Biochemical Pharmacology, Vol. 45, No. 1, pp. 113–122, 1993.
Printed in Great Britain.

0006-2952/93 $6.00+0.00

HEPATIC CYTOCHROME P450 INDUCTION IN GOATS

EFFECTS OF MODEL INDUCERS ON THE METABOLISM OF ALKOXYRESORUFINS, TESTOSTERONE AND ETHYLMORPHINE, AND ON APOPROTEIN AND mRNA LEVELS

GERBEN A. E. VAN'T KLOOSTER,*† G. J. M. JEAN HORBACH,‡
MASAHIRO NATSUHORI,*§ BAS J. BLAAUBOER,‡ JAN NOORDHOEK‖ and
ADELBERT S. J. P. A. M. VAN MIERT*

*Department of Veterinary Pharmacology, Pharmacy 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* 17 *June* 1992; *accepted* 21 *September* 1992)

Abstract—Male and female African dwarf goats were treated orally with phenobarbital (PB) or triacetyloleandomycin (TAO), or subcutaneously with β-naphthoflavone (BNF). Hepatic microsomal cytochrome P450 content was increased by PB and TAO, but not by BNF. PB effects on P450 activities were non-selective: ethoxyresorufin deethylase (EROD) and pentoxyresorufin depentylase (PROD), hydroxylation of testosterone (TST) and demethylation of ethylmorphine (ETM) were all induced by a factor of 2–3. A similar non-selective induction was observed with TAO, except for EROD and PROD (no effects). After PB and TAO treatment, increased levels of a protein cross-reactive with anti-sheep P450 3A and 2B were found. Thus, in dwarf goats, both PB and TAO appeared to be P450 3A inducers. Selective PB effects related to a P450 2B form on PROD are lacking but 16α-hydroxylation of TST was induced markedly. At the mRNA level, PB induced an mRNA that showed good sequence homology with a human P450 3A4 cDNA probe, rather than with a rat 3A1 probe. BNF selectively induced EROD, whereas TST hydroxylation and ETM dealkylation were inhibited. With BNF-treated animals, increased concentrations of a protein cross-reactive with anti-rat P450 1A1/1A2 and of an mRNA that showed homology with a human 1A1 cDNA probe, but not with a mouse 1A1/1A2 probe, were observed.

Cytochrome P450 (P450¶), the major catalytic component of the liver mixed-function oxidase system, plays a pivotal role in the metabolism of many endogenous and exogenous compounds. In the past decades, research efforts showed the existence of numerous forms of P450, each with its characteristic but often overlapping substrate specificity [1–3]. Thus, P450 enzymes have been grouped into several different families and subfamilies, depending on similarities in amino acid sequences or gene sequence similarities [4]. Families 1–4 cover the major catabolic drug-metabolizing P450s. These enzymes can be induced by selective substrates. Inducibility, in combination with selected metabolic activities, has provided a basis for classification and characterization. However, remarkable qualitative and quantitative differences in the presence, activity and inducibility of P450 enzymes have been reported for different species, such as rat, rabbit, mouse and humans [2–6].

To date, knowledge on the drug-metabolizing P450s and their regulation in ruminants, such as goats, sheep and cattle, is very limited. Nevertheless, such knowledge is of crucial importance within the field of applied veterinary pharmacotherapy, which may have an impact on public health as drug therapy in food-producing animals may result in the presence of residues of drugs and their metabolites in food of animal origin [7].

No information is available concerning the cattle P450 families 1–4, but based on microsomal metabolic activity, a 3-methylcholanthrene type of induction (related to P450 1A) has been reported in cattle treated with pentachlorophenol [8]. Phenobarbital (PB) appeared to be an effective inducer in *in vivo* pharmacokinetic studies in sheep and goats [9, 10]. In accordance, using microsomes from PB-treated goats, benzo[*a*]pyrene hydroxylase activity was induced [11]. Recently, a P450 from the 2B subfamily was isolated from sheep lung [12, 13] and liver [14]. Similarly, a triacetyloleandomycin (TAO)-inducible P450 from the 3A subfamily was purified from sheep liver [15]. Altogether, these results indicated a similar presence of inducible P450 enzymes in ruminants as observed in the rat.

In order to study characteristics of the P450 enzyme system in goats, animals were treated with

† Corresponding author: G. A. E. van't Klooster, Department of Veterinary Pharmacology, University of Utrecht, Yalelaan 2, 3584 CM Utrecht, The Netherlands. Tel. (31) 30 535400; FAX (31) 30 535077.

§ Present address: Department of Veterinary Medicine, Tokyo University of Agriculture and Technology, Fuchu, 183, Japan.

¶ Abbreviations: AND, androstenedione; BNF, β-naphthoflavone; EROD, ethoxyresorufin deethylase; ETM, ethylmorphine; 3-MC, 3-methylcholanthrene; OHT, hydroxytestosterone; P450, cytochrome P450; PB, phenobarbital; PROD, pentoxyresorufin depentylase; TAO, triacetyloleandomycin; TST, testosterone.

the P450 inducers PB, TAO and β-naphthoflavone (BNF). In rats and several other laboratory animal species, PB induces P450 2B, 2C and 3A enzymes, BNF induces P450 1A and TAO induces P450 3A [1, 3]. Induction effects were monitored through hepatic microsomal metabolism of selected substrates, such as ethoxy- and pentoxyresorufin, ethylmorphine (ETM) and testosterone (TST). A qualitative comparison between control and induced goats at the apoprotein and mRNA level was made.

MATERIALS AND METHODS

Chemicals. TAO was a kind gift from Pfizer (Rotterdam, The Netherlands). PB, 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). BNF, 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 & Co. (Skokie, IL, U.S.A.). 2β-, 6β-, 7α-, 11α-, 16β- and 19-OHT and 16-ketotestosterone wee purchased from Steraloids (Wilton, NH, U.S.A). Resorufin was from Eastern Kodak (Rochester, NY, U.S.A.). All other chemicals were of analytical grade.

Antibodies and RNA probes. Polyclonal antibodies against sheep P450 3A [15] and 2B [14] were generous gifts from Dr P. Galtier (INRA, Toulouse, France). A monoclonal antibody against P450 1A1/2 was a gift from Dr P. Kremers (Université de Liège, Belgium).

cDNA probes were kind gifts from Dr D. W. Nebert (Cincinatti, OH, U.S.A.): P450 1A1: mouse cDNA P1-P450 and human cDNA PhP_1450FL, and P450 1A2: mouse cDNA P3-P450; Dr M. Adesnik (New York, NY, U.S.A.): P450 2B1/2: rat cDNA R17; Dr F. J. Gonzalez (Bethesda, MD, U.S.A.): P450 2E1: rat cDNA IIE1, and P450 3A1: rat cDNA IIIA1; Dr F. P. Guengerich (Nashville, TN, U.S.A.): P450 2C9: human cDNA mp8-m13mp9; and Dr D. Pompon Gif-sur-Yvette, France): P450 3A4: human cDNA pYeDP1/8-2-NF25.

Animal treatment and isolation of microsomes. Three months prior to the experiment, groups of 3–6 healthy male (13–17 kg) and female (11–15 kg) African dwarf goats, 8–10 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. All experiments were performed during the mating season (October–December).

PB (dose: 50 mg/kg/day) and TAO (100 mg/kg/day) were administered orally, by means of two daily administrations. BNF (20 mg/kg), dissolved in arachid oil, was administered by daily subcutaneous injections at four different and changing locations in flanks and neck region. Treatment with inducers was performed for 6 consecutive days, including the day of termination. Without further pretreatment, animals were shot using a slaughter gun and exsanguinated. Within 10 min, livers were removed and the right lobe cut off. It was perfused with approx. 250 mL ice-cold saline via the main veins at the cut surface. Representative cross-sections of the right liver lobe (approx. 40 g slices) were frozen in liquid nitrogen.

The slices were thawed and homogenized in 0.15 M KCl, containing 0.1 mM EDTA, using a Potter–Elvehjem homogenizer. Microsomes were prepared by differential centrifugation: 2 × 20 min at 9000 *g*, supernatant 60 min at 105,000 *g*. The microsomal pellet was resuspended in 0.125 M phosphate buffer (pH 7.4), containing 0.1 mM EDTA and 20% (v/v) glycerol, quickly frozen in liquid nitrogen and stored in aliquots at −80° until use.

The Animals Ethics Committee of the Veterinary Faculty has approved the use of the animals in the present experiment.

Enzyme assays. Contents of protein and total P450 were determined according to Rutten *et al.* [16], using a double-beam spectrophotometer (PU 8800, Pye Unicam, Eindhoven, The Netherlands).

Microsomal incubations were performed in an incubation buffer (50 mM potassium phosphate, pH 7.4), containing 0.5 mM $NADP^+$, 5 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase (1 U/mL), 2.5 mM $MgCl_2$, at 37°.

Microsomes from TAO-induced animals were preincubated with 100 μM $K_3Fe(CN)_6$ at 4° for 15 min prior to use; incubations were performed in the presence of 50 μM $K_3Fe(CN)_6$ in order to release TAO metabolites bound to P450s [17].

TST hydroxylation activity was determined according to Sonderfan *et al.* [18], as modified by Wortelboer *et al.* [19], using 0.3 mg of microsomal protein in an incubation volume of 1 mL, containing 0.5 mM TST. After 15 min, incubations were stopped by addition of 6 mL dichloromethane, the extraction solvent. Dichloromethane fractions were evaporated to dryness under a flow of nitrogen. Residues were reconstituted in methanol–water (50:50, v/v) and analysed by HPLC, essentially as described by Wortelboer *et al.* [19]. In short, 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 0.8 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. OHTs were detected at 254 nm and quantified by comparing peak areas to those of authentic standards. TST and its metabolites were eluted at (retention times in min): 15β-OHT: 15.8, 6β-OHT: 19.4, 15α-OHT: 21.7, 7α-OHT: 22.8, 16α-OHT: 27.2, 16β-OHT: 28.1, 11α-OHT: 29.9, 12β-OHT: 30.8, 11β-OHT: 31.6, 2α-OHT: 33.4, 2β-OHT: 34.7, AND: 40.9 and TST: 42.8.

Fluorimetric determinations of microsomal 7-ethoxyresorufin deethylation (EROD) and 7-pentoxyresorufin depentylation (PROD) activities were performed as described by Rutten *et al.* [20]. Incubations (5 min) were performed with 50–200 μg protein in 2.5 mL 5 μM ethoxyresorufin or pentoxyresorufin.

N-Demethylation and O-deethylation of ETM were determined in 2.0 mL incubation mixtures,

containing 1 mg protein. Microsomes were incubated with 2.5 mM ETM for 30 min. Sample pretreatment and HPLC analysis have been described previously [21]. In short, samples were deproteinized using 0.35 mL 40% $ZnSO_4$ and 0.65 mL saturated $Ba(OH)_2$ solutions. Morphine, norethylmorphine and ETM were separated on a 100 × 8 mm Lichrosorb CN Radial PAK column (Waters, Etten-Leur, The Netherlands). Isocratic elution was performed with acetonitril–phosphate buffer (0.05 M, pH 2.5) 10:90 (v/v), containing 0.2% (v/v) triethylamine, at a flow rate of 2 mL/min. Norethylmorphine and morphine were quantified at 210 nm by comparing peak areas to those of authentic standards.

Gel electrophoresis and western blots. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) of microsomal proteins was performed in a Protein II Mini Cell (Biorad, Veenendaal, The Netherlands), using the discontinuous system as described by Laemmli [22]. The resolved proteins were blotted on polyvinylidenedifluoride sheets (Millipore, Etten-Leur, The Netherlands), according to the method of Towbin *et al.* [23]. Antibodies raised to sheep and rat P450 forms were used for immunochemical staining. Details have been described by Wortelboer *et al.* [24]. In each gel a set of prestained M_r markers (Low Weight Range, Biorad) was run.

RNA isolation and northern blots. RNA was isolated from saline-perfused, liquid N_2-frozen dwarf goat or rat liver using the LiCl/urea procedure as modified by Clemens [25], with an additional phenolic extraction step. Equal amounts of total RNA (15 μg per lane) were size-fractionated by electrophoresis in a 1.5% agarose gel containing 2.2 M formaldehyde followed by transfer to a Hybond-N^+ membrane (Amersham, Buckinghamshire, U.K.). Hybridization was carried out at 65° in the presence of 7% (w/v) SDS in 0.5 M phosphate buffer (pH 7.2), using random-primed ^{32}P-labelled probes. Membranes were either washed using low stringency [4.5 mM sodium citrate pH 7, containing 45 mM sodium chloride ("0.3*SSC") and 0.1% SDS] or high stringency [1.5 mM sodium citrate pH 7, containing 15 mM sodium chloride ("0.1*SSC") and 0.1% SDS] buffers at 65°. Autoradiography was performed at −80° using Fuji New RX-NIF X-ray films.

Statistics. Mean values were statistically evaluated using a double-sided Student's *t*-test, and were considered significantly different at $P < 0.01$ or $P < 0.05$ as denoted. Where appropriate, the number of animals or observations is given in text and tables.

RESULTS

Inducer treatment of goats

During the treatment of goats with inducers no clinical changes were observed in any of the animals. Especially for the six animals that received subcutaneous injections of BNF, this finding is of significance, as intraperitoneal injection of BNF in oil, the more generally used route of administration (e.g. Refs 24, 26 and 27), causes (sterile) peritonitis. At necropsy, after subcutaneous treatment, injection sites were obvious, but visible signs of local inflammation were not found and corresponding lymph nodes were unaltered.

Total exsanguinated liver weights ranged from 1.9 to 2.1% of body weight and were not significantly influenced by inducer treatment (results not shown).

Table 1. Total P450 content and metabolic activities in microsomes from control and induced male dwarf goats

	Control (6)	PB-induced (3)	BNF-induced (3)	TAO-induced (3)
P450 content (pmol/mg protein)	598 ± 149	1395 ± 114‡	633 ± 146	993 ± 221†
EROD	103 ± 28	239 ± 63‡	593 ± 173‡	109 ± 10
PROD	15.4 ± 2.9	42.3 ± 8.5‡	36.0 ± 14.6†	15.0 ± 3.2
ETM demethylation	5650 ± 2730§	17100 ± 1350‡	5725 ± 115§	18950 ± 780‡§
ETM deethylation	690 ± 200	165 ± 25‡	370 ± 20‡	260 ± 21‡
TST hydroxylation*				
2β	569 ± 122§	952 ± 129‡	522 ± 49	1422 ± 371‡
6β	421 ± 171§	2509 ± 865‡	511 ± 123	4814 ± 784‡
11α	115 ± 44§	382 ± 83‡	162 ± 25	406 ± 87‡
12β	228 ± 46§	342 ± 70†	210 ± 40	364 ± 87†
15α	47 ± 17	129 ± 26‡	141 ± 27‡	81 ± 54
15β	59 ± 27§	141 ± 25‡	91 ± 20†	154 ± 49‡
16α	10.9 ± 7.6§	206 ± 25‡	14.5 ± 3.4	21.3 ± 9.0†
16β	12.3 ± 4.6§	75 ± 26‡	30 ± 12†	68 ± 27‡
19	11.5 ± 3.9§	26 ± 4‡	9.9 ± 1.4	29 ± 4‡
AND	273 ± 84	797 ± 304‡	190 ± 56	524 ± 191‡

Activities (mean ± SD) are expressed as product formation in pmol/mg protein/min.
Numbers of animals are given in parentheses.
* Position of steroid hydroxylation is given.
† Significant ($P < 0.05$) difference vs control.
‡ Significant ($P < 0.01$) difference vs control.
§ Significant ($P < 0.05$) sex difference.

Table 2. Total P450 content and metabolic activities in microsomes from control and induced female dwarf goats

	Control (6)	PB-induced (3)	BNF-induced (3)	TAO-induced(3)
P450 content (nmol/mg protein)	747 ± 110	1667 ± 350†	801 ± 130	1027 ± 105†
EROD	146 ± 49	216 ± 40†	525 ± 79‡	104 ± 42
PROD	16.8 ± 4.5	37.3 ± 3.6‡	28.0 ± 9.7	16.8 ± 2.7
ETM demethylation	9050 ± 2300§	18700 ± 1050‡	8225 ± 1250§	21850 ± 1100‡§
ETM deethylation	620 ± 150	140 ± 23‡	280 ± 56‡	180 ± 55‡
TST hydroxylation*				
2β	876 ± 274§	854 ± 261	407 ± 151†	1253 ± 144†
6β	1152 ± 377§	2899 ± 1556†	653 ± 255†	6703 ± 628‡
11α	233 ± 59§	396 ± 152†	147 ± 55†	456 ± 50‡
12β	403 ± 102§	323 ± 110	167 ± 44‡	321 ± 41†
15α	63 ± 14	145 ± 5‡	98 ± 30	103 ± 20‡
15β	93 ± 23§	158 ± 28‡	115 ± 37	199 ± 19‡
16α	48 ± 15§	181 ± 37‡	29 ± 3†	18.0 ± 3.2†
16β	29 ± 10§	84 ± 43†	35 ± 20	80 ± 9‡
19	33 ± 10§	24 ± 10	10.5 ± 3.0‡	33 ± 4
AND	797 ± 304§	765 ± 47	311 ± 63‡	354 ± 97†

Activities (mean ± SD) are expressed as product formation in pmol/mg protein/min.
Numbers of animals are given in parentheses.
* Position of steroid hydroxylation is given.
† Significant ($P < 0.05$) difference vs control.
‡ Significant ($P < 0.01$) difference vs control.
§ Significant ($P < 0.05$) sex difference.

Microsomal P450 content and enzymatic activities

The contents of total P450 in microsomes from male and female African dwarf goats are presented in Tables 1 and 2, respectively. Although a trend was observed that showed mean P450 content in female goats to be >20% higher than in males, it was not significantly different. P450 content in microsomes from BNF-treated animals was equal to that in microsomes from non-treated goats. TAO and, especially, PB treatment significantly increased the microsomal P450 content in a similar way to that reported for rats [16, 23].

Microsomal EROD and PROD activity, ETM dealkylation and TST hydroxylation activities are presented in Tables 1 and 2 for male and female dwarf goats, respectively. EROD and PROD activities were nearly equal in animals of either gender. Sex differences were observed for N-demethylation of ETM and hydroxylation of TST, the female being metabolically more active than the male. The microsomal ETM O-deethylation activity, resulting in morphine formation, was low and was not inducible by PB, BNF or TAO.

Major metabolites resulting from microsomal hydroxylation of TST were 2β-, 6β-, 11α- and 12β-OHT (Tables 1 and 2). In all animals, 15α-, 15β-, 16α-, 16β- and 19-OHT were minor metabolites. Formation of these metabolites was inducible in both male and female animals. Detectable amounts of 2α- and 7α-OHT were formed but the relative contribution to total TST metabolism was less than 0.5% (results not shown). AND was a major metabolite, especially in female goats.

Induction effects of PB were highly significant in both male and female goats for EROD, PROD, ETM demethylation and TST hydroxylation in most positions (Tables 1 and 2). Many of the induced activities were enhanced to the same extent as the PB-related increase (2.5-fold) in total microsomal P450 content. However, TST-16α- (4–20-fold), 6β- (2.5–6-fold) and 16β- (3–6-fold) hydroxylation activities were relatively strongly increased.

BNF appeared to be an effective inducer for EROD and, to some extent, PROD. Inhibitory effects were observed for ETM dealkylation and TST hydroxylations, except for hydroxylations at the TST-15- and TST-16-positions. A similar suppression or down-regulation of steroid metabolism by P450 1A inducers has been described for rat [23, 27]. Obviously, BNF exerted a more selective effect than both PB and TAO in goat hepatic P450.

TAO significantly induced ETM N-demethylation and virtually all different OHT activities. After preincubation of microsomes with 100 μM potassium ferricyanide, incubations were performed in the presence of 50 μM potassium ferricyanide [17]; compared to microsomes from TAO-treated animals incubated without ferricyanide, a 30% increase was observed for ETM N-demethylation and TST hydroxylation. It did not increase microsomal EROD or PROD activity in TAO-treated animals (results not shown).

Western blots

Apoproteins of both PB- and TAO-treated male and female goats appeared induced in western blots immunostained with anti-sheep P450 3A (Fig. 1). BNF did not induce a P450 3A-related protein.

Immunostaining with anti-sheep P450 2B (Fig. 2) yielded two well-separated bands, the lower one

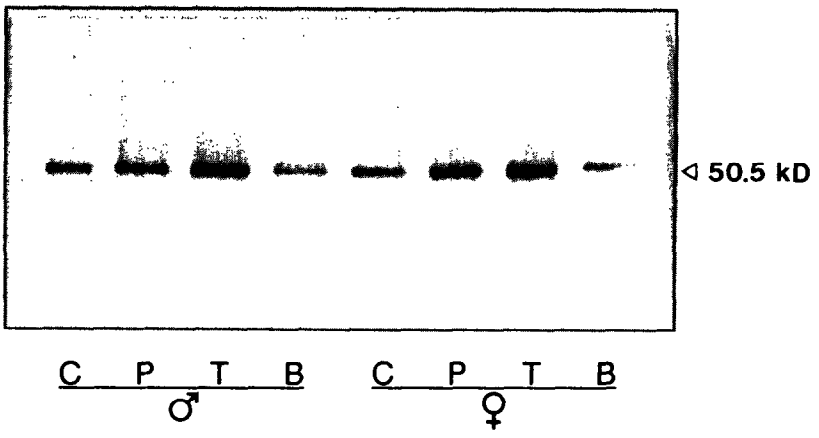

Fig. 1. Western blot of microsomes prepared from control and induced goat liver, immunochemically stained with sheep anti-P450 3A (2.5 μg protein/lane). Left four lanes: liver microsomes from male dwarf goats; right four lanes: liver microsomes from female dwarf goats. C, control; P, PB-treated; T, TAO-treated; B, BNF-treated.

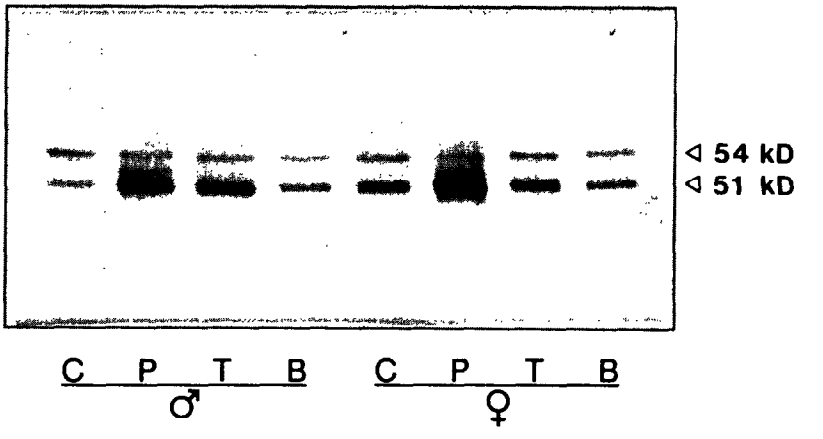

Fig. 2. Western blot of microsomes prepared from control and induced goat liver, immunochemically stained with sheep anti-P450 2B (2.5 μg protein/lane). Left four lanes: liver microsomes from male dwarf goats; right four lanes: liver microsomes from female dwarf goats. C, control; P, PB-treated; T, TAO-treated; B, BNF-treated.

showing mobility similar to that of the band observed with anti-sheep P450 3A immunodetection. It is this band that showed an inducing effect of PB and TAO. Again, no effects of BNF were observed. The upper band (M_r approx. 54,000) was found to be equally strong in control, PB-, TAO- and BNF-treated animals. It is unknown whether this band corresponds to another P450 form. No differences between samples from male and female goats were observed.

Figure 3 shows anti-rat P450 1A1/2 immunostaining in goat microsomal proteins. With this antibody, one distinct protein was detected for which only BNF was an effective inducing agent. The BNF-induced protein was present in approximately equally low amounts in control, PB- and TAO-treated animals. In microsomal proteins from a BNF-induced rat, two separate bands were observed, the upper one being P450 1A1 and the lower one being 1A2 [26]. The protein in goat samples and rat P450 1A2 had similar mobilities.

Northern blots

Liver RNA of control, PB- and BNF-treated male and female goats and male rats were separated on agarose gels, and tested for the presence of homologous sequences with several rat, mouse and human P450 cDNA probes (Figs 4 and 5). TAO-induced liver material was not screened, as the mechanism of TAO induction is primarily through protein stabilization and not at the transcriptional level [3, 17].

Hybridization with a human P450 1A1 cDNA probe resulted, after low stringency washing (0.3* SSC), in two clear bands in the lanes containing RNA from BNF-treated male and female goats and in BNF-treated rats (Fig. 4). In the rat, the lower band corresponds to P450 1A1 and the upper one is

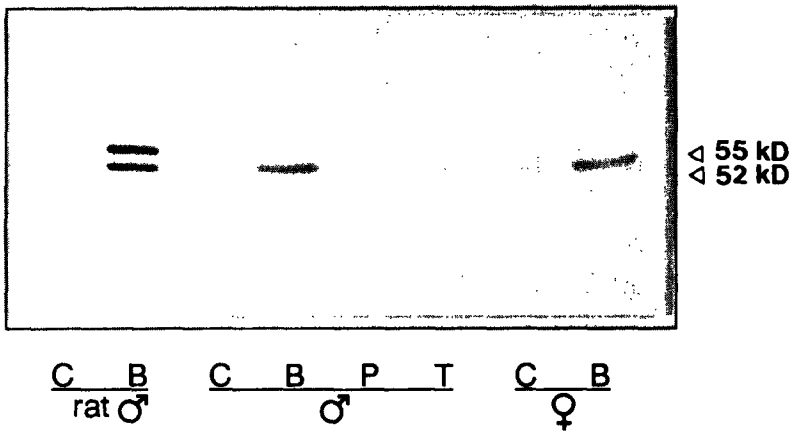

Fig. 3. Western blot of microsomes prepared from control and induced goat and rat liver, immunochemically stained with rat anti-P450 1A1/2. Lanes 1 and 2: control and BNF-induced male rat, respectively (1 μg protein/lane). Lanes 3–6: microsomes from male dwarf goats (5 μg protein/lane); lanes 7 and 8: microsomes from female dwarf goats (5 μg protein/lane). C, control; P, PB-treated; T, TAO-treated; B, BNF-treated.

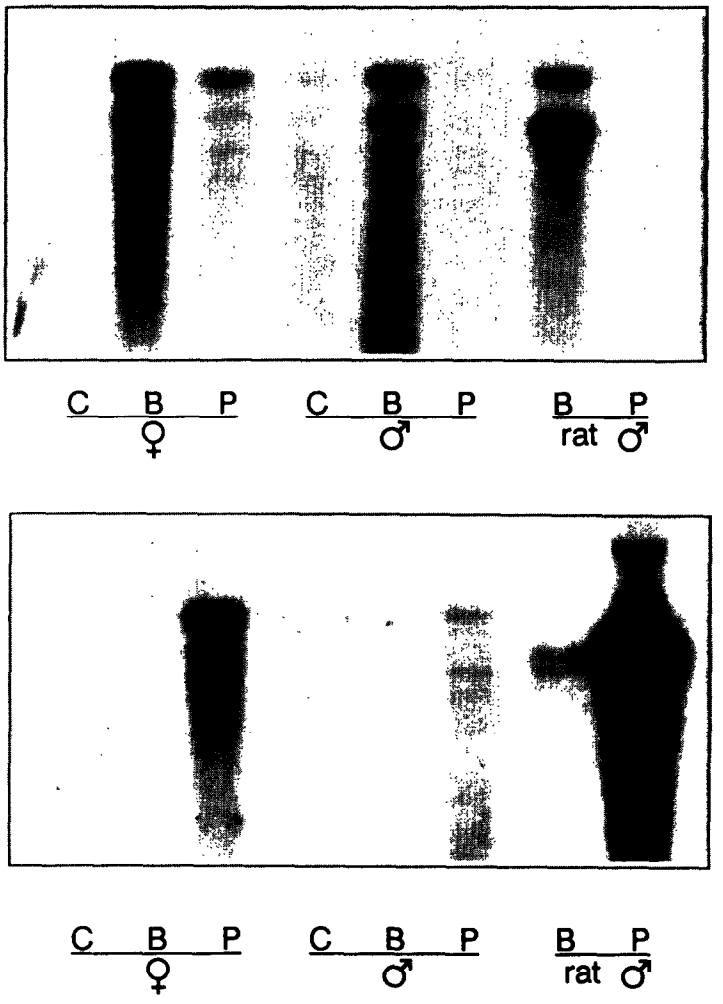

Fig. 4. Northern analysis of female- (lanes 1–3) and male goats (lanes 4–6), and male rat (lanes 7 and 8) liver microsomal RNA. The upper blot shows results of hybridization with a human P450 1A1 cDNA probe and subsequent low stringency (0.3*SSC) washing. The lower blot was hybridized with a rat P450 2B1 cDNA probe and high stringency washed (0.1*SSC). Autoradiography films were exposed for 48 hr. C, control; B, BNF-treated; P, PB-treated.

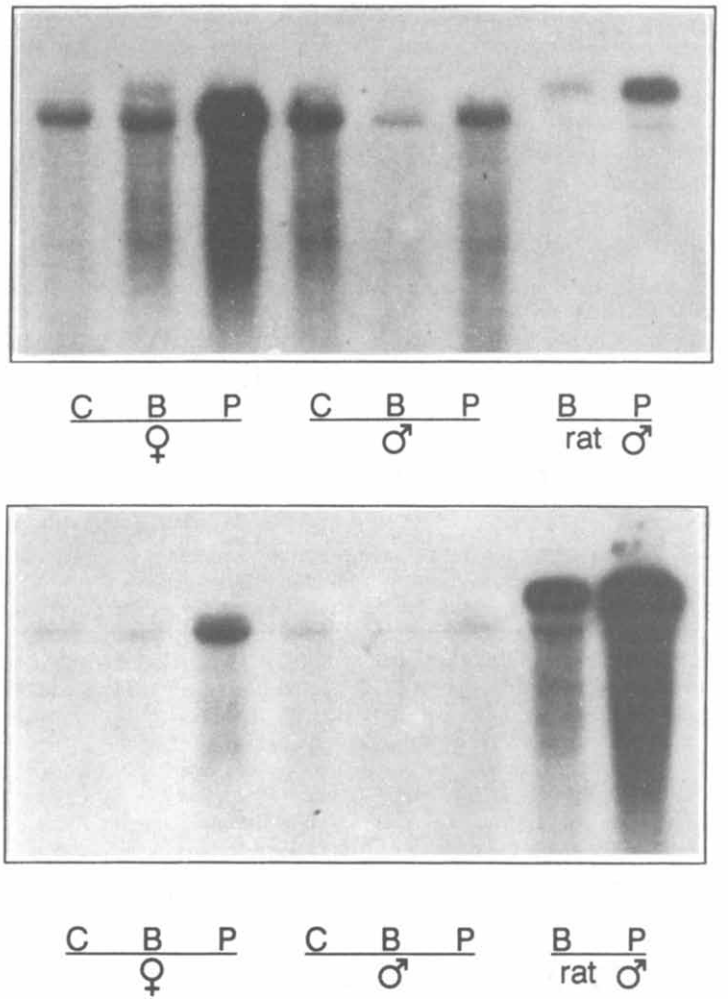

Fig. 5. Northern analysis of female- (lanes 1–3) and male goat (lanes 4–6), and male rat (lanes 7 and 8) liver microsomal RNA, hybridized with a human P450 3A4 cDNA probe (upper blot) and a rat P450 3A1 cDNA probe (lower blot). Blots were high stringency washed (0.1*SSC); autoradiography film was exposed for 48 hr. C, control; B, BNF-treated; P, PB-treated.

a result from cross-hybridization with P450 1A2 mRNA. After high stringency washing (0.1*SSC) the signal was diminished but not lost. Signals in control or PB-induced animals were very weak, indicating a selective induction at the RNA level by BNF. No hybridization could be observed with goat RNA when two mouse cDNA probes against P450 1A1 and 1A2 mRNA were used (results not shown). A rat P450 2B1 cDNA probe gave a clear signal in PB-induced female goat RNA, but only a weak signal with PB-induced male goat RNA (Fig. 4).

In Fig. 5, hybridization with a human P450 3A4 and a rat P450 3A1 cDNA probe is shown. For goat RNA from control and PB-induced animals, strong signals were found with the human probe, whereas with the rat probe only RNA from a PB-induced female goat showed hybridization. Thus, a better homology between goat mRNA and the human P450 3A cDNA probe, as compared to the rat P450 3A cDNA probe, could be demonstrated.

In addition, sequence homologies were found with rat P450 2E1 and human 2C9 cDNA probes (results not shown). The 2E1 signal persisted after high stringency washing (0.1*SSC), whereas the 2C9 signal did not. No induction effects were observed.

DISCUSSION

To achieve some basic insight into the nature and regulation of drug-metabolizing P450s in ruminant species, the present study was undertaken using African dwarf goats as model animal species, representing the class of ruminants. In contrast to genetically standardized laboratory animals, most farm animal species show a broad variability in genetic type, feeding condition, husbandry and disease history. This is reflected in largely varying microsomal activities and subtle changes in drug-metabolizing enzyme activities may be hard to demonstrate. In order to minimize variability in the present study, animals were housed and fed under controlled conditions and all experiments were performed in autumn to prevent cycle-related hormonal variation.

BNF treatment of goats was performed through subcutaneous administration. In general, intraperitoneal administration has been described, both for induction of laboratory and agricultural animal species [24, 27]. However, following this treatment, abdominal influx of inflammatory cells and secretion of inflammatory fluid, indicating a (sterile) peritonitis, were observed in rats and pigs. During the acute phase response to inflammation, cytokines are released. Such a release is known to decrease total liver P450 content [28] and to modulate activities of selected P450 enzymes [29–31]. Moreover, cytokines appear to interact with the induction of P450 2B and 1A enzymes [31, 32] which should be taken into account in interpretation of results of intraperitoneal BNF induction. In goats, subcutaneous administration of BNF does not result in visible signs of inflammation and, based upon the results on enhanced activities and apoprotein levels described above, is an effective way to induce P450.

A slice of the right liver lobe from each goat was used for preparation of microsomes. At least for control animals, the sample was considered representative for the liver as a whole, since Wisniewski *et al.* [33] reported that only minor differences exist in the intralobular distribution of selected xenobiotic-metabolizing enzyme activities. Whether or not a liver lobe-differentiated response to P450 inducers may occur remains unknown.

Control goat microsomal activity towards alkoxyresorufins, ETM and TST was of the same order as reported for several laboratory animal species, including rat [17, 24, 34, 35], mouse [36] and hamster* [37]. EROD and PROD activity in dwarf goat microsomes was similar to that for hamsters, half that in mice and at least double that in rat microsomes. In all studied species, EROD activity was high when compared to PROD activity (goats, rats and mice, 9:1; hamsters, 20:1).

For the N-demethylation of ETM, a marker activity to study sex-differentiated drug metabolism in rats and mice [38], a significant sex difference was observed with goat microsomes. In contrast with rats and similar to mice, activity in female dwarf goat microsomes was significantly higher than in males, not only with respect to ETM N-demethylation but also for TST hydroxylation. In hamsters [37], TST hydroxylation activity was significantly higher in female than in male microsomes, whereas no sex difference was reported for ETM demethylation. The latter activity was approx. five times higher than that observed with male goats or male sheep [15].

ETM O-deethylation, a P450 2D-mediated dealkylation in various species [5], appeared to be not sex dependent and not inducible in the experiments with goats. This is in accordance with data on P450 2D in other species, including rat and humans [5], which indicates that similar to those species, a P450 2D form is involved in ETM O-deethylation in African dwarf goats.

Over all, TST hydroxylation activity in hepatic microsomes from goats was similar to that in rats [18, 24], mice [36], hamsters* [37] and humans [39, 40], but considerably higher (3-fold) than in sheep microsomes [39]. With microsomes from non-induced animals of all species, 6β-hydroxylation was the predominant route of biotransformation. Other major metabolites in goats were 2β-, 11α- and 12β-OHT. The latter two have not been studied thoroughly in other species, but 2β-OHT was a major metabolite in sheep [39], mice [36] and humans [40]. Reports on hydroxylation at position 2 by rat and hamster microsomes are conflicting; it has been shown quantitatively important but both 2β- and 2α-OHT have been reported as the major product. In non-treated goat microsomes, formation of 2α-OHT was negligible, as is the case for 7α-OHT and 16α-OHT. These are major metabolites in rats, mice and hamsters [18, 24, 34–37], but much less so in sheep and humans [39, 40], yielding rather similar metabolite patterns for untreated goats, sheep and humans.

In rats, induction by PB (primarily related to the P450 2B subfamily) and 3-methylcholanthrene (3-MC) (or BNF, related to the P450 1A subfamily) is related to an increased microsomal dealkylation activity towards pentoxyresorufin [41] and ethoxyresorufin [42], respectively. In non-induced animals, however, these biotransformations take place primarily through other—constitutive—enzymes such as the male-specific P450 2C11 [43]. The 3-MC type of induction is species dependent, the rat being more responsive when compared to hamster and mouse [26]. We found a 5-fold induction on dwarf goat hepatic microsomal EROD by BNF and a 2-fold induction by PB. In goats, similar to hamsters,* PB lacks a specific inductive effect on PROD. In contrast, in two mouse strains, PB effects on PROD (30-fold induction) were reported, whereas 3-MC effects on EROD were lacking [36]. Therefore, PROD may be a selective activity marker for PB induction in species other than rats, but it was not for goats.

PB non-selectively induced most of the determined goat hepatic P450 activities. The observed enhancement by a factor of 2–3 is only slightly higher than the increase in microsomal P450 content. Specific PB effects in rat are related to strong induction of PROD [41] and TST 16β-hydroxylation [34]. The absence of these effects might indicate the lack of an inducible hepatic P450 2B form as observed in rats. Nevertheless, at the RNA level, some homology with rat P450 2B1 existed, and an enhanced hybridization was observed after PB treatment (Fig. 4). Furthermore, a P450 2B form has also recently been purified from sheep lung [12, 13]. An immunochemically related protein was detected in sheep liver [13, 14]. Possibly, such a P450 form is involved in 16α-OHT formation, which was strongly induced by PB.

Upon treatment with PB, BNF or TAO, both male and female goats can be induced to a similar "maximum level of activity". Since microsomal ETM N-demethylation and TST hydroxylation activity in control males was only half that in females, this resulted in significant sex differences in induction

* Mennes WC, Vogel LJ, Wortelboer HM, Horbach GJMJ, Noordhoek J and Blaauboer BJ, Differences in the effects of model inducers of cytochrome P450 on biotransformation and P450 protein pattern in hamster liver microsomes, submitted.

factors. Presumably, the apparent sex differences in inducer effects reflect down-regulation of sex-specific or sex-predominant P450 enzymes (2C and 3A subfamilies), as shown by Emi and Omura [44] for PB- and 3-MC-induced rats. In the present study, the observed down-regulation, especially in female goats, is concurrent with induction of (a) P450 form(s) that is not involved in ETM N-demethylation or most TST hydroxylations. Similarly, the inhibitory effect of BNF on several TST hydroxylations and ETM dealkylation in dwarf goat microsomes can be explained by down-regulation. Interestingly, 15α-OHT and, to a lesser extent, 15β- and 16β-OHT formation were induced by BNF treatment. It indicates that in dwarf goats, BNF-inducible P450 enzymes may be involved in TST hydroxylation, as is the case for 7α-OHT in rats [24, 34].

We could demonstrate that after TAO induction, 6β-hydroxylation of TST is still significantly higher in female goats, possibly because TAO stabilizes the protein already present rather than having an inductive effect at the transcriptional level [3, 17]. TAO is a P450 3A subfamily inducer [3, 17] and 6β-OHT has been shown to be primarily a P450 3A metabolite in several species, including rats [34], sheep [39] and humans [40]. This enzyme was also reported to be involved in ETM N-demethylation in sheep [15], a metabolic pathway that showed a persistent sex difference in TAO-induced goat microsomes, too. The similar regulation for both biotransformations indicates involvement of the same P450 enzyme, which should be a P450 3A form. An apparently quantitative important presence of a P450 3A enzyme (as is the case for humans [5]) in African dwarf goat liver resulted in the clear signals in western (Fig. 1) and northern (Fig. 5) blots, even in microsomes from non-treated animals. Interestingly, with goat mRNA, human P450 3A4 cDNA showed a much larger sequence homology than rat P450 3A1 cDNA.

Since no antibodies directed against goat P450 are known to exist, immunostaining was performed with heterologous antibodies, raised against P450s from rat and sheep. Phylogenetically, the latter species is relatively closely related to goats. For immunostaining of P450 1A enzymes, a monoclonal antibody directed against rat P450s was used. In an experiment with rat, hamster and mouse liver microsomes, Thomas *et al.* [26] detected two bands, related to P450 1A1 and P450 1A2, in all species but one mouse strain. Similar with the latter, only one cross-reactive and BNF-inducible protein was observed with goat microsomes (Fig. 3). Since the relative mobility of P450 1A1 and 1A2 was shown to be species dependent [26], the nature of the goat P450 1A enzyme remains unclear. At the RNA level it showed stronger homology with human P450 1A1 (Fig. 4) than with mouse 1A1 or 1A2 (not shown).

In conclusion, the present study indicates the presence of P450 3A and P450 1A enzymes in goat liver microsomes. P450 3A enzymes can be induced by TAO and PB, and P450 1A by BNF. A P450 2B form may be involved in 16α-hydroxylation of TST, but not in PROD. In comparative studies at the level of selected activities, presence of constitutive and inducible enzymes, and mRNA homologies, goat liver microsomes appear considerably different from those of rodents. However, similarities with human microsomes are obvious. This should have its consequences for toxicological risk assessment, especially with regard to the use of veterinary drugs and the occurrence of their metabolites in food of animal origin.

REFERENCES

1. 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 & Francis, London, 1987.
2. Nebert DW, Nelson DR and Feyereisen R, Evolution of the cytochrome P450 genes. *Xenobiotica* **19**: 1149–1160, 1989.
3. Gonzalez FJ, The molecular biology of cytochrome P450s. *Pharmacol Rev* **40**: 243–288, 1989.
4. 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.
5. Smith DA, Species differences in metabolism and pharmacokinetics: are we close to an understanding? *Drug Metab Rev* **23**: 355–373, 1991.
6. Boobis AR, Sesardic D, Murray BP, Edwards RJ, Singleton AM, Rich KJ, Murray S, de la Torres R, Segura J, Pelkonen O, Pasanen M, Kobayashi S, Zhi-Guang T and Davies DS, Species variation in the response of the cytochrome P-450-dependent monooxygenase system to inducers and inhibitors. *Xenobiotica* **20**: 1139–1161, 1990.
7. Juskevich JC, Comparative metabolism in food-producing animals: programs sponsored by the center for veterinary medicine. *Drug Metab Rev* **18**: 345–362, 1987.
8. Shull LR, Olson BA, Hughes BJ, McKenzie RM and Kinzell JH, Effect of pentachlorophenol on microsomal mixed-function oxidases in cattle. *Pestic Biochem Physiol* **25**: 31–39, 1986.
9. Berecky I, Interakcia fenobarbitali resp. hydrokortizonu so sulfadimidinom u oviec. *Biol chem Vet (Praha)* **XIX** (XXV): 379–384, 1983.
10. 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*, in press.
11. Burley FE and Bray TM, Effect of rumen developments and pre-exposure to chemicals on the activity of the mixed function oxidase system in goats. *Comp Biochem Physiol* **75C**: 137–140, 1983.
12. Adali O and Arinç E, Electrophoretic, spectal, catalytic and immunochemical properties of highly purified cytochrome P-450 from sheep lung. *Int J Biochem* **22**: 1433–1444, 1990.
13. Williams DE, Dutchuk M and Lee M-Y, Purification and characterization of a microsomal cytochrome P450IIB enzyme from sheep lung. *Xenobiotica* **21**: 979–989, 1991.
14. Kaddouri M, Brasset N, Alvinerie M, Eeckhoutte C, Bonfils C, Derancourt J and Galtier P, Ontogenic development of liver progesterone metabolism in female sheep. Contribution of cytochrome P450 2B and P450 3A subfamilies. *J Steroid Biochem Mol Biol* **42**: 499–508, 1992.
15. 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 Pharmacol* **39**: 901–909, 1990.
16. 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.
17. 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* (Eds. Bridges JW, Chasseaud LF and Gibson GG), pp. 61–98, 1989.
18. 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.
19. 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.
20. Rutten AAJ, Falke HE, Catsburg JF, Wortelboer HM, Blaauboer BJ, Doorn L, van Leeuwen FXR, Theelen R, and Rietjens I, Interlaboratory comparison of microsomal ethoxyresorufin and pentoxyresorufin O-dealkylation determinations: standardization of assay conditions. *Arch Toxicol* **66**: 237–244, 1992.
21. van't Klooster GAE, Kolker HJ, Woutersen-van Nijnanten FMA, Noordhoek J and van Miert ASJPAM, High performance liquid chromatography ethylmorphine and its metabolites in cell culture media and microsomal incubation mixtures. *J Chromatogr (Biomed Appl)* **579**: 158–164, 1992.
22. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227**: 680–685, 1970.
23. 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.
24. 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.
25. Clemens MJ, Purification of eukariotic mRNA. In: *Transcription and Translation. A Practical Approach* (Eds. Hames BD and Higgins SJ), pp. 211–230. IRL Press, Oxford, 1984.
26. Thomas PE, Reidy J, Reik LM, Ryan DE, Koop DR and Levin W, Use of monoclonal antibody probes against rat hepatic cytochromes P-450c and P-450d to detect immunochemically related isozymes in liver microsomes from different species. *Arch Biochem Biophys* **235**: 239–253, 1984.
27. Krogsgaard Thomsen M, Friis C and Nielsen P, Purification and characterization of hepatic microsomal cytochrome P-450 in phenobarbital and β-naftoflavone-treated pigs. *Pharmacol Toxicol* **69**: 381–385, 1991.
28. Andus T, Bauer J and Gerok W, Effects of cytokines on the liver. *Hepatology* **13**: 364–375, 1991.
29. Wright K and Morgan ET, Regulation of cytochrome P450IIC12 expression by interleukin-1α, interleukin-6, and dexamethasone. *Mol Pharmacol* **39**: 468–474, 1991.
30. Ansher SS, Puri RK, Thompson WC and Habig WH, The effects of interleukin-2 and α-interferon administration on hepatic drug metabolism in mice. *Cancer Res* **52**: 262–266, 1992.
31. Williams JF, Bement WJ, Sinclair JF and Sinclair PR, Effect of interleukin-6 on phenobarbital induction of cytochrome P-450IIB in cultured rat hepatocytes. *Biochem Biophys Res Commun* **178**: 1049–1055, 1991.
32. Barker CW, Fagan JB and Pasco DS, Interleukin-1β suppresses the induction of P450 1A1 and P450 1A2 mRNAs in isolated hepatocytes. *J Biol Chem* **267**: 8050–8055, 1992.
33. Wisniewski JA, Moody DE, Hammock BD and Shull LR, Interlobular distribution of hepatic xenobiotic-metabolizing enzyme activities in cattle, goats and sheep. *J Anim Sci* **64**: 210–215, 1987.
34. 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.
35. Lee DW and Park KH, Testosterone metabolism by microsomal cytochrome P-450 in liver of rats treated with some inducers. *Int J Biochem* **21**: 49–57, 1989.
36. Kelley M, Womack J and Safe S, Effects of cytochrome P-450 monooxygenase inducers on mouse hepatic microsomal metabolism of testosterone and alkoxyresorufins. *Biochem Pharmacol* **39**: 1991–1998, 1990.
37. Miura T, Komori M, Iwasaki M, Kurozomi K, Ohta K, Ohmori S, Kitada M and Kamataki T, Sex-related difference in oxidative metabolism of testosterone and erythromycine by hamster liver microsomes. *FEBS Lett* **231**: 183–186, 1988.
38. Skett P, Biochemical basis of sex differences in drug metabolism. *Pharmacol Ther* **38**: 269–304, 1988.
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. 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.
41. Lubet RA, Meyer 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.
42. 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.
43. Nakajima T, Elovaara E, Park SS, Gelboin H, 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.
44. Emi Y and Omura T, Synthesis of sex-specific forms of cytochrome P-450 in rat liver is transiently suppressed by hepatic monoxygenase inducers. *J Biochem* **104**: 40–43, 1989.