Cytochrome P450 3A expression and function in liver and intestinal mucosa from dexamethasone-treated sheep

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The effects of repeated administrations of dexamethasone (DEX) (3 mg/kg/day by IM route for 7 days) on the gene expression profile of a cytochrome P450 (CYP) 3A28-like isoenzyme, on the expression of a CYP3A-immunoreactive protein and on CYP3A-dependent metabolic activities in sheep liver and small intestinal mucosa were evaluated in the current work. CYP 3A-dependent metabolic activities (erythromycin and triacetyl-oleandomycin N-demethylations) were assessed in microsomal fractions. The mRNA expression of CYP3A28-like, glucocorticoid receptor, constitutive androstane receptor, pregnane X receptor and retinoic X receptor alpha (RXRα) was determined by quantitative real-time PCR. The expression of a CYP3A-immunoreactive protein was measured by Western blot analyses. In the liver, DEX treatment increased CYP3A28-like mRNA levels (2.67-fold, $P < 0.01$) and CYP3A apoprotein expression (1.34-fold, $P < 0.05$) and stimulated CYP3A-dependent metabolism. High and significant correlation coefficients between CYP3A-dependent activities and CYP3A28-like gene ($r = 0.835–0.856$, $P < 0.01$) or protein ($r = 0.728–0.855$, $P < 0.05$) expression profiles were observed. Among the transcriptional factors, DEX only stimulated (2.1-fold, $P < 0.01$) the mRNA expression of RXRα. In sheep small intestine, DEX caused a slight increment (34.6%, $P < 0.05$) in erythromycin N-demethylase activity in the jejunal mucosa and a significant enhancement ($P < 0.05$) of CYP3A apoprotein level in the duodenal mucosa.

INTRODUCTION

Xenobiotic-metabolizing enzymes (XMEs) play a major role in detoxification and bioactivation of chemicals (xenobiotics) entering the animal's body, as well as certain endogenous compounds (endobiotics) such as steroids and bile acids. Metabolism of endobiotics and xenobiotics takes place predominantly in the liver although extrahepatic tissues such as the gastrointestinal (GI) tract display numerous phase 1 and phase 2 metabolic reactions. Among XMEs involved in phase 1 metabolism, the microsomal mixed-function oxidase system is located in the smooth endoplasmic reticulum of most tissues. Cytochrome P450 (CYP) and flavin monooxygenase (FMO) are mixed-function oxidases of major interest in veterinary pharma-cology and toxicology (Nebbia, 2001; Ioannides, 2006; Fink-Gremmels, 2008).

In mammals, most members of the CYP enzyme superfamily are constitutively expressed, and a large number of factors (i.e. diet, gender, breed, physiological state and exposure to xenobiotics) may modulate their expression and/or metabolic activity. For instance, sustained exposure to certain xenobiotics may increase the expression level of different enzymes within the CYP system (Xu *et al.*, 2005; Tompkins & Wallace, 2007). Consequently, the induction of CYP-mediated metabolism is considered a tightly regulated defensive process within the cell and thus an adaptive response to the xenobiotic (Williams *et al.*, 2005). A wide array of chemicals, such as environmental pollutants (i.e. polycyclic aromatic hydrocarbons) or therapeutically used drugs

like phenobarbital and dexamethasone (DEX) are able to induce both mRNA and protein expression of different CYP isoforms and, therefore, their metabolic activities (Pascussi *et al.*, 2000a,b; Tompkins & Wallace, 2007). As far as ruminant species are considered, it has been shown that a sustained exposure to phenobarbital increased the expression levels of CYP2B, CYP2C and CYP3A apoproteins and their respective catalytic activities in cattle liver (Cantiello *et al.*, 2006). Furthermore, phenobarbital also induced CYP2B and CYP3A protein expression in sheep liver, as well as their respective benzphetamine and erythromycin N-demethylase activities (Dupuy *et al.*, 2001). Increased CYP1A1 and CYP1B1 mRNA levels were observed in primary cultured bovine hepatocytes exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, polybrominated dibenzo-p-dioxins and purified fish oil extracts used as feed additives (Guruge *et al.*, 2009).

The CYP3A subfamily is composed of multiple enzymes mostly involved in the oxidation of a wide array of xenobiotics including therapeutically used drugs in food-producing animals (Ioannides, 2006). Different members within the CYP3A subfamily are known to be induced by a variety of compounds including natural and synthetic glucocorticoids (GCs). Owing to their anti-inflammatory and immunosuppressive properties, synthetic GC derivatives such as DEX are among the most widely prescribed drugs in large animal's therapy (Ferguson *et al.*, 2009). Overall, DEX is marketed as a phosphate disodium salt or as a propionate ester (a long-acting formulation) for the treatment for inflammation, allergies, shock, stress and metabolic diseases (i.e. ketosis) in ruminant species. The classical genomic mechanism of action of DEX involves its interaction with the GC receptor (GR), a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors (Yudt & Cidlowski, 2002). In addition to its primary mechanism of action, DEX can enhance the mRNA transcription and translation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR), both members of the nuclear receptor superfamily of transcription factors, which are known to be involved in the coordinated transcriptional control of certain CYP isoforms like CYP3A4 in humans (Pascussi *et al.*, 2000a,b; Quattrochi & Guzelian, 2001). In cattle production systems, subtherapeutic doses of DEX are often illegally administered for growth-promoting purposes, either alone or in combination with other growth promoters such as 17β-oestradiol and clenbuterol. Therefore, the effects of illicit treatment protocols with DEX on the expression levels of different CYP subfamilies and some transcriptional factors involved in their regulation have been studied in cattle liver (Cantiello *et al.*, 2008, Cantiello *et al.*, 2009; Giantin *et al.*, 2010). For instance, decreased hepatic CYP3A28 mRNA, apoprotein content and CYP3A-dependent catalytic activities were observed when DEX sodium phosphate was orally administered (as a growth promoter) to veal calves for 23 days (Cantiello *et al.*, 2009). Conversely, upregulation of CYP3A28, CAR and retinoic X receptor alpha (RXRα) mRNAs in cattle liver was observed after the oral administration of a subtherapeutic dose of DEX for 50 days, while CYP3A28 and RXRα mRNAs were increased when DEX was administered by the oral route for

43 days in combination with three intramuscular doses of 17β-oestradiol at 15-day intervals (Giantin *et al.*, 2010). Interestingly, these authors also observed that both CYP2B22 and CYP2E1 mRNAs were downregulated regardless of the illicit schedule used.

The above-mentioned investigations, aimed to the identification of biomarkers of response for the screening of growth promoters misuse in cattle farming, clearly showed the effects of DEX upon certain members within the CYP system. On the other hand, the use of DEX as an enzyme inducer has been a powerful tool to investigate the molecular mechanisms involved on CYPs expression and regulation. Therefore, an inducing schedule with DEX was validated in the current work. We investigated the effects of repeated administrations of DEX (3 mg/kg/day by IM route for 7 days) on (i) the gene expression profile of a CYP3A28-like isoenzyme, (ii) the expression of a CYP3A-immunoreactive protein and (iii) on CYP3A-dependent metabolic activities in sheep liver and small intestinal mucosa. The highest recommended dose of DEX in sheep, usually employed for shock episodes (Escudero Pastor *et al.*, 2002), was selected for the inductive protocol evaluated in the current research. Additionally, the impact of the DEX treatment on the expression of transcriptional factors presumably involved on CYP3A28-like gene regulation was also assessed.

MATERIALS AND METHODS

Chemicals

Dexamethasone sodium phosphate was purchased from Richmond Vet Pharma (Grand Bourg, Buenos Aires, Argentina). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Roche Applied Science (Buenos Aires, Argentina). Erythromycin (ERTM) was purchased from Parafarm, Buenos Aires, Argentina. Buffer salts (KCl, NaHCO$_3$, Na$_2$HPO$_4$, NaH$_2$PO$_4$, K$_2$HPO$_4$, KH$_2$PO$_4$ and CH$_3$COONH$_4$) were purchased from Baker Inc. (Phillipsburg, NJ, USA) while all other chemicals were from Sigma-Aldrich Chemical Company (St Louis, MO, USA).

Animals

Seventeen (17) clinically healthy adult Corriedale male sheep (25–30 kg) were kept on a rye grass/white and red clover pasture. Additionally, the animals received 200 g/day/animal of a conventional concentrate supplement (89.4% dry matter, 16.0% neutral detergent fibre, 11.2% crude protein, 74.2% dry matter digestibility and 11.21 MJ/kg dry matter). Water was provided *ad libitum*.

Animals were randomly divided into two experimental groups. The sheep in the first group ($n = 8$) were used as controls, whereas animals in the second group ($n = 9$) were intramuscularly injected with DEX sodium phosphate at 3 mg/kg per day over 7 days. Animal procedures and management protocols were carried out according to the Animal Welfare Policy of the

Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina (Academic Council Resolution 087/02, http://www.vet.unicen.edu.ar), and internationally accepted animal welfare guidelines (American Veterinary Medical Association, 2007). After a 12-h fasting period, sheep were stunned and immediately exsanguinated in agreement with these institutional and internationally accepted animal welfare guidelines. The DEX-treated animals were sacrificed 24 h after the last dose.

Preparation of subcellular fractions

After sacrifice, a piece (10 g) of liver parenchyma (caudate lobe) was rinsed with ice-cold KCl 1.15% to remove haemoglobin, covered with an aluminium foil and immediately frozen in liquid N_2. The whole small intestine was removed to obtain 1.5 m of each segment: duodenum (10 cm posterior to the pyloric sphincter), middle jejunum and the terminal ileum (1 m before the ileo-caecal valve). Samples of liver parenchyma were collected from all control and DEX-treated sheep. Intestinal segments (duodenum, jejunum and ileum) were taken from five animals of each experimental group, opened through a longitudinal incision and immediately submerged into an ice-cold saline solution to remove residues of gut contents. Each tissue sample was shortly dried between two paper towels, and small pieces (about 50 mg each other) of liver parenchyma and mucosal tissue from duodenum, jejunum and ileum were collected for total RNA extraction, immediately snap-frozen in liquid N_2 and then stored at −80 °C until use.

Microsomal and cytosolic fractions from both hepatic tissue and intestinal mucosa were isolated by differential ultracentrifugation as previously described (Virkel *et al.*, 2010). An aliquot of each subcellular fraction was used to determine protein content according to Lowry *et al.* (1951).

Enzyme assays

The total amount of CYP in liver microsomes was determined as the carbon monoxide difference spectrum of sodium dithionite-reduced microsomal suspensions (Rutten *et al.*, 1987). The rates of the *in vitro* N-demethylations of triacetyl-oleandomycin (TAO) and ERTM were measured for the evaluation of CYP3A-dependent metabolism in liver microsomal preparations, whereas only ERTM N-demethylase activity was assayed in microsomes obtained from the small intestine. Enzyme assays were carried out under aerobic conditions as previously described (Maté *et al.*, 2010).

Isolation of RNA and reverse transcription

Total RNA was isolated from about 50 mg of frozen control and DEX-treated sheep hepatic and intestinal samples using Pure Link™ RNA Mini kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To control the RNA for integrity and for DNA contamination, 5 μL of total RNA extracted was run on a SYBR safe (Invitrogen)-stained 0.8%

agarose gel at 80 V for 60 min. Total RNA purity and concentration were determined spectrophotometrically by recording the absorbance at 260 and 280 nm after a 1:500 sample dilution in RNase-free water. In all samples, the 260/280 ratio (purity) was higher than 1.8.

The reverse transcription was performed with 2 μg of total RNA in a final volume of 20 μL, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems S.A, Foster City, CA, USA) following the purchaser's protocol. The reaction was performed in a water bath for 10 min at 25 °C, 120 min at 37 °C and finally 5 sec at 85 °C for enzyme inactivation.

Quantitative real-time PCR (qPCR)

The sets of primers used for quantitative real-time PCR (qPCR) of CYP3A28-like GR, PXR, RXRα and CAR were obtained from Cantiello *et al.* (2009) and Giantin *et al.* (2010). As bovine primer sequences were used, their gene specificities were assayed by means of PCR amplification and agarose gel electrophoresis before starting the qPCR analysis. For each primer set analysed, a unique PCR amplification product with an expected size was observed.

The qPCR was carried out in an ABI Prism 7500 Real Time PCR System (Applied Biosystems S.A). The reaction mixture included 10 μL of PCR SYBR Green Master Mix 2X (Applied Biosystems S.A), 2 μL of each primer set, 1 μL of cDNA diluted 1:250 and 7 μL of water to obtain a final volume of 20 μL. The amplification reaction was performed in a 96-well plates. The qPCR was carried out using the following thermal profile: 2 min at 50 °C followed by 10 min at 95 °C (Holding Stage) and then 95 °C over 15 sec followed by 1 min at 60 °C (40 cycles). Each primer set was assayed under these conditions in a 200–500 nM range to identify the concentration providing the highest sensitivity and specificity for each target sequence. Calibration curves were performed after the amplification of decreasing amounts of a cDNA pool, making dilutions at fivefold intervals, to evaluate the qPCR efficiency for a given gene in each tissue. Standard curves displayed slope values between −3.6 and −3.1 and r^2 higher than 0.985 as it is recommended in the ABI Prism 7500 Real Time PCR System guidelines (Applied Biosystems S.A). Slope and r^2 values, qPCR efficiency and dynamic range for each target gene in each tissue are shown in Table 1. The selected reference gene was β-actin (ACTB, NM_001009784); its amplification efficiency was approximately equal to target genes, and no difference in the expression of this gene was observed between control and DEX-treated groups. The mRNA relative quantification was carried out using the $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001).

SDS–polyacrylamide gel electrophoresis and Western blot analyses

Aliquots of hepatic (five samples from both control and DEX-treated animals randomly selected) and intestinal microsomal preparations were diluted in protein sample loading buffer (2% SDS, 10% glycerol, 5% 2-β-mercaptoethanol and 0.25% bromophenol blue) in Tris–HCl 0.0625 M (pH 6.8). SDS–polyacrylamide gel electrophoresis of proteins and Western

Table 1. Validation parameters for the quantitative PCR assays of each target gene studied in sheep liver and intestinal mucosa

Gene	Tissue	Slope	r^2	E_x	Dynamic range*
CYP3A28-like	Liver	−3.32	0.998	2.00	0.0016–1
CYP3A28-like	Small intestine	−3.60	0.989	1.90	0.0016–1
GR	Liver	−3.50	0.985	1.93	0.0016–1
PXR	Liver	−3.27	0.985	2.02	0.008–1
CAR	Liver	−3.28	0.996	2.02	0.0016–1
RXRα	Liver	−3.10	0.992	2.10	0.0016–1
ACTB	Liver	−3.20	0.998	2.05	0.0016–1
ACTB	Small intestine	−3.36	0.998	1.98	0.0016–1

E_x, PCR efficiency ($E_x = 10^{-1/\mathrm{slope}}$); CAR, constitutive androstane receptor; CYP, cytochrome P450; GR, GC receptor; PXR, pregnane X receptor; RXRα, retinoic X receptor alpha.
*Range of target RNA serial dilutions.

blotting were carried out following the methodology described by Laemmli (1970) and Towbin *et al.* (1979). One hundred micrograms (100 μg) of microsomal proteins was loaded in each gel line and separated in a 10% SDS–polyacrylamide gel using a Mini PROTEAN Tetra System (Bio-Rad Life Science, Hercules, CA, USA). Then, proteins were transferred onto nitrocellulose membranes (Thermo Scientific, Rockford, IL, USA) which, after blocking with BSA, were first incubated with a primary antibody raised against human CYP3A4 (polyclonal rabbit IgGs, 1/1000; Oxford Biomedical Research, Rochester Hill, Michigan, USA). The membranes were then incubated with a secondary peroxidase-labelled goat anti-rabbit antibody (1/2500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proteins were detected using Pierce ECL Western blotting Substrate (Thermo Scientific).

Data and statistical analysis

Metabolic activities (mean ± SD) were expressed in nmol of formed metabolic products per min per mg of microsomal protein (nmol/min/mg). Hepatic CYP-dependent enzyme activities were also expressed in nmol of metabolic products formed per min per nmol of CYP (turnover number: maximal amount of substrate molecules converted per unit of time per catalytic site). The relative abundance of a specific mRNA (mean ± SEM) was normalized to the expression of *β*-actin and given as fold-change compared to control values. Protein expression was quantified from integrated optical densities (IODs) using a Bio-Rad software Quantity One (version 4.5.2) and expressed as arbitrary intensity

units (mean ± SD). As equal amounts of protein were loaded on the gel prior to electrophoresis and blotting, no internal standard was used for normalization of the Western blot analysis.

Statistical comparisons between the results obtained in both control and DEX-treated groups were made using the unpaired Student's *t*-test with the Welch correction (Instat 3.0; Graph Pad Software Inc., San Diego, CA, USA). A *P* < 0.05 was considered significant.

RESULTS

CYP content and enzyme activities

Mean protein concentration in liver microsomes from DEX-treated sheep (51.4 ± 5.8 mg/mL) was similar compared to that measured in liver microsomal fractions from control animals (56.8 ± 5.6 mg/mL). However, the hepatic CYP content was 34% lower (*P* < 0.01) in DEX-treated (0.43 ± 0.11 nmol/mg of microsomal protein) compared to control sheep (0.64 ± 0.13 nmol/mg). Table 2 shows phase 1 enzyme reactions measured in sheep liver microsomes. When expressed in nmol/min per mg of microsomal protein, both TAO and ERTM N-demethylations were approximately threefold higher (*P* < 0.01) in DEX-treated compared to control animals. Moreover, being expressed as turnover number (nmol/min/nmol CYP), both metabolic activities showed a stronger enhancement (approximately fivefold higher, *P* < 0.01) which confirmed the inductive effect of DEX.

Table 2. Cytochrome P450 (CYP) 3A-dependent metabolic activities measured in liver microsomes obtained from control and dexamethasone (DEX)-treated sheep

Enzyme reaction	Catalytic activity (nmol/min/mg)		Turnover number (nmol/min/nmol P450)	
	Control	DEX	Control	DEX
TAO N-demethylase	0.88 ± 0.54	2.74 ± 1.26**	1.44 ± 0.95	6.46 ± 2.49**
ERTM N-demethylase	0.88 ± 0.27	2.94 ± 1.20**	1.42 ± 0.57	7.08 ± 2.97**

TAO, triacetyl-oleandomycin; ERTM, erythromycin; DEX, dexamethasone.
Data are expressed as mean ± SD of eight (control) or nine (DEX) liver samples.
**Significantly different (*P* < 0.01) from its respective control value.

Table 3 shows ERTM N-demethylase activities measured in microsomes obtained from sheep small intestinal mucosa. A slight increment (34.6%, $P < 0.05$) in this enzyme activity was observed only in the jejunal mucosa of DEX-treated animals.

Expression of enzymes and transcription factors

Figure 1 shows CYP3A28-like gene expression profiles in the liver and in different small intestinal segments (expressed as fold change in DEX-treated compared to control sheep). In the liver, the CYP3A28-like mRNA showed a 2.67-fold increase ($P < 0.01$) in DEX-treated compared to control animals (Fig. 1a). Conversely, the expression level of intestinal CYP3A28-like mRNA was not affected by the repeated administration of DEX (Fig. 1b).

The effect of DEX treatment on mRNA levels of the most important transcription factors presumably involved in CYP3A expression and regulation in sheep liver (GR, CAR, PXR and RXRα) is shown in Fig. 2. The DEX treatment did not affect the expression profiles of GR, CAR and PXR but caused a 2.1-fold ($P < 0.01$) increase in the mRNA level of RXRα.

The Western blot analysis of a CYP3A-immunoreactive protein in microsomes from liver and small intestinal mucosa is shown in Fig. 3. An overall significant enhancement of the apoprotein level was observed in both the liver and duodenal mucosa from DEX-treated sheep.

Pearson correlation analysis

Pearson correlations between CYP3A-dependent metabolic activities and CYP3A28-like gene expression or CYP3A apoprotein levels are shown in Table 4. High and significant ($P < 0.01$) correlation coefficients were obtained between gene or protein expression profiles and TAO and ERTM N-demethylations when both metabolic activities were expressed in nmol/min/mg and also in nmol/min/nmol CYP (turnover number).

DISCUSSION

The effects of repeated administrations of therapeutic doses of DEX on the gene expression profile of a CYP3A28-like isoenzyme, on the expression of a CYP3A-immunoreactive protein and on the CYP3A-dependent metabolic activities in sheep liver and small intestine were assessed in the current work. Because DEX is considered to be an inducer of certain CYP3A isoenzymes in various species (van 't Klooster *et al.*, 1993; Pascussi *et al.*, 2000a,b; Quattrochi & Guzelian, 2001), the expression of the transcription factors presumably involved in CYP3A28-like gene regulation in sheep liver was also characterized.

The CYP3A is believed to be the most abundant CYP enzyme subfamily in the liver of humans and laboratory animals. In humans, this subfamily is involved in the metabolism of approximately half of the most frequently prescribed drugs (Luo *et al.*, 2004). There is also now extensive experimental evidence for the expression of this subfamily in the liver of food-producing animals (reviewed in Ioannides, 2006; Fink-Gremmels, 2008). Proteins cross-reacting with primary antibodies raised against rat, rabbit or human CYP3A isoenzymes have been identified in liver microsomes from cattle (Sivapathasundaram *et al.*, 2001; Nebbia *et al.*, 2003; Virkel *et al.*, 2010), sheep (Pineau *et al.*, 1990) and goats (Szotáková *et al.*, 2004). In cattle liver, the expression of a CYP3A28 protein was

Table 3. Erythromycin (ERTM) N-demethylase activities measured in microsomes obtained from the small intestinal mucosa of control and dexamethasone (DEX)-treated sheep

	ERTM N-demethylase nmol/min/mg	
Intestinal segment	Control	DEX
Duodenum	0.22 ± 0.05	0.32 ± 0.17
Jejunum	0.26 ± 0.01	0.35 ± 0.05*
Ileum	0.31 ± 0.19	0.22 ± 0.06

Data are expressed as mean ± SD of five samples taken from each small intestinal segment.
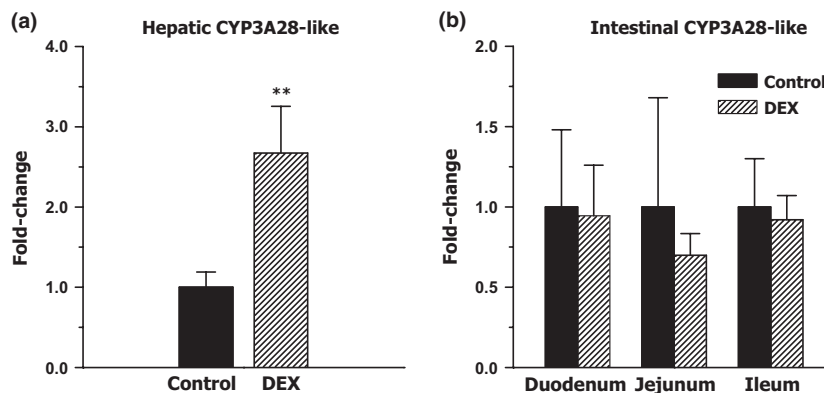*Significantly different ($P < 0.05$) from its respective control value.

Fig. 1. Effect of dexamethasone repeated administration on the hepatic (a) and intestinal (b) gene expression profiles for CYP 3A28-like in sheep. **Significantly different ($P < 0.01$) from control.
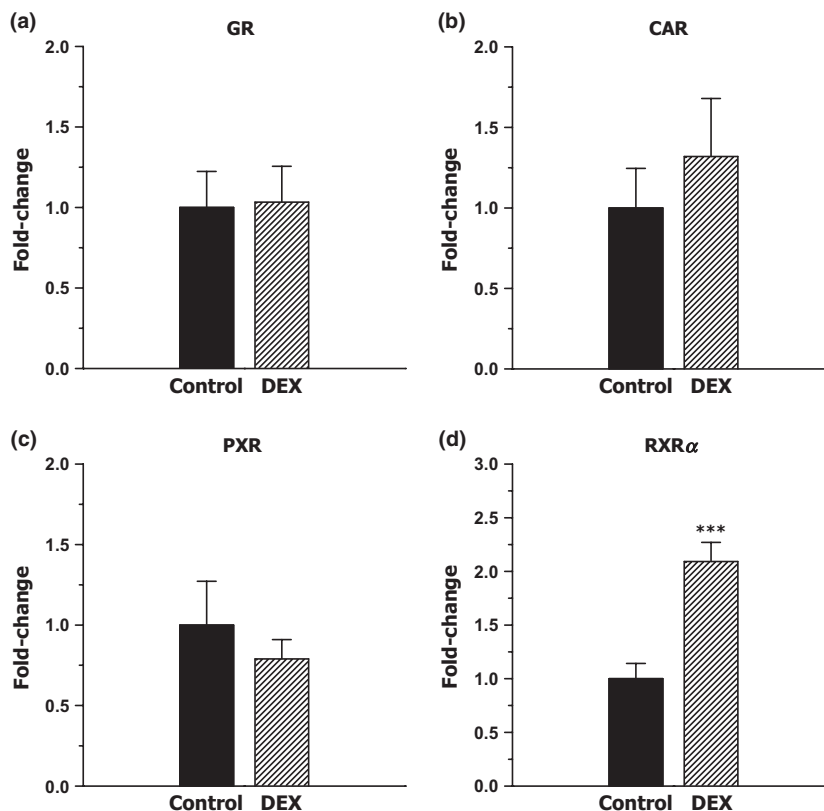
Fig. 2. Effect of repeated administrations of dexamethasone on the gene expression profiles of glucocorticoid receptor (a), constitutive androstane receptor (b), pregnane X receptor (c) and retinoic X receptor alpha (d) in sheep liver. ***Significantly different ($P < 0.001$) from control.

characterized at post-transcriptional and post-translational levels (Cantiello et al., 2008a,b; Giantin et al., 2008, 2010; Zancarella et al., 2010). TAO and ERTM are among the preferred substrates to test the metabolic activity of this subfamily in food-producing species (Ioannides, 2006). In sheep, a TAO-inducible CYP3A enzyme has been purified from sheep liver, displaying a remarkable activity in the N-demethylation of veterinary drugs such as ERTM, chlorpromazine, chlorpheniramine and bromhexine (Pineau et al., 1990).

In the current work, the repeated administration of DEX increased the CYP3A28-like mRNA content (assessed by qPCR) and the level of a CYP3A-immunoreactive protein in the liver (see Figs 1a & 3a). In line with this observation, DEX also stimulated the CYP3A-dependent metabolism in sheep liver microsomes. Although the hepatic CYP content was 34% lower, both TAO and ERTM N-demethylations were increased (approximately threefold, $P < 0.01$) in liver microsomes from DEX-treated compared to control animals. In addition, a stronger inductive effect (approximately fivefold, $P < 0.01$) was observed for both N-demethylations (see Table 1) when these metabolic activities were expressed as turnover number (nmol/min/nmol CYP). It is clear that members of other CYP subfamilies, not studied here (such as CYP2B and CYP2C), may have been induced by DEX as well. A DEX-dependent stimulation was demonstrated in primary cultures of human hepatocytes for CYP2B6 (Pascussi et al., 2000b; Wang et al., 2003), CYP2C8,

CYP2C9 and CYP2C19 (Gerbal-Chaloin et al., 2001; Raucy et al., 2002; Ferguson et al., 2005). Therefore, an unobserved stimulation of these isoenzymes may contribute to some extent to the observed increase in these N-demethylase activities in DEX-treated animals. However, the obtained high and significant correlation coefficients between gene/protein expression profiles and TAO and ERTM N-demethylations (see Table 4) may indicate that the enhanced CYP3A-dependent metabolism is consequence of an induced CYP3A28-like isoenzyme. Further to this speculation, additional comparative studies with model CYP3A inducers are needed for a complete understanding of the expression and function of this subfamily in sheep liver.

Glucocorticoids exert their actions through the interaction with the GR, a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors (Yudt & Cidlowski, 2002). Binding to the GR ultimately regulates GC-responsive genes and, consequently, the expression and function of different proteins synthesized by target tissues (Ferguson et al., 2009). In addition to their classical mechanism of action, GCs have other complex molecular mechanisms involved in CYP3A upregulation in humans and rodents. This may involve (i) a GR-dependent activation of the CYP3A promoter region; (ii) a nonclassical pathway involving the direct activation of PXR by GCs; and (iii) the interaction of GCs with the GR followed by induction of other transcription factors (such as PXR and CAR) interacting with the CYP3A promoter
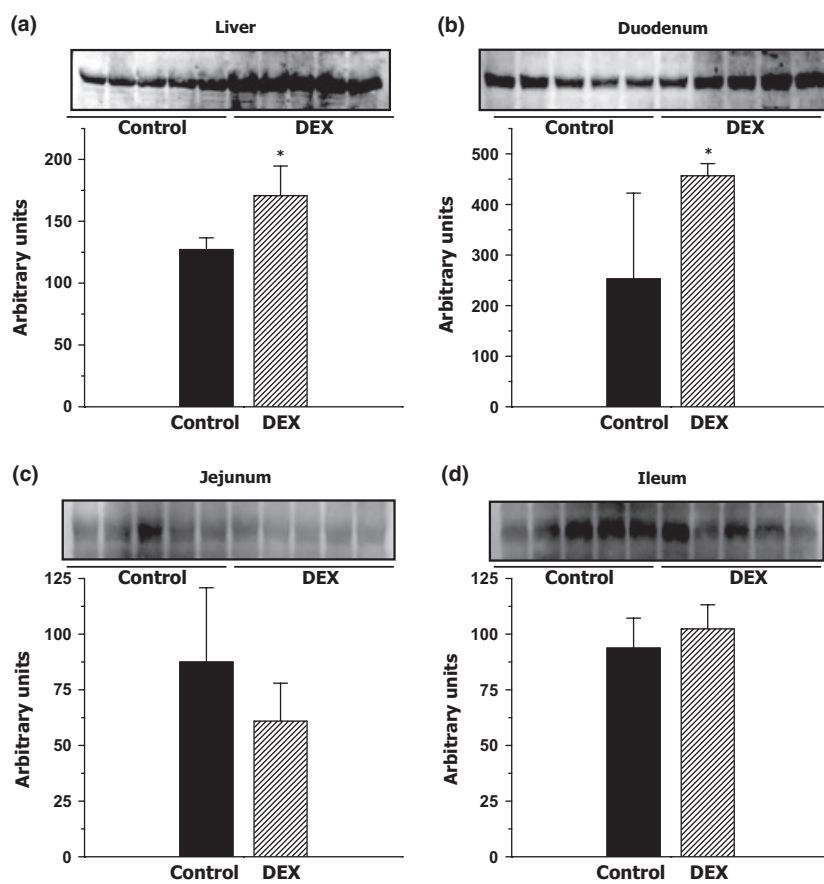
Fig. 3. Representative Western blot analysis of the expression of a CYP 3A immunoreactive protein in the liver (a) and the small intestinal mucosa (b, c and d) of control and dexamethasone (DEX)-treated sheep. For the analysis of liver microsomal preparations, five (5) samples from both control and DEX-treated animals were randomly selected. Membranes were first incubated with a primary antibody raised against human CYP3A4 (polyclonal rabbit IgGs, 1∕1000) and then with a secondary peroxidase-labelled goat anti rabbit antibody (1∕2500) (see Materials and Methods). *Significantly different ($P < 0.05$) from control.

(El-Sankary *et al.*, 2000; Pascussi *et al.*, 2000a,b; Quattrochi & Guzelian, 2001). Both CAR and PXR mRNA transcription and translation are known to be enhanced by different GCs including DEX (Pascussi *et al.*, 2000a,b). In addition, a dual effect of DEX on human CYP3A4 and rat CYP3A23 gene expression profiles

Table 4. Pearson correlation analysis between CYP3A-dependent metabolic activities and cytochrome P450 (CYP) 3A28-like mRNA abundance or CYP3A apoprotein levels in sheep liver

Metabolic activity	Gene∕protein	Pearson correlation coefficient	
		nmol/min/ mg	nmol/min/mg P450
TAO N-demethylase	CYP3A28-like	0.8493**	0.8569**
ERTM N-demethylase	CYP3A28-like	0.8352**	0.8459**
TAO N-demethylase	CYP3A protein	0.8550**	0.7623*
ERTM N-demethylase	CYP3A protein	0.7977**	0.7284*

TAO, triacetyl-oleandomycin; ERTM, erythromycin.
The Pearson's correlation coefficients were significant at: *$P < 0.05$ and **$P < 0.01$.

has been observed in primary hepatocyte cultures from both species (Pascussi *et al.*, 2001; Quattrochi & Guzelian, 2001). These authors showed that under physiological conditions (nanomolar concentrations), DEX modulates the induction of PXR, CAR and RXRα through the classical GR pathway. Conversely, at supramicromolar concentrations, such as those occurring under stress conditions or therapeutic use, the inductive response is because of DEX binding and activation of PXR. Once in the nucleus, these transcription factors bind to RXRα; the heterodimers PXR-RXRα and∕or CAR-RXRα can interact with specific response elements in CYP3A4 or CYP3A23 gene promoter regions (Pascussi *et al.*, 2001; Quattrochi & Guzelian, 2001). The expression profiles of these transcription factors in cattle liver were studied after administration of subtherapeutic doses of DEX. For instance, calves receiving intramuscular DEX (30 μg/kg body weight, given twice a day during 4 days) showed a 40% lower mRNA abundance of both CAR and PXR compared to untreated animals (Greger & Blum, 2007). On the other hand, increased levels of both CAR and RXRα mRNAs were observed after the oral administration of DEX to beef cattle for 50 days (0.75 mg per animal per day), whereas

only RXRα mRNA abundance was higher when DEX was administered in combination with 17β-oestradiol (Giantin *et al.*, 2010). In the current research, the repeated administration of DEX significantly induced RXRα mRNA in sheep liver (approximately twofold, $P < 0.001$), while no differences in the gene expression profiles of GR, PXR and CAR were observed between control and DEX-treated animals. Altogether, these observations made in ruminant species, compared to humans or rodents, may indicate species differences in the regulation of these transcription factors and, consequently, in the expression of the CYP3A subfamily as previously suggested (Greger & Blum, 2007; Giantin *et al.*, 2010).

The intestinal mucosa of ruminants also displays a CYP3A-dependent metabolism (Dupuy *et al.*, 2001; Maté *et al.*, 2010; Virkel *et al.*, 2010). In addition, the presence of CYP3A-immunoreactive proteins was observed in the small intestinal mucosa of these species. For instance, this enzyme subfamily was identified in duodenal microsomes from sheep by means of purified antibodies raised against sheep liver CYP3A (Dupuy *et al.*, 2001). The administration of DEX moderately increased (34.6%) the ERTM N-demethylase activity in the jejunum but not in duodenum and ileum (see Table 3). Although no change in the intestinal CYP3A28-like mRNA content was observed between both experimental groups (see Fig. 1), a significant increase ($P < 0.05$) in CYP3A apoprotein level was only observed in the duodenal mucosa of DEX-treated animals (see Fig. 3). Taken together, these results indicate only a slight inductive effect of DEX on CYP3A expression and catalytic activity in the small intestinal mucosa, which may be a consequence of the reduced concentrations of the drug reaching the small intestine after its intramuscular administration. Owing to their high lipid solubility, GCs are widely distributed from the systemic circulation to different tissues, particularly to those involved in their metabolism and excretion. For instance, accumulation of DEX has been observed in bovine liver and kidney after its intramuscular administration at either 40 or 200 μg/kg dosage (Van den Hauwe *et al.*, 2005). In camels and cattle, metabolism of DEX occurs predominantly in the liver and includes (i) the reduction in the 3-carbonyl group in ring A, mediated by hydroxysteroid dehydrogenases, and (ii) the hydroxylation at position 6 of the same ring (Al Katheeri *et al.*, 2006; Vanhaecke *et al.*, 2011). Moreover, the parent compound and its metabolites may be conjugated with both glucuronic acid and sulphate (Antignac *et al.*, 2002; Al Katheeri *et al.*, 2006). In cattle, the urine was found as the main excretion route of DEX, administered by the intramuscular route, predominantly in its unchanged form (Antignac *et al.*, 2002; Vincenti *et al.*, 2009; Vanhaecke *et al.*, 2011). Conversely, the biliary-faecal excretion (the main route by which DEX may reach the GI-tract) was found notably lower than renal elimination (Vanhaecke *et al.*, 2011). In addition, faecal samples from DEX-treated calves contained higher amounts of conjugated (inactive) metabolites than free parent drug (Vanhaecke *et al.*, 2011). Altogether these observations suggest a low availability of DEX in the gut. Despite these kinetic data were taken from experiments carried out in other species, they may help to explain the slight inductive effect

of DEX on the expression and function of intestinal CYP3A in the current work.

In conclusion, an inductive protocol using therapeutic doses of DEX was evaluated in the current work. A sustained exposure to DEX should also be expected after the administration of a long-acting formulation of this GC (i.e. formulated as a propionate ester). Treatment of DEX increased the CYP3A28-like mRNA content and the level of a CYP3A-immunoreactive protein and induced the CYP3A-dependent metabolism in sheep liver. High and significant correlations between gene or protein expression profiles and CYP3A-dependent metabolic activities were observed. This observation may suggest that the enhanced CYP3A-dependent metabolism is consequence of an induced expression of a CYP3A28-like isoenzyme. Among the transcriptional factors involved in CYP3A regulation, only the expression of RXRα mRNA was induced by DEX. The slight induction of intestinal CYP3A28-like expression/activity observed in DEX-treated animals may be consequence of the reduced availability of the drug in the small intestine after its intramuscular administration. Finally, these results are a further contribution to the understanding of those factors involved in the regulation of XMEs in food-producing species.

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