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COMPARISON OF CYTOCHROME P450 ISOENZYME PROFILES IN RAT LIVER AND HEPATOCYTE CULTURES

THE EFFECTS OF MODEL INDUCERS ON APOPROTEINS AND BIOTRANSFORMATION ACTIVITIES

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Abstract—The metabolic profile of seven subfamilies of cytochrome P450 (P450IA, IIA, IIB, IIC, IIE, IIIA, IVA) was studied in rat liver (*in vivo*) and in primary hepatocyte cultures (*in vitro*) after treatment with various inducers. The dealkylation of 7-ethoxyresorufin (EROD) and 7-pentoxyresorufin (PROD), aniline 4-hydroxylation and the regio- and stereoselective hydroxylation of testosterone were measured to characterize the isoenzyme pattern in intact hepatocytes and in liver microsomes. Occurrence of isoenzyme apoproteins was determined using Western blotting. Primary cultures of rat hepatocytes retain the capacity to respond to inducers of isoenzymes belonging to six different subfamilies (P450IA, IIA, IIB, IIC, IIIA and IVA). Treatment of cells with β-naphthoflavone revealed a P450-activity profile similar to *in vivo*, namely a highly induced EROD (P450IA1), a small enhancement of testosterone 7α-hydroxylation (P450IIA) and a marked reduction in 2α- and 16α-hydroxylation (P450IIC11). Exposure of cultured cells to phenobarbital resulted in a higher testosterone 16β-hydroxylation (reflecting P450IIB), though to a lesser extent than *in vivo*. The induction of P450IIIA due to both phenobarbital and dexamethasone, as mirrored by 6β- and 15β-hydroxylation of testosterone, was the same in cultured hepatocytes and *in vivo*. Treatment of cells with clofibric acid resulted in an induction profile similar to the one observed in liver microsomes from clofibrate-treated rats: the apoprotein P450IVA as well as the apoprotein P450IIB1/2 and its associated activities (PROD and testosterone 16β-hydroxylation) were induced. Isoniazid, a known *in vivo* inducer of P450IIE1 and aniline 4-hydroxylation, did not change any of the determined P450-dependent activities *in vitro*.

Cytochrome P450 comprises a family of haemoproteins that are involved in the metabolism of a wide variety of endogenous and exogenous compounds. Some forms of this enzyme are present constitutively, whereas others are induced to high levels of expression upon exposure to certain foreign compounds [1–4]. The multigenic superfamily of cytochrome P450 has been categorized into nine different subfamilies according to resemblance between their amino acid and gene sequences [5]. Each subfamily can be characterized by its inducibility [1, 2]. For example, P450IA1 and P450IA2 are induced by polycyclic hydrocarbons, whereas P450IIB is the major subfamily induced by phenobarbital (PB§). Treatment of animals with glucocorticoids like dexamethasone (DEX) results in an induction of a third subfamily, P450IIIA. In untreated male rats P450IA1 and P450IIB are barely detectable whereas relatively high levels of IA2, IIC6, IIC11, IIIA1 and IIE have been found [1, 6]. The isoenzymes differ from each other in substrate specificity and catalytic activity [2, 7, 8]. Therefore, changes in the isoenzyme pattern of cytochrome P450 have major implications for the metabolism and toxicity of many compounds [2].

Primary cultures of hepatocytes are widely used to study various aspects of cytochrome P450 regulation, drug metabolism and related cytotoxicity [9–11]. Although there is a rapid decline of the basal level of cytochrome P450 during the first 24 hr in culture (for review see Ref. 11), primary cultures of rat hepatocytes retain the capacity to respond to some P450-inducers by increasing the synthesis of specific mRNAs and proteins and by enhancing associated catalytic activities [12–16]. Therefore, primary hepatocyte cultures may be used to study the capacity of various xenobiotic compounds to change the isoenzyme profile and the subsequent effects of these changes on biological processes. A more detailed comparison not only of the major induced forms of P450, but also of the total P450-dependent metabolic profile, is needed in order to establish if primary cultures represent the *in vivo* situation.

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§ Abbreviations: AH, aniline 4-hydroxylation; BNF, β-naphthoflavone; CLOF, clofibrate; CLOFA, clofibric acid; DEX, dexamethasone; DMSO, dimethyl sulfoxide; EROD, 7-ethoxyresorufin O-deethylation; HBSS, Hanks' balanced salt solution; INH, isoniazid; OHT, hydroxytestosterone; PB, phenobarbital; PBS, phosphate-buffered saline; PROD, 7-pentoxyresorufin O-depentylation; PVDF, polyvinylidene difluoride; TBST, Tris-buffered saline containing 0.3% Tween 20.

Recently, we studied the time-related decrease in culture of several P450 dependent activities and compared the activities as measured in intact monolayers and in microsomes prepared from parallel cultured cells [17]. In the present study we used five different model inducers, known to induce specifically one P450 subfamily (β-naphthoflavone, IA; phenobarbital, IIB; isoniazid, IIE; dexamethasone, IIIA; and clofibrate, IVA) to describe their influences on the metabolic profile of P450 isoenzymes. We compared the induction ratio of the dealkylation of 7-ethoxyresorufin (EROD) and pentoxyresorufin (PROD), and the regio- and stereoselective hydroxylation of testosterone as measured in intact monolayers of hepatocytes and in liver microsomes from *in vivo* treated rats. Western blotting was used to compare enzyme activities to the occurrence of P450 isoenzyme apoproteins.

MATERIALS AND METHODS

Materials

PB and corn oil were obtained from O.P.G. (Utrecht, The Netherlands). Clofibrate (2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester, CLOF) was supplied by Centra Farm (Belgium). Isoniazid (INH) and clofibric acid (ethyl-2-(4-chlorophenoxy)-2-methylpropionic acid, CLOFA) were purchased from Janssen Chimica (Beerse, Belgium). Newborn calf serum was obtained from Gibco Europe (Breda, The Netherlands). Williams' E medium, β-naphthoflavone (BNF), androstenedione, DEX, 11β- and 16α-hydroxytestosterone (11β- and 16α-OHT) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 2α-OHT was a gift from Prof. D. N. Kirk (Queen Mary College, University of London). 15β- and 12β-OHT were gifts from G. D. Searle and Co. (Skokie, IL, U.S.A.), 6β-, 7α-, 16β-and 19-OHT were obtained from Steraloids (Wilton, NH, U.S.A.). All other chemicals were of analytical grade. Monoclonal antibodies towards P450IA1/2, P450IIB1/2 and P450IIIA were kind gifts from Dr P. J. Kremers, Université de Liège, Belgium. A polyclonal antibody towards P450IIE1 was a gift from Dr I. Johansson, Karolinska Institutet, Stockholm, Sweden and a polyclonal antibody towards P450IVA was a gift from Dr G. G. Gibson, University of Surrey, Guildford, U.K. Secondary antibodies were obtained from Dakopatts a/s, Glostrup, Denmark.

Animals

Male Wistar rats (Riv.Tox(M), RIVM, Bilthoven, The Netherlands), weighing 180–250 g, were fed a TNO-CIVO Institute grain based open-formula diet (TNO-CIVO, Zeist, The Netherlands) (*ad lib.*) and had free access to drinking water. PB and INH were given as 0.1% solution in drinking water for 5 and 10 days, respectively. BNF (40 mg/kg) was given by i.p. injection once daily for 4 days in 0.5 mL corn oil. DEX (300 mg/kg) and CLOF (250 mg/kg) were given by gastric intubation once daily for 3 days in 0.5 mL corn oil. Control rats received either corn oil i.p. for 4 days or corn oil p.o. for 3 days or no treatment at all.

Cell isolation and culture

Hepatocytes were isolated from untreated male rats (same strain and diet as in *in vivo* studies) using a two step collagenase perfusion technique [18]. The cells were plated on 6- or 9-cm tissue culture dishes (Sterilin) at a density of 4 or 8×10^6 cells/dish in 4 or 10 mL Williams' E medium, respectively. Media were supplemented with 3% (v/v) newborn calf serum, 1 μM insulin, 10 μM hydrocortisone and 50 mg/L gentamycin. Cells were incubated in a humidified atmosphere of air (95%) and CO_2 (5%) at 37°. After 4 hr in culture, medium was replaced. Thereafter, media were refreshed every 24 hr.

After a total preincubation period of 24 hr, the inducers, suspended or dissolved in dimethyl sulfoxide (DMSO), were added to the culture media to give final concentrations of 1.5 mM PB, 50 μM BNF, 0.6 μM DEX, 100 μM INH or 1 mM clofibric acid (CLOFA), the latter being the active analogue of CLOF. An equal amount of DMSO was added to the control cultures (final DMSO concentration 0.1% v/v).

Preparation of microsomes

Whole livers. Livers from control and treated animals were perfused with ice-cold saline and homogenized in 0.15 M KCl containing 0.1 mM EDTA using a Potter-Elvehjem glass–Teflon homogenizer. Microsomes were prepared by centrifugation (2×20 min, 9000 *g*; supernatant 60 min, 105,000 *g*). The microsomal pellet was resuspended in sodium phosphate buffer (0.1 M, pH 7.8) containing 0.1 mM EDTA, frozen quickly in liquid N_2 and stored at −70°.

Cultured hepatocytes. Per treatment group seven 9-cm culture dishes were washed with ice-cold PBS and scraped with a rubber policeman in 1 mL PBS/dish. Cells were centrifuged (3 min, 50 *g*), the supernatant was replaced by 2.5 mL 0.15 M KCl containing 0.1 mM EDTA and kept at −70°. Microsomes were prepared as described previously [17].

Biochemical determinations

Cytochrome P450 and protein. Contents of protein and total cytochrome P450 were determined according to Rutten *et al.* [19].

Ethoxy- and pentoxyresorufin O-dealkylation (EROD and PROD). Fluorimetric determinations of liver and hepatocyte microsomal EROD and PROD activities were performed according to the method of Burke *et al.* [7] using a Cobas-Bio centrifugal analyser, equipped with a spectrofluorimeter. Less than 200 μg microsomal protein was used in the incubation mixture of 0.1 M phosphate buffer (pH 7.4) with a final volume of 320 μL. Substrate was first dissolved in DMSO, diluted 1:10 with buffer, and added to the incubation mixture (final concentration DMSO 0.1% v/v). Substrate concentrations of 5 μM were used.

Determinations directly in intact hepatocytes were as previously described [17]. In short, hepatocyte monolayers were washed twice with Hanks' balanced salt solution (HBSS) gassed with carbogen (95% O_2/5% CO_2, v/v). The incubation was initiated by

adding 4 mL HBSS (37°) containing 5 μM substrate and 10 μM dicumarol. Fluorescence was determined after 5 and 15 min (EROD) or 10 and 20 min (PROD) using a Kontron SFM 25 fluorimeter.

Aniline 4-hydroxylation (AH). The AH activity in liver and hepatocyte microsomes was determined according to Chhabra *et al.* [20], using an aniline concentration of 15 mM. The reaction was stopped after 45 min at 37°, by adding 0.5 mL 20% TCA on ice. After centrifugation (5 min, 100 *g*), the formation of 4-aminophenol was determined spectrophotometrically at 630 nm, according to Imai *et al.* [21].

Testosterone hydroxylation. Determinations in microsomal preparations were carried out in 1 mL incubation mixture containing potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), $NADP^+$ (1 mM), glucose 6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit/mL), testosterone (250 μM) and 200–300 μg microsomal protein. Incubation mixture and microsomes were mixed while standing on ice. Reactions were started by heating the mixture in a 37° water bath and stopped after 15 min by addition of 6 mL dichloromethane. Extraction and subsequent analysis of metabolites by HPLC were performed as previously described [17]. 11β-OHT was used as internal standard.

Determinations of testosterone hydroxylation activity directly in intact monolayers were as previously described [17]. In brief, cells were washed twice with HBSS, and incubated with 4 mL HBSS containing 250 μM testosterone. After 15 min, testosterone and its metabolites were extracted with dichloromethane and subsequently analysed using HPLC.

Gel electrophoresis and immunoblotting. Separation of microsomal proteins was carried out on a Biorad mini Protean II cell applying the sodium dodecyl sulphate–polyacrylamide gel electrophoresis discontinuous system of Laemmli [22], using a 4% stacking gel and a 12.5% separating gel (whole livers: 2 μg microsomal protein/lane; cultured cells; 4 μg microsomal protein/lane). The resolved proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) sheets (Millipore) at 30 V overnight according to Towbin *et al.* [23] and immunochemically stained using antibodies directed to P450IA1/2, P450IIB1, P450IIE, P450IIIA1/2 and P450IVA1. PVDF sheets were rinsed in Tris-buffered saline containing 0.3% Tween 20 (TBST) for 5 min, blocked in TBST containing 1% bovine serum albumin for 1 hr at room temperature, and thereafter incubated with primary antibody. After 1 hr, the sheets were washed five times in TBST and incubated with either anti-mouse or anti-rabbit IgG for 1 hr. Both IgG preparations were conjugated with alkaline phosphatase and colour was developed with a mixture of 5-bromo-,4-chloro,3-indole toluidine phosphate and *p*-nitrobluetetrazolium chloride.

Statistical analysis. Results have been expressed as means ± SD, where appropriate. Statistical analysis was performed by Student's *t*-test, for unpaired samples. The null-hypothesis was rejected at $P < 0.05$.

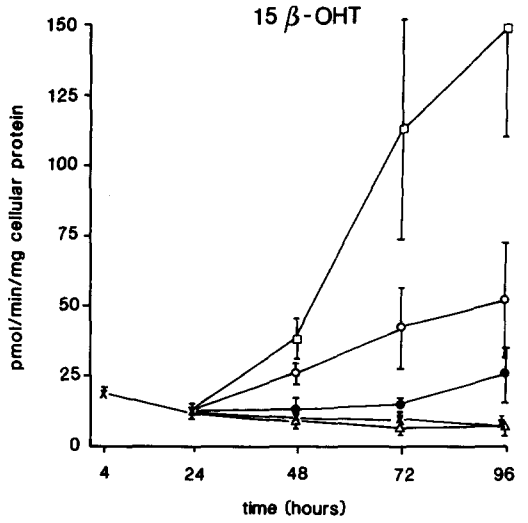

Fig. 1. Change in 15β-hydroxylation of testosterone measured in intact hepatocyte monolayers after exposure to vehicle (DMSO) only (×), β-naphthoflavone (△), phenobarbital (○), clofibric acid (●) and dexamethasone (□). After a preincubation of 24 hr, hepatocytes were exposed to the inducing compound for another 72 hr. Data are means ± SD from three different experiments.

RESULTS

In Fig. 1 the change in testosterone 15β-hydroxylation after exposure to different model inducers during culture is shown as a representative example. In this study *in vitro* induction ratios are based on the activity measured in 96-hr-old intact hepatocytes treated for 72 hr with inducer, compared to the activity in parallel cultured cells treated for 72 hr with DMSO alone.

Figure 2 shows the elution profiles of testosterone metabolites obtained using liver microsomes from untreated rats (A,C) and intact monolayers of 96-hr-old rat hepatocytes (B,D). In untreated intact cells (Fig. 2B) relatively high levels of androstenedione (peak 9) and relatively low levels of 7α-OHT (peak 3) are formed compared to the activity in liver microsomes from control rats (Fig. 2A). We reported earlier that the testosterone metabolite profile using liver microsomes from untreated rats was similar to the metabolite profile when microsomes prepared from cultured cells were used in the incubation [17]. Treatment with model inducers did not affect the basal differences between microsomes and intact cells towards the formation of 7α-OHT and androstenedione. As an example, the testosterone metabolite profile after exposure to clofibrate *in vivo* and clofibric acid *in vitro* are shown in Fig. 2C and D. These results indicate that induction ratios can be compared only as calculated from the activity in treated rats and hepatocytes, divided by the corresponding activities in their matching controls.

In Fig. 3 an overview is given of the induction ratios of several biotransformation activities *in vivo* and *in vitro*. Table 1 shows semi-quantitative determinations of apoprotein levels of several P450s

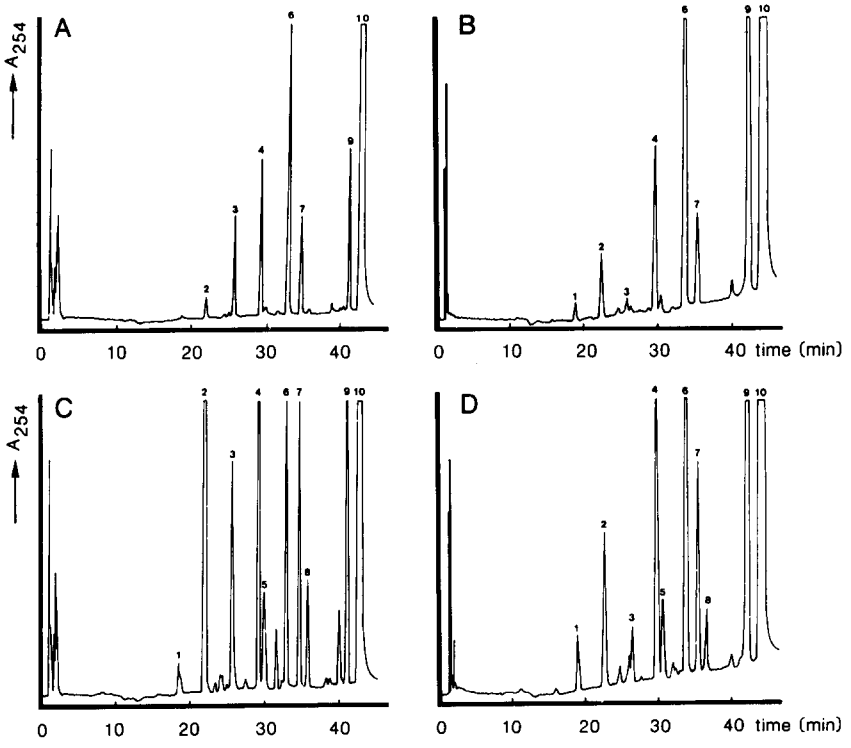

Fig. 2. Elution profiles of testosterone metabolites determined in microsomes from rat liver and in intact monolayers of cells. (A) *In vivo* control; (B) control 96-hr-old hepatocytes; (C) *in vivo* rats treated with clofibrate for three days; (D) 96-hr-old hepatocytes treated with clofibric acid for 72 hr. Peak identification: (1) 15β-OHT; (2) 6β-OHT; (3) 7α-OHT; (4) 16α-OHT; (5) 16β-OHT; (6) 11β-OHT (internal standard); (7) 2α-OHT; (8) X, unknown metabolite; (9) androstenedione; (10) testosterone.

in microsomes prepared from cultured hepatocytes and whole liver. The original data concerning the P450-dependent activities, including the statistical test results, are given in Table 2 (*in vivo*) and Table 3 (*in vitro*).

Exposure to β-naphthoflavone

Treatment of rats with BNF *in vivo* resulted in the well-known induction of EROD activity (24-fold; Fig. 3a), related to an enhanced level of P450IA apoproteins (Table 1). BNF increased the 7α-hydroxylation of testosterone 3.5-fold, but decreased testosterone 2α- and 16α-hydroxylation in the liver. The PROD activity in microsomes from BNF-treated rats was enhanced (3-fold), although no P450IIB1/2 apoprotein levels could be detected (Table 1). Exposure of hepatocyte cultures to BNF resulted in a similar metabolic profile to the one observed after exposure to BNF *in vivo* (EROD 24- and 27-fold enhanced, *in vivo* and *in vitro*, respectively), though no enhancement of PROD activity was measured in intact monolayers after BNF treatment. In contrast, in microsomes prepared from BNF-treated cells a similar enhancement of PROD activity was measured as *in vivo* microsomes (results not shown). The characteristic decrease in 2α- and 16α-hydroxylation of testosterone after BNF treatment was also seen *in vitro*. Immunochemical analysis showed the presence of both P450IA1 and IA2, although the level of IA2 is lower in culture than *in vivo* (Table 1).

Exposure to phenobarbital

Treatment of rats with PB caused a large induction of cytochrome P450IIB *in vivo* (Table 1) and resulted in an associated increase in liver microsomal PROD activity and 16β-hydroxylation of testosterone (67- and 36-fold, respectively; Fig. 3b). EROD activity was enhanced 5-fold, whereas no P450IA could be detected immunochemically in PB-microsomes (Table 1). The production of 15β-, 7α-, 6β- and 16α-OHT and an unknown metabolite with a relative retention time towards testosterone of 0.83 (probably 18-OHT [21], to be called X in this text) were all increased after PB treatment. In contrast to PB treatment *in vivo*, exposure of hepatocytes to PB revealed only minor levels of P450IIB1/2 proteins, which was mirrored in low PROD, 16β- and 16α-testosterone hydroxylation activities (Fig. 3b). On the other hand, PB induced P450IIIA apoprotein levels both in cell culture and *in vivo* (Table 1), associated with enhanced production of 6β-OHT, 15β-OHT and compound X, the latter two even more enhanced *in vitro* compared to the induction *in vivo*. The enhancement of EROD activity in PB-treated hepatocytes was to a similar extent as *in vivo*.

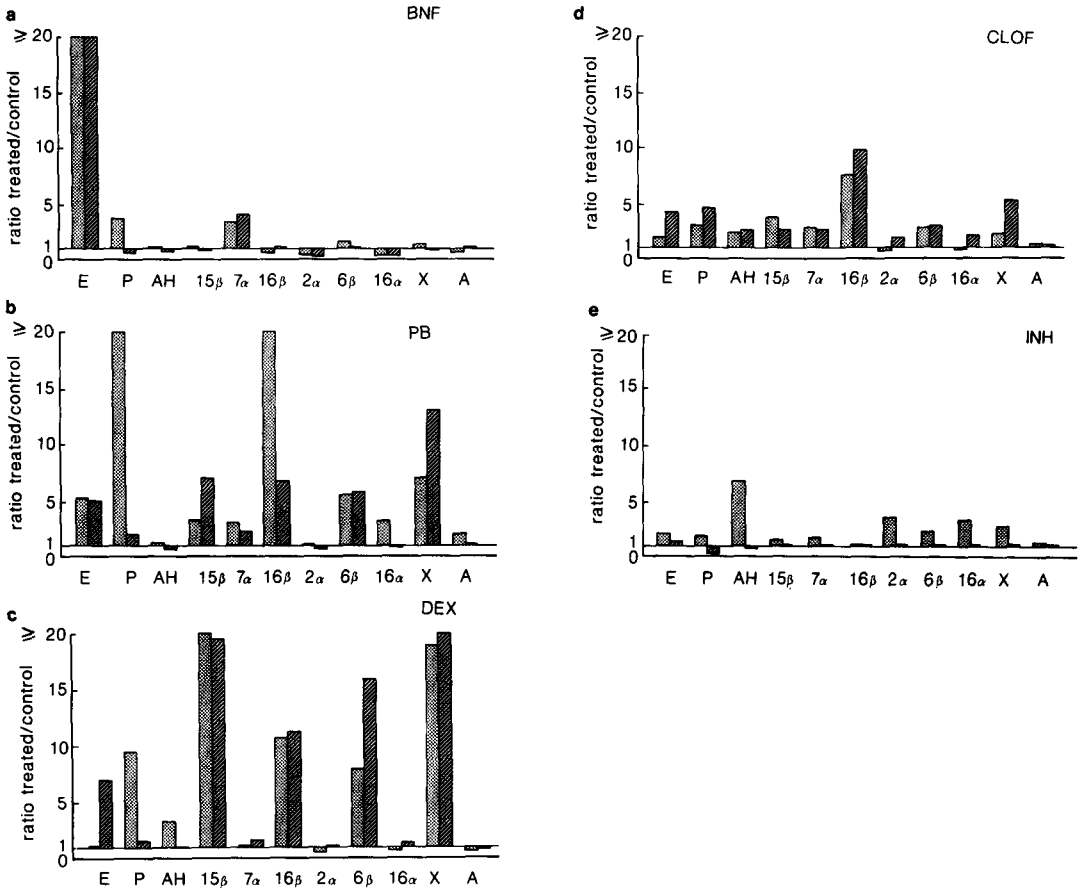

Fig. 3. Induction ratios (treated/matching control) of cytochrome P450-dependent activities measured in liver microsomes from rats treated with different inducers *in vivo* and in intact cultured hepatocytes after exposure to the same inducers. (a) β-naphthoflavone (BNF); (b) phenobarbital (PB); (c) dexamethasone (DEX); (d) clofibrate (CLOF(A)); (e) isoniazid (INH). (▨) *in vivo*, (▨) *in vitro*. ≥ 20 indicates an induction ratio of 20 or higher. For duration of treatment see Materials and Methods. Because of a 16β-OHT activity below the detection limit in 96-hr-old control cultures, minimal ratios for this activity *in vitro* are given.

Table 1. Immunochemical detection of apoprotein levels of different P450 isoenzymes, *in vivo* and *in vitro* after treatment with phenobarbital (PB), β-naphthoflavone (BNF), dexamethasone (DEX), clofibrate/clofibric acid (CLOF(A); CLOF, *in vivo*; CLOFA, *in vitro*) and isoniazid (INH)

	Control	BNF	PB	DEX	CLOF(A)	INH
In vivo treatment						
P450IA1	−	+++	−	−	−	−
P450IA2	+	++	−	−	−	+
P450IIB1/2	−	−	+++	++	++	−
P450IIE	+	+	+	+	++	+++
P450IIIA	+	−	+	+++	+	−
P450IVA	−	−	−	−	+++	−
In vitro treatment						
P450IA1	−	+++	−	+	+	−
P450IA2	±	+	−	−	−	−
P450IIB1/2	−	−	±	±	+	−
P450IIE	+	+	+	+	+	+
P450IIIA	±	−	±	+++	±	−
P450IVA	−	−	−	−	+++	−

In vivo, 2 μg microsomal protein per lane; *in vitro*, 4 μg microsomal protein per lane. Key: −, isoenzyme not detected; ±, isoenzyme weakly detected; +, isoenzyme easily detected; ++, isoenzyme strongly detected; +++, isoenzyme very strongly detected.

Table 2. Profile of P450-dependent activities in liver from rats treated with different inducers

	Control	co-ip	co-po	BNF	PB	DEX	CLOF	INH
P450	372 ± 165	350 ± 23	290 ± 36	879 ± 138*	661 ± 151*	1211 ± 120*	782 ± 54*	417 ± 23
EROD	44 ± 18	110 ± 21*	53 ± 11	2601 ± 389*	241 ± 24*	106 ± 23*	113 ± 37*	98 ± 46*
PROD	5 ± 3	11 ± 2*	8 ± 1	43 ± 9*	322 ± 109*	76 ± 5*	24 ± 3*	9 ± 3*
AH	446 ± 194	633 ± 206	424 ± 83	761 ± 97	580 ± 250	1477 ± 472*	1055 ± 94*	3206 ± 790*
Testosterone								
15β	9 ± 4	14 ± 7	9 ± 4	14 ± 2	32 ± 7*	541 ± 72*	34 ± 14*	15 ± 7
7α	76 ± 51	143 ± 77	107 ± 53	506 ± 140*	237 ± 93*	125 ± 47	323 ± 95*	141 ± 50
16β	18 ± 6	16 ± 9	10 ± 2	10 ± 3	671 ± 135*	116 ± 26*	81 ± 13*	20 ± 6
2α	254 ± 57	964 ± 617	551 ± 55*	393 ± 171	287 ± 39	313 ± 66*	424 ± 112	963 ± 239*
6β	223 ± 147	537 ± 85	449 ± 146	923 ± 151*	1272 ± 107*	4136 ± 961*	1462 ± 701	558 ± 177*
16α	460 ± 108	1554 ± 1025	927 ± 110*	649 ± 278	1541 ± 280*	708 ± 84*	794 ± 251	1598 ± 385*
X	19 ± 8	48 ± 2	44 ± 10	68 ± 5*	139 ± 21*	871 ± 137*	105 ± 44*	55 ± 12*
Androst	529 ± 229	692 ± 252	508 ± 60	481 ± 67	1142 ± 265*	349 ± 38*	674 ± 106*	760 ± 110

The values are expressed as pmol/min/mg microsomal protein and are means ± SD (N = 3 rats, except control N = 5 rats).

* $P < 0.05$ when compared to matching control; inducers phenobarbital (PB) and isoniazid (INH) compared to control, β-naphthoflavone (BNF) compared to control-ip (co-ip), clofibrate (CLOF) and dexamethasone (DEX) compared to control-po (co-po).

Table 3. Profile of P450-dependent activities in primary rat hepatocyte cultures after treatment with various inducers for 3 days

	Control (24 hr)	Control (96 hr)	BNF	PB	DEX	CLOFA	INH
P450†	169.6 ± 53.2	136.4 ± 65.4	173.5 ± 3.1	145.3 ± 24.0	170.4 ± 115.2	130.9 ± 115.7	122.1 ± 55.0
EROD	7.0 ± 1.9	3.9 ± 1.5	104.1 ± 7.6*	20.1 ± 3.3*	29.0 ± 4.0*	18.2 ± 3.2*	5.9 ± 1.8
PROD	18.4 ± 3.2	5.4 ± 1.7	3.2 ± 1.3	11.1 ± 2.5*	8.9 ± 2.2*	24.2 ± 3.7*	1.7 ± 1.0*
AH†	237.9 ± 96.7	63.6 ± 30.3	31.3 ± 1.5	37.5 ± 5.4	69.8 ± 4.7	115.1 ± 6.3	90.8 ± 13.8
Testosterone							
15β	11.3 ± 1.3	7.3 ± 2.3	7.5 ± 2.1	53.5 ± 20.2*	149.7 ± 39.6*	25.4 ± 10.6	9.5 ± 1.6
7α	12.9 ± 5.3	9.8 ± 5.3	41.3 ± 10.1	23.2 ± 7.5*	17.3 ± 5.1	16.3 ± 7.2	10.7 ± 4.2
16β	5.4 ± 2.0	<2	<2	13.9 ± 0.6	21.8 ± 1.5	22.1 ± 1.0	<2
2α	80.2 ± 6.6	29.8 ± 5.8	9.4 ± 3.4*	19.8 ± 5.4*	35.0 ± 7.3	59.1 ± 7.1*	31.0 ± 9.9
6β	159.1 ± 11.5	40.8 ± 16.6	42.3 ± 11.3	245.4 ± 98.7*	681.9 ± 220.6*	126.4 ± 62.6	44.8 ± 13.6
16α	154.9 ± 11.4	65.3 ± 6.0	28.5 ± 4.6*	55.7 ± 12.1	96.0 ± 18.1*	142.8 ± 6.6*	65.7 ± 7.4
X	14.7 ± 1.2	5.2 ± 4.1	3.5 ± 0.1	70.9 ± 31.0*	235.5 ± 68.0*	26.6 ± 16.8*	4.1 ± 1.1
Androst	493.2 ± 48.7	359.6 ± 86.9	446.7 ± 104.2	392.9 ± 111.5	305.0 ± 90.4	443.7 ± 37.5	396.6 ± 85.7

The values are expressed as pmol/min/mg cellular protein and are means ± SD (N = 3 rats).

* $P < 0.05$ when compared to 96-hr-old hepatocytes; inducers: BNF, β-naphthoflavone; PB, phenobarbital; DEX, dexamethasone; CLOFA, clofibric acid; INH, isoniazid. After a preincubation period of 24 hr, hepatocytes were exposed to the inducing compound for another 72 hr.

† P450 content and aniline 4-hydroxylation (AH) were determined in microsomes prepared from cultured hepatocytes and are both expressed per mg microsomal protein. P450 content in freshly isolated cells was 343 ± 84 pmol/mg microsomal protein.

Exposure to dexamethasone

DEX, an inducer of subfamily P450IIIA (Table 1), increased the production of 15β-OHT (63-fold) and compound X (20-fold) *in vivo* (Fig. 3c). The PROD activity as well as the hydroxylation activity towards the 16β- and the 6β-site of testosterone were highly induced, 10-, 11- and 8-fold, respectively. Immunochemical studies of DEX-microsomes revealed an induction of both P450IIIA and P450IIB apoprotein levels *in vivo* (Table 1). Treatment of cultured hepatocytes with DEX resulted in a similar induction profile concerning the testosterone metabolites, although the induction ratios of testosterone 6β-hydroxylation and the formation of compound X (50-fold) were higher *in vitro* compared to the activity in liver after *in vivo* exposure to DEX (Fig. 3c). Unlike PB, in DEX-treated hepatocytes the production of 16β-OHT was induced to the same extent compared to the induction of this activity *in vivo*. However, the PROD activity was not enhanced *in vitro*. A remarkable difference *in vivo* was found concerning the EROD activity after DEX exposure, since in DEX-treated cells (measured in intact cells as well as in microsomes prepared from these cells) this activity was induced 7-fold, which was consistent with a small but distinct induction of P450IA1 apoprotein *in vitro* (Table 1).

Exposure to clofibrate and clofibric acid

Treatment of rats with CLOF resulted in an induction of the apoprotein level of P450IVA, which was not found with any of the other model inducers (Table 1). Moreover, an 8-fold induction of 16β-hydroxylation of testosterone was measured (Fig. 3d), which was associated with a substantial increase in the level of apoprotein P450IIB1 (Table 1). A more modest increase (3–4-fold) was determined for PROD, 15β-, 7α- and 6β-OHT activities. Treatment of primary cultures with clofibric acid resulted in a metabolic profile almost similar to the one obtained after exposure *in vivo* (Fig. 3d), and in the same characteristic induction of P450IVA apoprotein (Table 1). An interesting point was the relatively high induction of P450IIB1/2 due to clofibric acid in hepatocyte culture as compared to the induction of this isoenzyme(s) in PB-treated cells (Fig. 4). In agreement, both PROD and testosterone 16β-hydroxylation were enhanced *in vitro* to a similar level as in rat liver after treatment with CLOF. In comparison to DEX exposure, treatment of cells with CLOFA revealed also a small induction of P450IA1 apoprotein levels (Table 1) and higher EROD activity.

Exposure to isoniazid

Isoniazid increased the rate of aniline 4-hydroxylation (7-fold) *in vivo* (Fig. 3e). This activity is known to be preferentially catalysed by P450IIE and P450IA2 [2, 22]. Towards testosterone a 2–4-fold increase was measured in the production of 2α-, 16α-, 6β-OHT and the unknown metabolite X. Both EROD and PROD activities were significantly enhanced (2-fold) as well. However, in isoniazid-treated cells only the EROD activity was increased whereas even a decrease in PROD activity was measured. No enhancement of aniline 4-hydroxylation (Fig. 3e) or P450IIE apoprotein levels (Table 1) could be detected *in vitro*.

DISCUSSION

So far, most studies of the effects of inducers on cytochrome P450 *in vitro* have concentrated on the enhancement of one or few specific P450 isoenzymes [13–16]. A direct comparison to study whether the *in vitro* changes of a broad spectrum of P450 activities, due to exposure to various model inducers, mimic the *in vivo* situation, has not been described yet. We used different specific catalytic activities supported with immunochemical detection of P450 apoproteins, to study the change in metabolic profile.

Treatment of rats *in vivo* with BNF, PB, DEX, CLOF and INH resulted in changes in P450 apoprotein pattern and associated catalytic activities which are consistent with data reported by others [2, 24–26]. Treatment of hepatocytes with the same model inducers resulted to a large extent in similar metabolic profiles and induction ratios. However some marked differences can be observed.

Exposure of cultured hepatocytes to BNF resulted in a catalytic profile very similar to the *in vivo* profile: a high EROD activity (which is preferentially catalysed by P450IA1 [7]), a moderate increase of 7α-hydroxylation (which represents P450IIA [26]) and a decrease in testosterone 2α- and 16α-hydroxylation. This decrease to *ca.* 40% of the activity measured in controls (*in vivo* as well as *in vitro*) could only be detected after treatment with BNF. P450IIC11 is known to catalyse testosterone hydroxylation at the 2α-site and to a lesser extent at the 16α-site [4]. A decrease of P450IIC11 is also observed in rats after exposure to other P450IA inducers [1]. These results indicate a comparable degree of down regulation of P450IIC11 *in vivo* and *in vitro* after BNF treatment.

PB is known to increase the *de novo* synthesis of apocytochrome P450IIB1/2 and P450IIIA in rat liver [2, 27]. P450IIB1/2 catalyses the conversion of testosterone mainly to 16β-OHT, and to a lesser extent to 16α-OHT and androstenedione [8]. However, in cultured hepatocytes P450IIB1/2 apoprotein levels and associated activities after PB treatment is low compared to the *in vivo* situation. For many years, several investigators [11, 14, 27, 28] have been concentrating on identifying culture conditions which would permit P450IIB1/2 to be induced in cultured hepatocytes. Recently, Schuetz *et al.* [29] reported a higher enhancement of P450IIB1/2 mRNA and apoprotein level after PB treatment when hepatocytes were cultured on a reconstituted basement membrane gel. However, no associated activities i.e. formation of 16β-OHT or PROD, were reported. In a preliminary study we could detect a small induction of P450IIB1/2 mRNA levels in PB- and CLOFA-treated cells (8- and 32-fold, respectively; results not shown). Therefore, in addition to the small induction of the apoprotein P450IIB1/2 and an enhancement of the associated

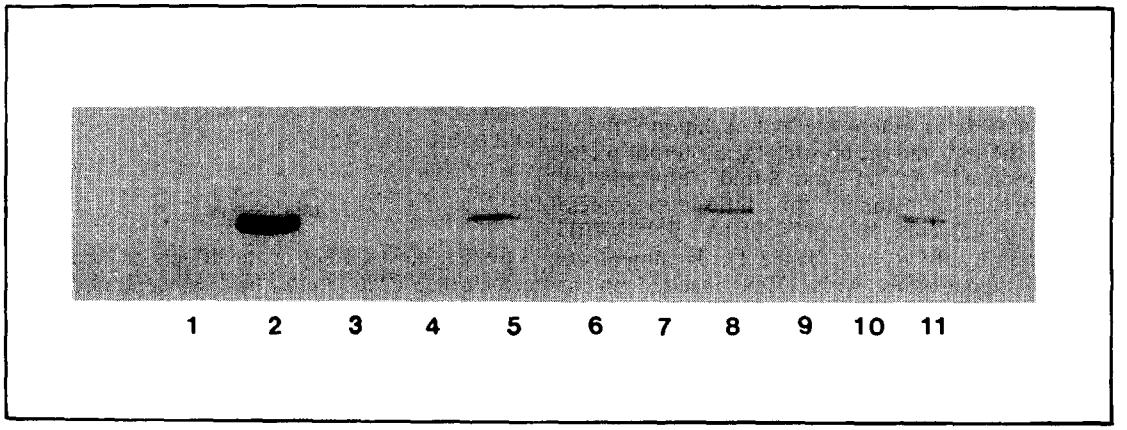

Fig. 4. Immunoblot of microsomes prepared from rat liver and cultured hepatocytes, immunochemically stained with anti-P450IIB1/2. Lanes 1–5, liver microsomes from rats treated *in vivo* (2 ±g protein/lane): (1) control; (2) phenobarbital; (3) β-naphthoflavone; (4) isoniazid; (5) clofibrate. Lanes 6–11, microsomes from cultured hepatocytes (4 μg protein/lane): (6) and (9) 24-hr-old hepatocytes; (7) and (10) 96-hr-old hepatocytes treated with DMSO; (8) 96-hr-old hepatocytes treated with clofibric acid; (11) 96-hr-old hepatocytes treated with phenobarbital.

enzyme activities (16β-hydroxylation of testosterone and PROD), we conclude that cultured hepatocytes do respond to P450IIB inducers, especially CLOFA, with an enhanced level of mRNA, apoprotein and associated enzyme activity, although in the case of PB to a much lower level than *in vivo*. A recent study of Waxman *et al.* [30] describes a comparable *in vivo* level of P450IIB and associated enzymatic activities in PB-treated hepatocytes when cells were cultured for 10 or more days.

In contrast to the low P450IIB1/2 levels in PB-treated cells, the P450IIIA apoprotein level and its associated [4] activities towards the 6β- and 15β-site of testosterone, were induced. P450IIIA is also known to catalyse the formation of 18-hydroxy-testosterone [31, 32]. Based on the enhanced production of compound X after PB and DEX treatment and a comparable relative retention time in a similar HPLC elution profile [31], we deduce that compound X most probably is identical to 18-hydroxytestosterone.

Exposure of cultured hepatocytes to CLOFA resulted in the characteristic enhancement of the P450IVA apoprotein level as observed *in vivo* [3]. Induction of this isoenzyme *in vitro* was also reported by Lake *et al.* [16], who, in addition, determined the corresponding higher activity in 12-hydroxylation of lauric acid. No lauric acid hydroxylation was measured in this study. Beside induction of P450IVA, exposure to CLOF(A) induced almost every P450-dependent activity measured in our study *in vivo* as well as *in vitro*. These results are in contrast with data of Sharma *et al.* [3], who measured a reduced dealkylation of both ethoxyresorufin and benzphetamine in rat liver after treatment with CLOF. However, other investigators [12, 16, 24] reported the enhancement of several P450 isoenzymes after CLOF treatment which is consistent with our data. The reason for this discrepancy is not clear.

P450IIE is different from many other forms of P450 since it is induced by numerous structurally different compounds [32, 33], many of which are known to effectively bind to the enzyme. Eliasson *et al.* [34] reported a maintenance of P450IIE in primary cultures of rat hepatocytes, provided that enzyme ligands (preventing enzyme degradation) are present in the medium. The induction of this isoenzyme *in vivo* is correlated with ligand binding and stabilization of mRNA levels, resulting in higher protein levels [35]. However, in agreement with our study, no induction of this isoenzyme *in vitro* has been reported yet. Whether the lack of high induction of P450IIB and P450IIE *in vitro* has to be attributed to hormonal disbalance has been the subject of much research [4, 27, 36, 37].

In this study we monitored specific biotransformation activities to study the P450 isoenzyme profile. In this regard the O-dealkylation of 7-ethoxyresorufin is often used as a reflection of the activity of P450IA1 [7]. However, in control and PB-treated rats the EROD activity (in the absence of P450IA1) is mainly catalysed by two constitutive enzymes, P450IIC11 and P450IIC6 [38]. For this reason, we would emphasize the need of both immunochemical and catalytic activity determinations to study the P450 isoenzyme profile. The 4-fold higher PROD activity in liver microsomes from BNF-treated rats *in vivo* (in the absence of P450IIB), for example, is probably the result of a catalytic activity of P450IA1 towards this substrate. This is supported by the study of Nakajima *et al.* [38] who could inhibit PROD activity in liver microsomes from rats treated with 3-methylcholanthrene for 50% with a monoclonal antibody directed towards P450IA. In contrast, in intact BNF-treated cells no enhancement of PROD activity was measured, whereas in microsomes prepared from similar BNF-treated cells the PROD activity was enhanced to the same level as *in vivo*. Such discrepancies between the activity in intact cells compared to the same activity in microsomes

(prepared from cells) were also found for the formation of 7α-hydroxylation and androstenedione. These results may indicate that changes in substrate accessibility and/or changes in the efficiency for the electron transfer from NADPH P450-reductase to certain P450 isoenzymes are introduced during preparation of microsomes.

In conclusion, in primary rat hepatocytes several isoenzymes of cytochrome P450, namely P450IA1, IA2, IIA, IIC, IIIA and IVA, can be changed with the same inducing compounds and to the same extent as *in vivo*. The induction of P450IIB1/2 in rat hepatocytes, however, occurs to a much lower level compared to *in vivo*. It should be noted that two distinct ways of regulation could be possible for this isoenzyme because of the different level of induction after PB and CLOF treatment *in vitro*. No induction of P450IIE could be detected in primary hepatocytes. In spite of this incompleteness for some forms of P450 we conclude that primary cultures of hepatocytes can be a useful alternative in studying the changes in biotransformation activities catalysed by the P450IA, IIA, IIC, IIIA and IVA subfamilies as well as the effects on cytochrome P450-mediated cytotoxicity.

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