Biochemical Pharmacology, Vol. 46, No. 10, pp. 1781–1790, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

CYTOCHROME P450 INDUCTION AND METABOLISM OF ALKOXYRESORUFINS, ETHYLMORPHINE AND TESTOSTERONE IN CULTURED HEPATOCYTES FROM GOATS, SHEEP AND CATTLE

GERBEN A. E. VAN 'T KLOOSTER,* BAS J. BLAAUBOER,† JAN NOORDHOEK†‡ and ADELBERT S. J. P. A. M. VAN MIERT

Department of Veterinary Pharmacology and Toxicology, and †Research Institute of Toxicology, University of Utrecht, P.O. Box 80.176, 3508 TD Utrecht, and ‡Department of Toxicology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

(*Received* 12 *January* 1993; *accepted* 28 *July* 1993)

Abstract—Very little is known of cytochrome P450 (P450) patterns and enzyme characteristics in food-producing animal species. Oxidative metabolism of alkoxyresorufins, ethylmorphine (EtM) and testosterone (TST) was used to monitor the effects of the P450 inducers phenobarbital (PB), β-naphthoflavone (BNF), dexamethasone (DEX) and triacetyloleandomycin (TAO) in primary cultured hepatocytes from goats, sheep and cattle. BNF effectively and specifically induced ethoxyresorufin deethylase (>20-fold), indicating the presence of an inducible P450 1A form, and down-regulated EtM demethylation and most selected TST hydroxylations. In non-induced hepatocyte cultures, TST was metabolized to 6β-, 2β-, 12β-, and 11α-hydroxy-TST (OHT). PB and, to a lesser extent, DEX non-specifically induced all OHT formations, and EtM demethylation. TAO almost completely inhibited OHT formation and EtM demethylation. These results indicate the involvement of principally one P450 form, or a restricted number of related P450 forms, presumably belonging to the P450 3A subfamily. In western blot analysis, cross reactivity was found with rat anti-P450 3A1 and anti-sheep P450 3A. A more specific PB effect was observed for 16α-OHT, which may be formed though a ruminant P450 2B form. None of the inducers influenced pentoxyresorufin depentylase (PROD) or EtM O-deethylation. Metabolite patterns and inducibility of selected activities in ruminant hepatocytes are in accordance with previous findings in goats *in vivo*. Cytochrome P450 characteristics in ruminants appear to differ from those in rats whereas similarities to the situation in humans appear to exist.

Hepatic cytochrome P450 (P450§) enzymes catalyse the oxidative metabolism of many endogenous and exogenous compounds. They belong to a gene superfamily in which the haemoproteins have been classified into families and subfamilies, based on amino acid sequences and the genes coding for the enzymes [1, 2]. The P450 families 1–4 are primarily involved in drug metabolism [3]. These P450 forms are inducible as an adaptive response to the presence of xenobiotics. This inducibility, in combination with selected activities, has formerly served as a basis to characterize and classify the enzymes [3, 4]. Thus, the P450 inducers phenobarbital (PB), 3-methylcholanthrene (3-MC) and pregnenolone carbonitrile (PCN) gave their names to groups of similar P450s that are now known as subfamilies P450 2B, P450 1A and P450 3A, respectively.

Hepatic P450 induction studies are generally performed in intact animals or with microsomes prepared from livers of pretreated animals. However, cultured hepatocytes were shown to respond to xenobiotics in maintaining or even increasing total culture P450 content [5]. Similar to their effects *in vivo*, glucocorticoid-related compounds, such as PCN and dexamethasone (DEX), and rifampicin were thus reported to induce P450 3A enzymes in cultured rat, rabbit and human hepatocytes [6–9]. The P450 1A inducers 3-MC and β-naphthoflavone (BNF) were shown to be potent inducers of P450 1A enzymes *in vitro* [7–10], too, resulting in increased levels of P450 1A apoproteins and related activities such as ethoxyresorufin O-deethylation (EROD). In contrast, with PB, a very potent inducer of P450 2B in intact rats, only a weak induction was found in cultured hepatocytes [9, 11] that was medium-dependent [12]. Changing culture conditions, including supplementing the culture medium with haem and selenium [13] or glucocorticoids [14], and introducing attachment substrata such as Matrigel and Vitrogen [15] were suggested to overcome the lack of responsiveness to PB.

Despite apparent limitations, the model system proves very useful to gather rapidly some basic information on the inducibility of P450 enzymes. It provides an attractive alternative to categorize drug metabolizing enzymes and study some of their characteristics, especially when other approaches are either impractical or unethical as is the case in humans [16]. Moreover, the use of cultured cells may

* Corresponding author. Tel. (31) 30 535400; FAX (31) 30 535077.

§ Abbreviations: AND, androstenedione; BNF, β-naphthoflavone; DEX, dexamethasone; EROD, ethoxyresorufin deethylase; EtM, ethylmorphine; HBSS, Hank's balanced salt solution; MAb, monoclonal antibody; OHT, hydroxytestosterone; P450, cytochrome P450; PB, phenobarbital; PROD, pentoxyresorufin depentylase; TAO, triacetyloleandomycin; TST, testosterone; 3-MC, 3-methylcholanthrene.

circumvent the difficulties that result from large inter-individual variation as found in *in vivo* studies, especially with studies in animal species that cannot be obtained as genetically standardized specimens raised under controlled conditions. The latter obviously is the case for ruminants.

The vast majority of investigations for P450 characteristics, including inducibility, have been performed in laboratory animals such as rats and mice. In agricultural species, only very limited information is available, in spite of the obvious risks for applied veterinary pharmacotherapy and public health, considering the animal origin of many foods. In recent studies, we have reported on the *in vivo* induction of P450 in dwarf goats [17, 18]. The present study was performed with the aim to obtain a general outline of P450-mediated metabolism and its induction in primary cultured hepatocytes from three ruminant species: goats, sheep and cattle. Both male and female dwarf goats were included as in this animal species, sex differences in oxidative drug metabolism both *in vivo* and in isolated hepatocytes, and inducibility have previously been reported [18–20]. Female rather than male sheep and cattle were used, because of their economical importance.

MATERIALS AND METHODS

Chemicals. Sodium phenobarbital, ethylmorphine (ETM) and morphine were obtained from Brocacef (Maarssen, The Netherlands). Norethylmorphine was a generous gift from Dr B. Lindström and Prof. A. Rane (University of Uppsala, Sweden). Triacetyloleandomycin (TAO) was a kind gift from Pfizer (Rotterdam, The Netherlands). BNF, testosterone (TST), androstenedione (AND), 11β- and 16α-hydroxytestosterone (OHT) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 2α-, 6α- and 15α-OHT were gifts from Prof. D. N. Kirk (Steroid Reference Collection, London, U.K.), and 12β- and 15β-OHT from G. D. Searle and Co. (Skokie, IL, U.S.A.).

2β-, 6β-, 7α-, 11α-, 16β- and 19-OHT and 16-keto-TST were purchased from Steraloids (Wilton, NH, U.S.A.). Resorufin was from Eastman Kodak (Rochester, NY, U.S.A.). All other chemicals were of analytical grade.

Polyclonal antibodies against sheep P450 3A and 2B were kind gifts from Dr P. Galtier (INRA, Toulouse, France). Monoclonal antibodies (MAb) against P450 1A1/2 and 3A were gifts from Dr P. Kremers (University of Liège, Belgium).

Animals and isolation of hepatocytes. At least one month prior to the experiment, groups of four healthy male (13–15 kg) or female (11–13 kg) dwarf goats, 8–10 months of age, and three female Texel sheep (51–56 kg), 12–13 months of age, were housed under controlled conditions. Animals were fed a commercially available pelleted feed concentrate and had free access to hay and water. For isolation of cattle hepatocytes, four Meuse-Rhine-Yssel cows (570–650 kg), 3–4 years of age, were selected from a flock of slaughterhouse cattle, examined and found healthy.

Parenchymal cells were isolated from left liver lobes (goats, sheep) or caudate liver lobes (cattle), as described previously [21].

Hepatocyte cultures and induction regimen. Hepatocytes were cultured in a humidified atmosphere of air (95%) and CO_2 at 37°, at a density of approx. 4×10^6 cells (35–40 mg) per 6-cm tissue culture dish (Greiner, Breda, The Netherlands), in 4 mL Williams Medium E (WME) (Sigma). The medium was supplemented with 5% v/v newborn calf serum (Flow, Irvine, U.K.), 1.67 mM glutamine, 10 μM hydrocortisone-hemisuccinate, 1 μM bovine pancreas insulin, and gentamicin sulphate (50 μg/mL) (all from Sigma). Cells were preincubated for 24 hr. Then the medium was replaced by medium containing either 0.1% v/v DMSO (control), 1.5 mM PB, 50 μM BNF, or 1 μM DEX. As in the control medium, in the latter three media, 0.1% v/v DMSO was present as DMSO was used as solvent for the inducers. The induction period lasted 48 hr. Media were refreshed every 24 hr.

Enzyme assays. Determinations of EROD, pentoxyresorufin depentylase (PROD), TST hydroxylation and EtM dealkylation activities were performed directly in intact hepatocyte monolayers, as described by Wortelboer *et al.* [22] and van 't Klooster *et al.* [23], respectively. In short, monolayers were washed with Hank's balanced salt solution (HBSS) and incubated with ethoxyresorufin (5 μM, 20 min), pentoxyresorufin (5 μM, 30 min) or TST (0.5 mM, 15 min), in 4 mL HBSS, or with EtM (2.5 mM, 120 min), in 4 mL serum-free WME.

Fluorimetric determinations of EROD and PROD activities were performed as described by Wortelboer *et al.* [22], using 1-mL medium samples that were mixed with 1 mL 0.01 M aqueous sodium hydroxide.

For TST determination, 2.5-mL medium samples were extracted with 6 mL dichloromethane. Dichloromethane fractions were evaporated to dryness under a flow of nitrogen. Residues were reconstituted in methanol/water 50/50 v/v and used for HPLC analysis. TST hydroxylated metabolites (OHTs) were separated using a 200 × 3 mm Chromsep C_{18} column (Chrompack, Middelburg, The Netherlands), kept at 60°. Elution was performed at a flow rate of 0.9 mL/min, using water/methanol 75/25 v/v from 0 to 7 min, followed by a linear gradient to water/methanol/acetonitril 56/40/4 by vol. from 7 to 50 min. UV detection at 254 nm was used. OHTs were quantified by comparing peak areas to those of authentic standards.

EtM N-demethylation and O-deethylation was determined in 2.5 mL medium. Samples were extracted with ethyl acetate. Ethyl acetate fractions were evaporated to dryness under a flow of nitrogen. Residues were dissolved in 10% v/v acetonitrile and used for HPLC analysis [23]. Morphine, norethylmorphine and EtM were separated on a 100 × 8 mm Lichrosorb CN Radial PAK column (Waters, Etten-Leur, The Netherlands) by isocratic elution with 10% v/v acetonitril in a 50 μM phosphate buffer (pH = 2.5),containing 0.2% triethylamine, at a flow rate of 2 mL/min, and detected at 210 nm.

Contents of protein and total P450 were determined according to Rutten *et al.* [24] using a double-beam spectrophotometer (PU 8800, Pye Unicam, Eindhoven, The Netherlands).

Table 1. Effects of various inducers on P450-dependent activities in primary cultures of hepatocytes from male goats (N = 4)

	Control	PB	BNF	DEX
P450	111 ± 19	112 ± 24	106 ± 26	97 ± 25
EROD	1.4 ± 0.5	2.6 ± 1.3	62 ± 10†	4.5 ± 1.5
PROD	2.0 ± 0.7	1.9 ± 0.6	2.2 ± 0.6	2.3 ± 1.0
EtM-NDM	225 ± 48	306 ± 56*	162 ± 46	237 ± 55
EtM-ODE	44 ± 12	44 ± 6	47 ± 12	46 ± 16
2β-OHT	49 ± 24	71 ± 34	25 ± 15	54 ± 29
6β-OHT	80 ± 13	124 ± 25*	39 ± 16	84 ± 21
11α-OHT	9.5 ± 3.2	15 ± 3	6.7 ± 3.2	10.7 ± 3.2
12β-OHT	14 ± 6	19 ± 7	6.8 ± 3.4	14 ± 5
15α-OHT	3.9 ± 1.1	6.8 ± 1.4*	2.6 ± 0.8	4.4 ± 0.7
15β-OHT	3.5 ± 0.9	4.9 ± 1.6	2.2 ± 0.7	3.3 ± 0.5
16α-OHT	1.5 ± 0.3	7.0 ± 1.7*	1.7 ± 0.3	1.5 ± 0.3
AND	116 ± 24	135 ± 22	116 ± 22	119 ± 27

Data are expressed as mean ± SD (N = 4). P450 content in pmol/mg protein. Activities in pmol/mg protein/min.

* Significant vs control (Student's *t*-test, $P < 0.05$).

† Significant vs control (Student's *t*-test, $P < 0.01$).

Abbreviations: NDM, N-demethylation; ODE, O-deethylation.

Gel electrophoresis and western blots. Hepatocytes were harvested in PBS (40 mg cells/mL) and sonicated for 2 × 30 sec (Soniprep 500, MSE), and centrifuged at 10,000 *g* for 30 min. The supernatant, containing microsomal and cytosolic proteins, was used for electrophoresis.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of microsomal proteins was performed in a Protean II Mini Cell (Biorad, Veenendaal, The Netherlands), using the discontinuous system as described by Laemmli [25]. The resolved proteins and prestained molecular weight markers (Biorad) were blotted on polyvinylidenedifluoride (PVDF) sheets (Millipore, Etten-Leur, The Netherlands), according to the method of Towbin *et al.* [26]. Antibodies raised to sheep P450 3A and P450 2B, and to rat P450 1A1/2 and P450 3A were used for immunochemical staining as no homologous antibodies to goat and cattle P450 enzymes are available. Methodological details have been described by Wortelboer *et al.* [22].

RESULTS

Hepatocyte isolation and cultivation

For all three ruminant species, liver lobes (sheep and goats: left lobes, cattle: caudate lobes) with intact blood vessel systems of approx. 90–100 g were used. Perfusion yielded 22–31 g hepatocytes. Viabilities as assessed by Trypan blue exclusion ranged from 81 to 92%. The cell concentration per culture dish was based on weight rather than on cell count. As cattle hepatocytes are small when compared to sheep and goat hepatocytes (approx. 15 and 20 μm in diameter, respectively), seeding of approx. 32 mg of cattle hepatocytes or 40 mg of sheep or goat hepatocytes per 6-cm dish resulted in similar cell densities (4×10^6 cells per dish).

The average initial culture protein content, determined in freshly isolated hepatocytes, was 3.5 mg for cattle, 4.1 mg for sheep and 4.5 mg for goats (SD < 15%). The plating efficiency, expressed as the ratio of protein concentration in freshly isolated hepatocytes ($t = 0$ hr) and after the first changing of medium, was >90%, indicating a partial restoration of membrane damage as initially detected with Trypan blue. After 72 hr in culture, protein content was over 85% of the initial concentration. Initial ($t = 0$ hr) P450 content (pmol/mg protein, N = 3, relative SD = 15%) was 220 for female goats and sheep, 180 for male goats and 140 for cows. At 72 hr, 51–60% of total P450 was maintained in hepatocyte cultures from female cattle, sheep and goat, whereas in male hepatocytes, this number was 57–63%, thereby abolishing the initially significant sex difference in P450 content.

Comparative metabolism of alkoxyresorufins, EtM and TST

The results on the metabolism of alkoxyresorufins, EtM and TST are shown in Tables 1, 2, 3 and 4 for male goats, female goats, female sheep and female cattle, respectively. Comparing selected activities, we found activities to be of the same order of magnitude in all three species, even though culture P450 content in cattle hepatocytes was 65% of that in sheep and goat liver cell cultures. Another discrepancy between total P450 content and measured activities was found for male and female goat hepatocytes: after 72 hr in culture, total P450 content was equal in hepatocytes from either sex, whereas in cells from females, EtM dealkylation and TST hydroxylation were significantly higher than in male hepatocytes.

EROD activity was relatively low in cultured sheep hepatocytes. In one sheep, no activity was detectable in control cells although it was obvious after BNF induction. For EtM dealkylation, the O-

Table 2. Effects of various inducers on P450-dependent activities in primary cultures of hepatocytes from female goats (N = 4)

	Control	PB	BNF	DEX
P450	112 ± 18	119 ± 30	121 ± 19	104 ± 25
EROD	1.6 ± 0.6	2.6 ± 0.3	40 ± 8†	1.8 ± 0.6
PROD	1.4 ± 0.2	1.6 ± 0.5	1.9 ± 0.5	1.8 ± 0.5
EtM-NDM	257 ± 18	357 ± 26†	147 ± 62*	189 ± 20
EtM-ODE	28 ± 5	25 ± 5	23 ± 7	34 ± 11
2β-OHT	95 ± 53	115 ± 52	50 ± 28	107 ± 51
6β-OHT	99 ± 22	141 ± 32	52 ± 15*	118 ± 26
11α-OHT	23 ± 11	36 ± 12	13 ± 6	27 ± 11
12β-OHT	37 ± 14	46 ± 14	21 ± 10	41 ± 14
15α-OHT	6.5 ± 1.4	9.9 ± 2.3	3.9 ± 1.2	8.1 ± 1.7
15β-OHT	7.6 ± 2.4	10 ± 2	4.9 ± 2.2	8.2 ± 3.1
16α-OHT	3.3 ± 0.5	12 ± 4†	3.3 ± 1.0	3.3 ± 0.4
AND	107 ± 12	126 ± 18	105 ± 13	116 ± 18

Data are expressed as mean ± SD (N = 4).
P450 content in pmol/mg protein.
Activities in pmol/mg protein/min. Abbreviations: see Table 1.
* Significant vs control (Student's *t*-test, $P < 0.05$).
† Significant vs control (Student's *t*-test, $P < 0.01$).

Table 3. Effects of various inducers on P450-dependent activities in primary cultures of hepatocytes from female sheep (N = 3)

	Control	PB	BNF	DEX
P450	107 ± 16	133 ± 20	103 ± 19	110 ± 7
EROD	0.6 ± 0.4	2.2 ± 0.6	36 ± 14†	0.6 ± 0.2
PROD	0.8 ± 0.3	0.6 ± 0.5	2.1 ± 0.8	1.1 ± 0.4
EtM-NDM	203 ± 25	412 ± 102†	139 ± 22	223 ± 40
EtM-ODE	38 ± 4	32 ± 4	36 ± 3	37 ± 8
2β-OHT	17 ± 3	30 ± 3*	7 ± 2*	23 ± 2
6β-OHT	70 ± 12	126 ± 25*	41 ± 6*	96 ± 18
11α-OHT	6.0 ± 0.4	13 ± 2†	2.2 ± 0.4†	9.0 ± 1.1*
12β-OHT	7.8 ± 1.0	13 ± 1†	3.9 ± 1.3*	10 ± 2
15α-OHT	4.3 ± 1.0	9.3 ± 1.2†	3.0 ± 1.3	6.1 ± 1.1
15β-OHT	9.9 ± 9.0	12 ± 9	4.5 ± 4.0	9.5 ± 7.3
16α-OHT	5.2 ± 1.5	22 ± 10*	7.2 ± 1.7	8.4 ± 5.8
AND	166 ± 33	187 ± 32	162 ± 12	167 ± 25

Data are expressed as mean ± SD (N = 3).
P450 content in pmol/mg protein.
Activities in pmol/mg protein/min. Abbreviations: see Table 1.
* Significant vs control (Student's *t*-test, $P < 0.05$).
† Significant vs control (Student's *t*-test, $P < 0.01$).

deethylation activity was similar in hepatocytes from goats, sheep and cattle. In all animals, N-demethylation was the major pathway. For TST hydroxylations, metabolite patterns in ruminant hepatocytes were similar but quantitative species-dependent differences in metabolite formation were found. For all species AND was most important. In sheep hepatocytes, 6β-OHT was the only other major metabolite, whereas in cells from goats and cows, 2β-OHT and 12β-OHT were important as well. In Tables 1–4, minor metabolites (<0.5% of total metabolism), such as 2α-, 19- and 16β-OHT were not included. 7α-OHT, a quantitatively important metabolite in rats [27], was not detectable in ruminant hepatocyte cultures.

Effects of P450 inducers on enzyme activities

Effects of PB, BNF and DEX on total P450 content and selected activities are presented in Tables 1, 2, 3 and 4. Effects of TAO were not included because it did neither significantly influence P450 levels (although levels were elevated, SDs were rather large) nor activities of EROD and PROD. However, it very effectively (>95%) inhibited

Table 4. Effects of various inducers on P450-dependent activities in primary cultures of hepatocytes from female cattle (N = 4)

	Control	PB	BNF	DEX
P450	70 ± 12	81 ± 9	79 ± 16	73 ± 6
EROD	1.2± 0.2	2.3 ± 1.4	54 ± 19†	5.2 ± 1.3*
PROD	0.5 ± 0.2	0.9 ± 0.3	1.7 ± 0.3*	1.2 ± 0.5
EtM-NDM	189 ± 44	388 ± 74*	150 ± 24	217 ± 19
EtM-ODE	54 ± 10	45 ± 6	47 ± 9	51 ± 12
2β-OHT	31 ± 12	68 ± 18*	16 ± 7	44 ± 15
6β-OHT	71 ± 20	143 ± 19*	41 ± 11	94 ± 25
11α-OHT	3.3 ± 0.9	6.5 ± 1.1*	1.7 ± 0.1	4.7 ± 1.5
12β-OHT	46 ± 17	101 ± 24*	5 ± 11	73 ± 25
15α-OHT	4.0 ± 1.6	9.6 ± 1.9*	2.4 ± 1.0	6.3 ± 2.4
15β-OHT	5.9 ± 1.6	12 ± 3*	3.6 ± 1.4	7.8 ± 2.9
16α-OHT	0.4 ± 0.1	1.1 ± 0.2*	0.7 ± 0.1	0.7 ± 0.2
AND	157 ± 24	176 ± 31	149 ± 27	155 ± 29

Data are expressed as mean ± SD (N = 4).
P450 content in pmol/mg protein.
Activities in pmol/mg protein/min. Abbreviations: see Table 1.
* Significant vs control (Student's *t*-test, $P < 0.05$).
† Significant vs control (Student's *t*-test, $P < 0.01$).

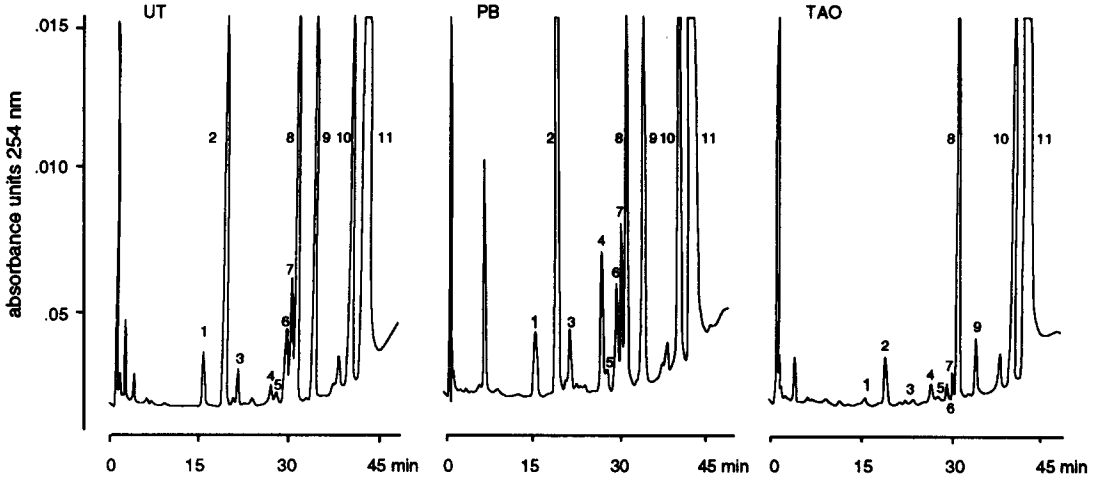

Fig. 1. Representative chromatograms of analysis of medium samples from untreated (UT) and 48-hr PB- or TAO-treated female goat hepatocyte cultures incubated with TST. Peaks: 1, 15β-OHT; 2, 6β-OHT; 3, 15α-OHT; 4, 16α-OHT; 5, 16β-OHT; 6, 11α-OHT; 7, 12β-OHT; 8, 11β-OHT (internal standard); 9, 2β-OHT; 10, AND; 11, TST.

EtM N-demethylation and all different TST hydroxylations. The latter is exemplified in the representative chromatogram shown in Fig. 1 from female goat hepatocytes incubated with TST; in TAO-treated cultures all peaks observed in control cultures are nearly eliminated (detailed results not shown).

In hepatocyte cultures from the three ruminant species, PB appeared a weak inducer for most selected activities. PROD, in rats related to P450 2B1 and PB induction [28], was not at all influenced by PB treatment. A remarkable induction (4-fold) was observed for 16α-OHT in goat and sheep hepatocytes (Tables 1–3, Fig. 1). In cattle hepatocytes, 16α-OHT was a very minor metabolite which hampered an exact determination of the induction factor. Total P450 level tended to increase although only in sheep hepatocytes was the increase significant.

BNF was the only selectively and strongly inducing compound. After BNF treatment, in all hepatocyte cultures, only EROD activity was 25–40 times increased. PROD activity was either not influenced or only weakly induced. Other measured activities were inhibited, with the exception of AND formation

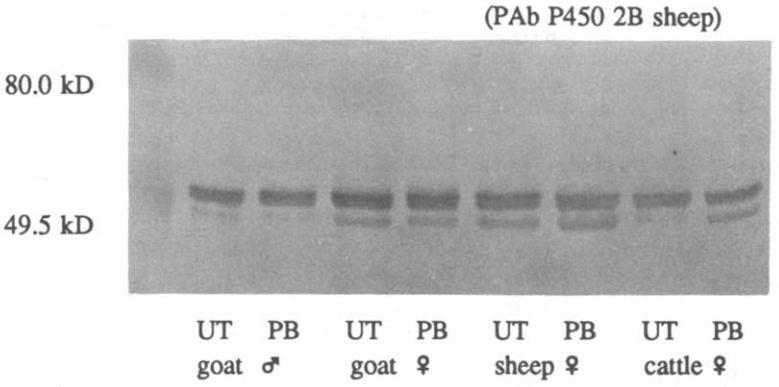

Fig. 2. SDS–PAGE immunodetection (15 μg protein/lane) of P450 2B apoproteins, stained with a polyclonal antibody (PAb) raised against sheep P450 2B, in sonicated hepatocytes from male and female goats, female sheep and female cattle. Hepatocytes were either untreated (UT) or treated for 48 hr with PB.

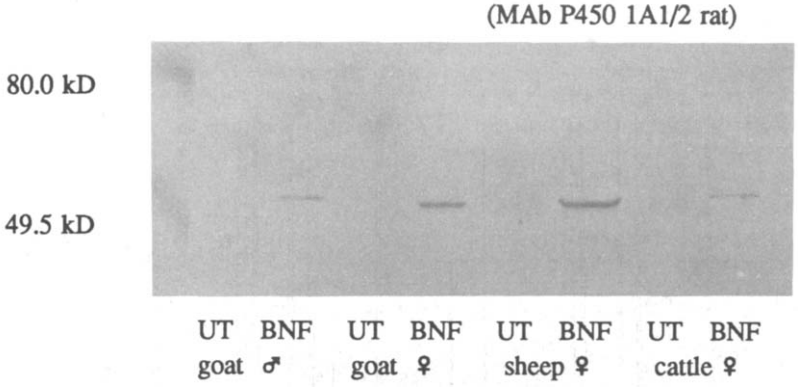

Fig. 3. SDS–PAGE immunodetection (15 μg protein/lane) of P450 1A apoproteins, stained with a polyclonal antibody (PAb) raised against rat P450 1A1/2, in sonicated hepatocytes from male and female goats, female sheep and female cattle. Hepatocytes were either untreated (UT) or treated for 48 hr with PB.

that was not influenced by any of the used inducers. In spite of the strong EROD induction, no increased levels of total P450 were observed.

In contrast to rat hepatocytes [6, 9], but, similar to human hepatocytes [16], DEX was not an effective *in vitro* inducer of cytochrome P450 activities in cultured ruminant hepatocytes. DEX induction appeared least effective in goats. In sheep and cattle, a weak induction (Tables 3 and 4) was observed for EtM N-demethylation and most TST hydroxylations. Similar to PB, all different TST hydroxylations and EtM N-demethylation were influenced to the same extent.

Western blots

Western blots immunostained with sheep anti-P450 2B, rat anti-P450 1A1/1A2, and sheep anti-P450 3A and rat anti-P450 3A1 are shown in Figs 2, 3 and 4, respectively. The polyclonal antibodies against sheep P450 2B and 3A stained several bands, even in sheep hepatocyte samples, indicating an incomplete specificity of the antibody and the presence of several immunoreactive proteins. The estimated apparent molecular weights of the stained proteins ranged from 48 to 58 kDa. It remains unknown whether these are all P450 forms. For the sheep anti-P450s, staining intensity was qualitatively equal in all three ruminant species, indicating a strong cross-reactivity across the P450s from the three ruminant species. The least staining reactivity was observed with rat anti-P450 1A1/1A2; to obtain the rather weak bands, especially with cows, in BNF-treated hepatocytes as shown in Fig. 2, staining time was double that for rat anti-P450 3A1 and the sheep P450 antibodies (Figs 2–4).

Induction effects on apoprotein levels were obvious for BNF: an immunoreactive protein was detected in BNF-treated hepatocyte cultures, although weakly in hepatocytes from cows, whereas no bands were stained in control cultures (Fig. 3).

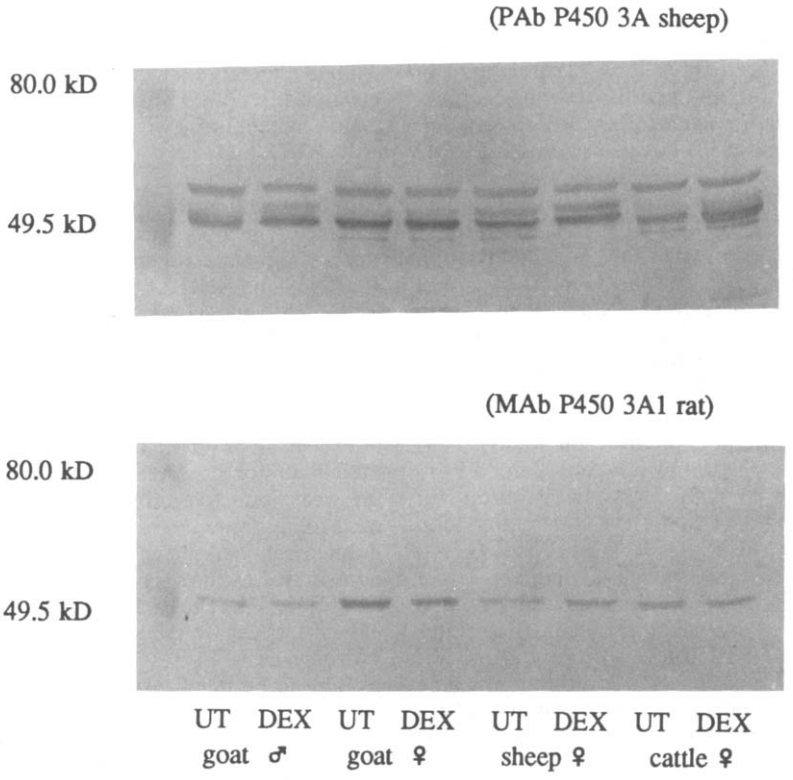

Fig. 4. SDS–PAGE immunodetection (15 μg protein/lane) of P450 3A apoproteins stained with sheep anti-P450 3A (upper blot), and stained with rat anti-P450 3A1 (lower blot) in sonicated hepatocytes from male and female goats, and female sheep and cattle. Hepatocytes were either untreated (UT) or treated for 48 hr with DEX. Weak inductive effects by DEX can be observed. In the upper blot, the lower band appears to be the inducible P450 3A. The nature of the other bands remains unknown.

Effects of PB or DEX treatment were much less obvious. Western blots immunostained with sheep anti-P450 2B (Fig. 2) yielded two hardly separated bands (approx. 51 and 52 kDa), for which no PB induction effects were observed. A third band (48 kDa) was present in female goat and sheep hepatocytes and in PB-treated cow hepatocytes. With sheep anti-P450 3A, a slight increase in apoprotein level could be observed in DEX-treated hepatocytes from sheep and cow, as compared to non-treated hepatocytes (Fig. 4). The slightly inducible protein in sheep and corresponding protein in goat hepatocytes had an estimated molecular weight of approx. 51 kDa, whereas in cow hepatocytes, it was approx. 50 kDa. The P450 3A form, cross-reactive with rat anti-P450 3A1 was relatively more abundant in female goats than in male goats, sheep or cows.

DISCUSSION

In BNF- and PB-treated rats, EROD and PROD activities are related to P450 1A and P450 2B, respectively [28, 29]. In non-induced animals however, both ethoxyresorufin and pentoxyresorufin are substrates for other, constitutive P450 forms, such as P450 2C11 in male rats [30]. In a previous paper [18], we reported on *in vivo* effects of BNF in male and female dwarf goats. Comparing microsomes from non-treated and BNF-treated animals, EROD was induced 6-fold. In cultured rat and rabbit hepatocytes, BNF was shown to be a potent inducer for P450 1A1/1A2 [7, 31] and EROD [9]. Thus, a strong induction by BNF of EROD activity in hepatocytes from goats, sheep and cattle (Tables 1–4) can readily be explained through a similar involvement of a BNF-inducible P450 1A enzyme. BNF induction is supported by western blots immunostained with rat-anti P450 1A1/2 (Fig. 2) showing an obvious increase at the apoprotein level. Apparently, either cross-reactivity of the MAb to the epitope of ruminant P450 1A is less than that of the anti-rat P450 3A1 MAb towards ruminant P450 3A, or the P450 1A apoprotein concentration in hepatocyte cultures remained relatively low, even after BNF treatment, as it took at least twice as long to obtain visible bands for P450 1A immunostaining as compared to P450 3A immunostaining.

In cultured ruminant hepatocytes, PB did not induce PROD. The same result was found in similarly cultured rat hepatocytes [9]. In rats, this finding could be explained by the well-established lack of PB effects *in vitro*, unless hepatocytes are cultured under modified conditions, including the use of a

matrigel substratum, medium supplementations or co-cultures [12–15]. However, in contrast to rats, PROD is not induced by *in vivo* PB treatment of goats [18], indicating that pentoxyresorufin is not a P450 2B substrate, even after PB treatment. Therefore, the lack of PB effects *in vitro* on PROD activity cannot be explained as for the rat. Is PROD dealkylated by a P450 3A form which would explain the weak PROD increase in DEX-treated cultures? Probably not as, in contrast to EtM N-demethylation and most TST hydroxylations, PROD was not inhibited by TAO. The weak induction seen with BNF is presumably associated with overlapping substrate specificities of P450 1A and 2A, both inducible by 3-MC, as has been reported for rats [2].

In rats, EtM N-demethylation and EtM deethylation are catalysed by a P450 3A and a P450 2D enzyme, respectively [32]. In cultured rat hepatocytes, P450 3A1 and EtM N-demethylation was inducible by PCN, DEX and TAO [33, 34]. Whereas DEX is an effective inducer at a concentration of 0.1–1 μM, the structurally related hydrocortisone (used as a culture medium constituent) does not affect P450 levels up to 100 μM [6]. DEX induces *de novo* P450 synthesis through transcriptional activation of the P450 3A gene, whereas TAO primarily blocks the degradation of the protein. A TAO metabolite binds to the active site of the P450 3A that catalyses its formation and, therefore, unless it is chemically removed (with ferricyanide), it inhibits rather than induces P450 activity [35]. In ruminant hepatocytes, EtM N-demethylation was inhibited by TAO but only slightly induced by DEX. Hydrocortisone did not influence P450 3A activity in cultured cattle and goat hepatocytes (results not shown). Incubation with PB, a P450 3A inducer as well, was more effective. Thus, P450 3A appears to be involved in EtM N-demethylation, but it is not a DEX-responsive P450 3A1. Remarkably, western blots immunostained with sheep anti-P450 3A, presumably a P450 3A6 like enzyme [36], showed an increase in apoprotein following DEX treatment (Fig. 4) and a lower molecular weight for cow P450 3A, as compared to sheep and goats. Rat anti-P450 3A1 immunostaining did not lead to similar observations, but it showed higher levels of a cross-reactive protein to be present in hepatocytes from female goats than in those from males, sheep and cattle. It remains unknown whether two different P450 3A forms were stained with the sheep and rat antibodies.

In goat, sheep and cow hepatocyte cultures, EtM O-deethylation was neither induced by PB, BNF or DEX, nor inhibitied by TAO. The constitutive P450 2D forms can be inhibited (e.g. by quinine or quinidine), but in addition, it is "characterized" by its non-inducibility [32]. It is therefore plausible that, similar to rat, a P450 2D is involved in EtM O-deethylation.

Regio- and stereoselective hydroxylation of TST and other steroids has generally been used to characterize P450s (for examples see Refs. 27, 37). In non-treated hepatocytes from male rats, AND, 6β-, 16α- and 2α-OHT were reported to be major metabolites [9, 14, 37]. In accordance with studies using liver microsomes, activities in female rats were much lower, AND and 7α-OHT being the major oxidative metabolites of TST [38]. In goat and cow hepatocytes, AND, 6β-, 2β- and 12β-OHT were major metabolites, whereas in sheep hepatocytes, only AND, 6β-, and, to a lesser extent, 2β-OHT (and 15β-OHT) were important. These results correlate well with previously reported results obtained with goat and sheep microsomes [18, 39]. In all three ruminant species, with the exception of 16α-OHT and AND, all different TST hydroxylations were slightly increased after PB and DEX treatment, whereas BNF down-regulated all activities. TAO practically eliminated all hydroxylation activity towards TST. The lack of specific effects indicates the involvement of only one or some related form(s) of P450 in all different TST hydroxylations. A nearly total inhibition by TAO points at a P450 3A, as does the weak inductive effect of PB. Remarkably, the metabolite patterns are similar to those found with purified rat P450 3A1 [40, 41], and the activity of human microsomes and cDNA-expressed human P450s [42, 43]. The lack, especially in goats, of an inducing effect by DEX, a potent inducer for TST hydroxylations in rats [9], indicates a P450 3A enzyme to be involved which differs from rat P450 3A1. With human microsomes, 6β-, 2β- and 15β-OHT were formed, and these hydroxylation activities were highly correlated with each other [42]. It was suggested that a single human liver P450 3A catalysed most hydroxylation pathways in TST metabolism. In the present study, a similar high correlation is found for all different OHTs, with regard to PB, BNF, DEX and TAO effects. If a single P450 3A (or several related forms) is involved in ruminant TST hydroxylation, it is more similar to the human than to the rat form. In a previous study [18], we reported on goat microsomal mRNA recognition with a human P450 3A4 cDNA probe, whereas a rat 3A1 probe yielded a much weaker signal.

PB treatment increased TST 16α-hydroxylation 4–5-fold in goats and sheep. With microsomes from PB-treated goats, a similar though more pronounced effect (up to 20-fold induction) has been observed [18]. If this activity is related to a P450 2B form, as is the case for rats [41], it appears inducible in ruminant hepatocytes, whereas in cultured rat hepatocytes, PB did not induce 16α-OHT formation [9]. The absence of down-regulation by BNF, on the other hand, might indicate the involvement of a P450 2A form, as P450 2A and 2B may be inducible by both PB and BNF [2].

AND was neither induced nor inhibited by PB, BNF, DEX or TAO. Possibly, mainly non-P450 enzymes (e.g. oxidoreductases [39]) are involved in its formation.

To compare species to species, results of the present study have been presented as means of three animals, which resulted in rather large standard deviations in measured activities. Culturing hepatocytes for several days under identical conditions minimizes differences caused by diet, physiological status etc., as observed *in vivo*, but not genetic differences. A clear example was seen with 2β-OHT formation which was very low in one male and one female goat, whereas in the other animals, it was at

least similar to 6β-OHT. Remarkably, the response to inducers and the magnitudes of induction appeared to be fairly constant in all individuals within one species, though overall activities differed widely. Thus, cultured ruminant hepatocyes may be used to circumvent interindividual differences if one is interested in effects of compounds with biotransformation enzymes, rather than levels of activity *per se*.

In conclusion, in cultured ruminant hepatocytes, a BNF-inducible P450 1A and at least one P450 3A appeared to be present. DEX is not a potent inducer for ruminant P450 3A. Indications for the presence of a P450 2A or 2B, and a 2D form have been found. Inter-ruminant species differences in metabolite patterns were found but inducibilities were similar. Both metabolite patterns and inducibilities are very different from those reported for rats, which should have its consequences for the use of this animal species in the risk evaluation for veterinary drugs and xenobiotic residues in food of animal origin.

REFERENCES

1. Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez F, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR and Waxman DJ, The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA Cell Biol* **10**: 1–14, 1991.
2. Gonzalez FJ, The molecular biology of cytochrome P450s. *Pharmacol Rev* **40**: 243–288, 1989.
3. Guengerich FP, Cytochrome P-450 enzymes and drug metabolism. In: *Progress in Drug Metabolism* 10 (Eds. Bridges JW, Chasseaud LF and Gibson GG), pp. 2–54. Taylor and Francis, London, 1987.
4. Okey AB, Enzyme induction in the cytochrome P-450 system. *Pharmacol Ther* **45**: 241–298, 1990.
5. Chenery RJ, The utility of hepatocytes in drug metabolism studies. In: *Progress in Drug Metabolism* 11 (Ed. Gibson GG), pp. 217–265. Taylor and Francis, London, 1988.
6. Schuetz EG, Wrighton SA, Barwick JL and Guzelian PS, Induction of cytochrome P-450 by glucocorticoids in rat liver. *J Biol Chem* **259**: 1999–2006, 1984.
7. Daujat M, Clair P, Astier C, Fabre I, Pineau T, Yerle M, Gellin J and Maurel P, Induction, regulation and messenger half-life of cytochromes P450, IA1, IA2 and IIIA6 in primary cultures of rabbit hepatocytes. *Eur J Biochem* **200**: 501–510, 1991.
8. Morel F, Beaune PH, Ratanasavanh D, Flinois J-P, Yang CS, Guengerich FP and Guillouzo A, Expression of cytochrome P450 enzymes in cultured human hepatocytes. *Eur J Biochem* **191**: 437–444, 1990.
9. Wortelboer HM, de Kruif CA, van Iersel AAJ, Falke HE, Noordhoek J and Blaauboer BJ, Comparison of cytochrome P450 isoenzyme profiles in rat liver and hepatocyte cultures: the effects of model inducers on apoproteins and biotransformation activities. *Biochem Pharmacol* **42**: 381–390, 1991.
10. Steward AR, Wrighton SA, Pasco DS, Fagan JB, Li D and Guzelian PS, Synthesis and degradation of 3-methylcholanthrene-inducible cytochrome P-450 and their mRNAs in primary monolayer cultures of adult rat hepatocytes. *Arch Biochem Biophys* **241**: 494–508, 1985.
11. Newman SL, Barwick JL, Elshourbagy NA and Guzelian PS, Measurement of the metabolism of cytochrome P-450 in cultured hepatocytes by a quantitative and specific immunochemical method. *Biochem J* **204**: 281–290, 1982.
12. Turner NA and Pitot HC, Dependence of the induction of cytochrome P-450 by phenobarbital in primary cultures of adult rat hepatocytes on the composition of the culture medium. *Biochem Pharmacol* **38**: 2247–2251, 1989.
13. Engelmann GL, Richardson AG and Fierer JA, Maintenance and induction of cytochrome P-450 in cultured rat hepatocytes. *Arch Biochem Biophys* **238**: 359–367, 1985.
14. Waxman DJ, Morrissey JJ, Naik S and Jauregui HO, High-level long-term responsiveness of primary rat hepatocyte cultures to drug induction, and glucocorticoid dependence of the phenobarbital response. *Biochem J* **271**: 113–119, 1990.
15. Kocarek TA, Schuetz EG and Guzelian PS, Differentiated induction of cytochrome P450b/e and P450p mRNAs by dose of phenobarbital in primary cultures of adult rat hepatocytes. *Mol Pharmacol* **38**: 440–444, 1990.
16. Donato MT, Gomez-Lechon MJ and Castell JV, Effects of xenobiotics on monooxygenase activities in cultured human hepatocytes. *Biochem Pharmacol* **39**: 1321–1326, 1990.
17. Natsuhori M, Witkamp RF, van 't Klooster GAE and van Miert ASJPAM, Metabolism of antipyrine and sulphadimidine in dwarf goats: effects of the enzyme inducing agents phenobarbital, troleandomycin and rifampicin. *Xenobiotica* **22**: 1243–1250,1992.
18. van 't Klooster GAE, Horbach GJMJ, Natsuhori M, Blaauboer BJ, Noordhoek J and van Miert ASJPAM, Hepatic cytochrome P450 induction in dwarf goats. Effects of model inducers on the metabolism of alkoxyresorufins, testosterone and ethylmorphine, and on apoprotein and mRNA levels. *Biochem Pharmacol* **45**: 113–122, 1993.
19. Witkamp RF, van 't Klooster GAE, Nijmeijer SM, Kolker HJ, Noordhoek J and van Miert ASJPAM, Hormonal regulation of oxidative drug metabolism in the dwarf goat. *J Vet Pharmacol Ther* **16**: 55–62, 1993.
20. van 't Klooster GAE, Blaauboer BJ, Noordhoek J and van Miert ASJPAM. Sulphadimidine metabolism *in vitro*: II. Comparative studies in isolated hepatocytes from rats, goats, sheep and cows. *J Vet Pharmacol Ther*, in press.
21. van 't Klooster GAE, Woutersen-Van Nijnanten FMA, Klein WR, Blaauboer BJ, Noordhoek J and van Miert ASJPAM, Effects of various medium formulations and attachment substrata on the performance of cultured ruminant hepatocytes in biotransformation studies. *Xenobiotica* **22**: 523–534, 1992.
22. Wortelboer HM, de Kruif CA, van Iersel AAJ, Falke HE, Noordhoek J and Blaauboer BJ, The isoenzyme pattern of cytochrome P450 in rat hepatocytes in primary culture, comparing different enzyme activities in microsomal incubations and in intact monolayers. *Biochem Pharmacol* **40**: 2525–2534,1990.
23. van 't Klooster GAE, Woutersen-Van Nijnanten FMA, Kolker HJ, Noordhoek J and van Miert ASJPAM, Improved high-performance liquid chromatographic method for the determination of ethylmorphine and its metabolites in microsomal incubations and cell culture media. *J Chromatogr* **579**: 158–164, 1992.
24. Rutten AAJL, Falke HE, Catsburg JF, Topp R, Blaauboer BJ, Van Holsteijn I, Doorn L and van Leeuwen FXR, Interlaboratory comparison of total cytochrome P450 and protein determinations in rat liver microsomes. *Arch Toxicol* **61**: 27–33, 1987.
25. Laemmli IK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
26. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to

nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
27. Waxman DJ, Interactions of hepatic cytochromes P-450 with steroid hormones. Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem Pharmacol* **37**: 71–84, 1988.
28. Lubet RA, Mayer RT, Cameron JW, Nims RW, Burke MD, Wolff T and Guengerich FP, Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat. *Arch Biochem Biophys* **238**: 43–48, 1985.
29. Iwasaki K, Lum PY, Ioannides C and Parke DV, Induction of cytochrome P-448 activity as exemplified by the O-deethylation of ethoxyresorufin. Effects of dose, sex, tissue and animal species. *Biochem Pharmacol* **35**: 3879–3884, 1986.
30. Nakajima T, Elovaara E, Park SS, Gelboin HV, Hietanen E and Vainio H, Monoclonal antibody-directed characterization of benzene, ethoxyresorufin and pentoxyresorufin metabolism in rat liver microsomes. *Biochem Pharmacol* **40**: 1255–1261, 1990.
31. Pasco DS, Boyuma KW, Merchant SN, Chalberg SC and Fagan JB, Transcriptional and post-transcriptional regulation of the genes encoding cytochromes P-450c and P-450d *in vivo* and in primary hepatocyte cultures. *J Biol Chem* **263**: 8671–8676, 1988.
32. Smith DA, Species differences in metabolism and phamacokinetics: are we close to an understanding? *Drug Metab Rev* **23**: 355–373, 1991.
33. Elshourbagy NA, Barwick JL and Guzelian PS, Induction of cytochrome P-450 by pregnenolone-16α-carbonitrile in primary cultures of adult rat hepatocytes and in a cell-free translation system. *J Biol Chem* **256**: 6060–6068, 1981.
34. Watkins PB, Wrighton SA, Schuetz EG, Maurel P and Guzelian PS, Macrolide antibiotics inhibit the degradation of the glucocorticoid-responsive cytochrome P-450p in rat hepatocytes *in vivo* and in primary monolayer culture. *J Biol Chem* **261**: 6264–6271, 1986.
35. Babany G, Larrey D and Pessayre D, Macrolide antibiotics as inducers and inhibitors of cytochrome P-450 in experimental animals and man. In: *Progress in Drug Metabolism* 11 (Ed. Gibson GG), pp. 61–98. Taylor and Francis, London, 1988.
36. Pineau T, Galtier P, Bonfils C, Derancourt J and Maurel P, Purification of a sheep liver cytochrome P-450 from the P450IIIA gene subfamily. Its contribution to the N-dealkylation of veterinary drugs. *Biochem Pharamacol* **39**: 901–909,1990.
37. Wood AW, Ryan DE, Thomas PE and Levin W, Regio- and stereoselective metabolism of two C_{19} steroids by five highly purified and reconstituted rat hepatic cytochrome P-450 isozymes. *J Biol Chem* **258**: 8839–8847, 1983.
38. Vind C, Dich J and Grunnet N, Effects of cytochrome P-450 inducing agents on the monooxygenation of testosterone in long-term cultures of hepatocytes from male and female rats. *Arch Biochem Biophys* **275**: 140–150, 1989.
39. Murray M, Microsomal cytochrome P450-dependent steroid metabolism in sheep liver. Quantitative importance of 6β-hydroxylation and evidence for the involvement of a P450 from the IIIA subfamily in the pathway. *J Steroid Biochem Mol Biol* **38**: 611–619, 1991.
40. Halvorson M, Greenway D, Eberhart D, Fitzgerald K and Parkinson A, Reconstitution of testosterone oxidation by purified rat cytochrome P450p (IIIA1). *Arch Biochem Biophys* **277**: 166–180, 1990.
41. Sonderfan AJ, Arlotto MP, Dutton DR, McMillen SK and Parkinson A, Regulation of testosterone hydroxylation by rat liver microsomal cytochrome P-450. *Arch Biochem Biophys* **255**: 27–41, 1987.
42. Waxman DJ, Attisano C, Guengerich FP and Lapenson DP, Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6β-hydroxylase cytochrome P450 enzyme. *Arch Biochem Biophys* **263**: 424–436, 1988.
43. Waxman DJ, Lapenson DP, Aoyama T, Gelboin HV, Gonzalez FJ and Korzekwa K, Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. *Arch Biochem Biophys* **290**: 160–166, 1991.