta 2002;473:71-82.
[18] Abraham G, Gottschalk J, Ungemach FR. Possible role of dexamethasone in sensitizing the beta-2-adrenergic receptor system in vivo in calves during concomitant treatment with clenbuterol. Pharmacology 2004;72:196-204.
[19] Odore R, Badino P, Barbero R, Cuniberti B, Pagliasso S, Girardi C, et al. Regulation of b-adrenergic, glucocorticoid and androgen receptors induced by repeated exposure to growth promoters in male veal calves. Res Vet Sci 2007;83:227-33.
[20] Serratosa J, Blass A, Rigau B, Mongrell B, Rigau T, Tortades M, et al. Residues from veterinary medicinal products, growth promoters and performance enhancers in food-producing animals: a European Union perspective. Rev Sci Tech OIE 2006;25:637-53.
[21] Plant N. The human cytochrome P450 sub-family: transcriptional regulation, inter-individual variation and interaction network. Biochim Biophys Acta 2007;1770: 478-88.
[22] Dacasto M, Eeckhoutte C, Capolongo F, DupuyJ, Carletti M, Calleja C, et al. Effect of breed and gender on bovine liver cytochrome P450 3A (CYP3A) expression and inter-species comparison with other domestic ruminants. Vet Res 2005;36:179-90.
[23] Giantin M, Carletti M, Capolongo F, Pegolo S, Lopparelli RM, Gusson F, et al. Effect of breed upon cytochrome P450s and phase II enzymes expression in cattle liver. Drug Metab Dispos 2008;36:1-9.
[24] Ioannides C. Cytochrome P450 expression in the liver of food-producing animals. Curr Drug Metab

2006;7:335^18.

[25] Machala M, Soucek P, Neca J, Ulrich R, Lamka J, Szotakova B, et al. Inter-species comparisons of hepatic cytochrome P450 enzyme levels in male ruminants. Arch Toxicol 2003;77:555-60.

[26] Natsuhori M, van Raak M, Ligtenberg M, Kleji L, ten Berge D, Zweers-Zeilmaker WM, et al. Isolation of a bovine full length cytochrome P450 (CYP3A) cDNA sequence and its functional expression in V79 cells. Environ Toxicol Pharmacol 1997;3:17-24.

[27] Nebbia C, Dacasto M, Rossetto Giaccherino A, Giuliano Albo A, Carletti M. Comparative expression of liver cytochrome P450- dependent monooxygenases in the horse and in other agricultural and laboratory species. Vet J 2003;165:53-64.

[28] Sivapathasundaram S, Magnisali P, Coldham NG, Howells LC, Sauer MJ, Ioannides C. A study of the expression of the xenobiotic-metabolising cytochrome P450 proteins and testosterone metabolism in bovine liver. Biochem Pharmacol 2001;62:635^15.

[29] Szotakova B, Baliharova V, Lamka J, Nozinova E, Wsol V, Velik J, et al. Comparison of in vitro activities of biotransformation enzymes in pig, cattle, goat and sheep. Res Vet Sci 2004;76:43-51.

[30] Greger DL, Philipona C, Blum JW. Ontogeny of mRNA abundance of nuclear receptors and nuclear receptor target genes in young cattle. Domest Anim Endocrinol 2006;31: 76-87.

[31] Krilger KA, Blum JW, Greger DL. Expression of nuclear receptor and target genes in liver and intestine of neonatal calves fed colostrum and vitamin A. J Dairy Sci 2005;88:3971-81.

[32] Lowry O, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.

[33] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402-8.

[34] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.

[35] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979;76:4350-4.

[36] Diamondstone IJ. Assay of TAT activity by conversion of pHPP to pHBA. Anal Biochem 1966;16:395^101.

[37] Dundjerski J, Butorovic B, Kipic J, Trajkovic D, Matic G. Cadmium affects the activity of rat liver tyrosine aminotransferase and its induction by dexamethasone. Arch Toxicol 1996;70:390-5.

[38] Capolongo F, Merlanti R, Cibin V, De Liguoro M, Montesissa C. In vivo hormone treatments in calves: effects on in vitro hydroxylation of testosterone. J Vet Pharmacol Ther 2003;26(Suppl. 1):159-60.

[39] Omura T, Sato R. The carbon monoxide binding pigment of liver microsomes. II. Solubilisation, purification, properties. J Biol Chem 1964;239:2379-85.

[40] Evans PJ. The regulation of hepatic tyrosine aminotransferase. Biochim Biophys Acta 1981;677:433-44.

[41] Jantzen HM, Strahle U, Gloss B, Stewart F, Schmid W, Boshart M, et al. Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell 1987;49:29-38.

[42] Ribarac-Stepic N, Vulovic M, Koricanac G, Isenovic E. Basal and glucorticoid induced changes of hepatic glucorticoid receptor during aging: relation to activities of tyrosine aminotransferase and tryptophan oxygenase. Biogerontology 2005;6:113-31.

[43] Furuta T, Suzuki A, Mori C, Shibasaki H, Yokokawa A, Kasuya Y. Evidence for the validity of cortisol 6b-hydroxylation clearance as a new index for in vivo cytochrome P450 3A phenotyping in humans. Drug Metab Dispos 2003;31:1283-7.

[44] Chen Z, Dunning LA, Anderson KE, Holtzman JL, Zheng W. Within-person variability of urinary 6b-hydroxycortisol to urinaryl ratios in Caucasian women. Steroids 2004;69: 67-70.

[45] Sakuma T, Kitajima K, Nishiyama M, Endo Y, Miyauchi K, Jarukamjorn K, et al. Collaborated regulation of female-specific murine Cyp3a41 gene expression by growth and glucocorticoid hormones. Biochem Biophys Res Comm 2004;314:495-500.

[46] Sakuma T, Kitajima K, Nishiyama M, Mashino M, Hashita T, Nemoto N. Suppression of female-specific Cyp2b9 gene expression by growth or glucocorticoid hormones. Biochem Biophys Res Comm 2004;323:776-81.

[47] Dupuy J, Eeckhoutte C, Sutra JF, Galtier P, Alvinerie M. In vitro metabolism of ^{14}C moxidectin by hepatic microsomes from various species. Vet Res Commun 2001;25:345-54.

[48] Zweers-Zeilmaker WM, Van Miert AS, Horbach GJ, Witkamp RF. In vitro complex formation and inhibition of cytochrome P450 activity by different macrolides and tiamulin in goats and cattle. Res Vet Sci 1999;66:51-5.

[49] Nebbia C, Ceppa L, Dacasto M, Nachtmann C, Carletti M. Oxidative monensin metabolism and cytochrome P450 3A content and functions in liver microsomes from horses, pigs, broiler chicks, cattle and rats. J Vet Pharmacol Ther 2001;24:399-403.

[50] El Desoky ES, Mohamed HO, Farghaly WMA, Hamed SA, Hedaya MA, Siest J-P. Study of urinary 6b-hydroxycortisol/ cortisol ratio in spot urine sample as a biomarker of 3A4 enzyme activity in healthy and epileptic subjects of Egyptian population. Pharmacol Res 2005;51:575-80.

[51] Romero DG, Zhou M-Y, Gomez-Sanchez CE. Cloning and expression of the bovine 11b-hydroxysteroid dehydrogenase type-2. J Steroid Mol Biol 2000;72:231-7.

[52] Caron E, Rioux N, Nicolas O, Lebel-Talbot H, Hamelin BA. Quantification of the expression and inducibility of 12 rat cytochrome P450 isoforms by quantitative RT-PCR. J Biochem Mol Toxicol 2005;19:368-78.

[53] Adcock IM. Molecular mechanisms of glucocorticoid actions. Pulm Pharmacol Ther 2000;13:115-23.

[54] El-Sankary W, Plant NJ, Gordon Gibson G, Moore DJ. Regulation of the CYP3A4 gene by hydrocortisone and xenobiotics: role of the glucocorticoid and pregnane X receptors. Drug Metab Dispos 2000;28:493-6.

[55] El-Sankary W, Bombail V, Gordon Gibson G, Plant N. Glucocorticoid-mediated induction of CYP3A is decreased by disruption of a protein: DNA interaction distinct from the pregnane X receptor response element. Drug Metab Dispos 2002;30:1029-34.

[56] Usui T, Saitoh Y, Komada F. Induction of CYP3A in HepG2 cells by several drugs—association between induction of CYP3A and expression of glucocorticoid receptor. Biol Pharm Bull 2003;26:510-7.

[57] PascussiJ-M, Drocourt L, Gerbal-Chaloin S, Fabre J-M, Maurel P, Vilarem M-J. Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes, sequential role of glucocorticoid receptor and pregnane X receptor. Eur J Biochem 2001;268:6346-58.

[58] Pascussi J-M, Gerbal-Chaloin S, Drocourt L, Assenat E, Larrey D, Pichard-Garcia L, et al. Cross-talk between xenobiotic detoxification and other signalling pathways: clinical and toxicological consequences. Xenobiotica 2004;34:633-64.

[59] Pascussi J-M, Gerbal-Chaloin S, Fabre J-M, Maurel P, Vilarem M-J. Dexamethasone enhances constitutive androstan receptor expression in human hepatocytes: consequences on cytochrome P450 gene regulation. Mol Pharmacol 2000;58:1441-50.

[60] Pascussi J-M, Drocourt L, Fabre J-M, Maurel P, Vilarem M-J. Dexamethasone induces pregnane X receptor and retinoid X receptor-a expression in human hepatocytes: synergistic increase of CYP3A induction by pregnane X receptor activators. Mol Pharmacol 2000;58:361-72.

[61] Pascussi J-M, Busson-Le Coniat M, Maurel P, Vilarem MJ. Transcriptional analysis of the orphan nuclear receptor constitutive androstan receptor (NR1I3) gene promoter: identification of a distal glucocorticoid response element. Mol Endocrinol 2003;17:42-55.

[62] Pascussi J-M, Dvorak Z, Gerbal-Chaloin S, Assenat E, Maurel P, Vilarem M-J. Pathophysiological factors affecting CAR gene expression. Drug Metab Rev 2003;35:255-68.

[63] Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. J Clin Invest 1998;102:1016-23.

[64] Quattrochi LC, Guzelian PS. CYP3A regulation: from pharmacology to nuclear receptors. Drug Metab Dispos 2001;29:615-22.

[65] Cole TJ, Blendy JA, Monaghan AP, Schmid W, Aguzzi A, Schiitz G. Molecular genetic analysis of glucocorticoid signaling during mouse development. Steroids 1995;60:93-6.

[66] Nitsch D, Boshart M, Schiitz G. Activation of the tyrosine aminotransferase gene is dependent on synergy between liver-specific and hormone-responsive elements. Proc Natl Acad Sci U S A 1993;90:5479-83.

[67] Dvorak Z, Modriansky M, Pichard-Garcia L, Balaguer P, Vilarem M-J, Ulrichova J, et al. Colchicine down-regulates cytochrome P450 2B6, 2C9, and CYP3A4 in human hepatocytes by affecting their glucorticoid receptor-mediated regulation. Mol Pharmacol 2003;64:160-9.

[68] Greger DL, Blum W. Effects of dexamethasone on mRNA abundance of nuclear receptors and hepatic nuclear receptor target genes in neonatal calves. J Anim Physiol Anim Nutr 2007;91:62-7.

[69] Ekins S, Reschly EJ, Hagey LR, Krasowski MD. Evolution of pharmacologic specificity of the pregnane X receptor. BMC Evol Biol 2008;8:103.

[70] Anakk S, Huang W, Staudinger JL, Tan K, Cole TJ, Moore DD, et al. Gender dictates the nuclear receptor-mediated regulation of CYP3A44. Drug Metab Dispos 2007;35:36-42.

[71] Cantiello M, Carletti M, Dacasto M, Pascal PGP, Pineau T, Capolongo F, et al. Cytochrome P450 inhibition profile in liver of veal calves administered a combination of *Uβ*-estradiol, clenbuterol and dexamethsone for growth-promoting purposes. Food Chem Toxicol 2008;46:2849-55.

[72] Huss JM, Wang SI, Kasper CB. Differential glucocorticoid responses of CYP3A23 and CYP3A2 are mediated by selective binding of orphan nuclear receptors. Arch Biochem Biophys 1999;372:321-32.

[73] van't Klooster GAE, Blaauboer BJ, Noordhoek J, van Miert ASJPAM. Cytochrome P450 induction and metabolism of alkoxyresorufins, ethylmorphine and testosterone in cultured hepatocytes from goats, sheep and cattle. Biochem Pharmacol 1993;46:1781-90.

[74] Chen Y-C, Gotzkowski SK, Nafziger AN, Kulawny RW, Rocci Jr ML, Bertino Jr JS, et al. Poor correlation between 6b-hydroxycortisol:cortisol molar ratio and midazolam clearance as measure

of hepatic CYP3A activity. Br J Clin Pharmacol 2006;62:187-95.

[75] Ohno M, Yamaguchi I, Ito T, Saiki K, Yamamoto I, Azuma J. Circadian variation of the urinary 6b-hydroxycortisol to cortisol ratio that would reflect hepatic CYP3A activity. Eur J Clin Pharmacol 2000;55:861-5.

[76] Tzeciak WH, LeHoux J-G, Waterman MR, Simpson ER. Dexamethasone inhibits corticotrophin-induced accumulation of CYP11 and CYP17 mRNAs in bovine adrenocortial cells. Mol Endocrinol 1993;7:206-13.

[77] Vascellari M, Pozza G, Poppi L, Capello K, Angeletti R, Ravarotto L, et al. Evaluation of indirect biomarkers for detecting corticosteroids used as illegal growth promoters in beef cattle. Vet Rec 2008;163:147-51.