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DRUG METABOLISM BY PERIPORTAL AND PERIVENOUS RAT HEPATOCYTES

COMPARISON OF PHASE I AND PHASE II REACTIONS AND THEIR INDUCIBILITY DURING CULTURE

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Abstract—Hepatocytes were aseptically isolated from either the periportal (pp; zone 1) or the perivenous (pv; zone 3) region by digitonin–collagenase perfusion and cultured on type I collagen for 4 to 9 days. In freshly isolated cells the pp:pv activity ratios of the acinar marker enzymes γ-glutamyltranspeptidase (γ-GT), alanine aminotransferase (ALAT) and glutamate dehydrogenase (GLDH) were 2.8, 1.6 and 0.76, respectively. During culture ALAT and GLDH activities gradually declined, but the pp–pv difference was retained for at least 4 days. In contrast, the difference in the γ-GT activity was rapidly lost, due to its fast initial activation in pv cells. The initial 7-ethoxycoumarin *O*-deethylase (ECDE) activity was higher in pv cells; this difference was retained for several days of culture and was increased by induction *in vitro* with either phenobarbital (PB) or β-naphthoflavone (βNF). Although the basal UDP-glucuronyltransferase (UDPGT) activity with either *p*-nitrophenol (*p*NP) or hydroxybiphenyl (HBP) as substrate did not differ significantly, the *in-vitro* PB- or βNF-induced activity was higher in pv cells. Both glucuronidation and sulfation of methylumbelliferone tended to be higher in pv cells. Glutathione *S*-transferase was initially significantly higher in pv cells and this difference was augmented after *in vitro* induction by PB or βNF. After six days in culture all the observed pp–pv differences had disappeared. These results suggest that hepatocytes isolated from the perivenous region seem to maintain their initially higher capacity for phase I and phase II drug reactions during culture and also respond more strongly than periportal cells to *in vitro* induction.

Several metabolic processes are heterogeneously distributed in the acinus [1, 2], the microcirculatory unit of the liver [3, 4]. A regional difference is also seen in the susceptibility of the liver cells to toxic chemicals. Most xenobiotics cause damage primarily to the perivenous cells, located in zone III surrounding the terminal hepatic vein [1, 5, 6]. Since the toxicity of most foreign chemicals only becomes overt after their metabolic activation to toxic intermediates, the regional differences in vulnerability have been suggested to result from a heterogeneous distribution of drug-metabolizing enzymes. Several studies [7–10] suggest that the perivenous cells have a high capacity to metabolize xenobiotics by cytochrome P-450 linked mono-oxygenases, especially after induction [11, 12]. These results, based on histochemistry, immunohistochemistry or analysis of microdissected tissue, have, however, yielded partly conflicting results, as have more recent perfusion studies [13, 14]. Attempts to approach this problem by separating cell populations according to density [11, 15, 16] have been hampered by the difficulties to assign the true origin of these cell preparations.

The present study uses a new approach to study the distribution of drug metabolizing enzymes and their inducibility. Populations highly enriched in either periportal (pp)‖ or perivenous (pv) cells are isolated with the digitonin–collagenase perfusion technique [17, 18]. Our results suggest that when the cell types are cultured under identical conditions where *in vivo* differences in oxygen, hormone and substrate concentrations are eliminated, cells from the perivenous region maintain a higher capacity for both phase I and phase II reactions and seem to react stronger to *in vitro* induction than cells from the periportal region.

MATERIALS AND METHODS

Isolation of hepatocytes. Livers from 6 to 10-week-old male rats (fed *ad lib.*) of the Alko mixed strain were used. Periportal or perivenous cells were isolated with the digitonin–collagenase perfusion technique as previously described [17], but under aseptic conditions. For preparation of periportal cells, 7 mM digitonin solution was infused via the venous cannula for 30–50 sec at 10 ml/min, to selectively destroy the

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‖ Abbreviations used: UDPGT, UDP-glucuronyltransferase (EC 2.4.1.17); γ-GT, γ-glutamyltranspeptidase (EC 2.3.2.2); ALAT, alanine aminotransferase (EC 2.6.1.2); GLDH, glutamate dehydrogenase (EC 1.4.1.2); GS, glutamine synthetase (EC 6.3.1.2); GST, glutathione *S*-transferase (EC 2.5.1.18); ECDE, 7-ethoxycoumarin *O*-deethylase (EC 1.14.14.1); *p*NP, *p*-nitrophenol; HBP, hydroxybiphenyl; PB, phenobarbital; βNF, β-naphthoflavone; CDNB, chlorodinitrobenzene; MUG, methylumbelliferone glucuronidation; MUS, methylumbelliferone sulfation; pp, periportal; and pv, perivenous.

perivenous zone. Intact cells from the periportal region were then released by conventional collagenase perfusion. For preparation of cells from the perivenous zone digitonin was infused via the portal vein. All perfusion solutions were sterilized on-line with a 0.22 μm Sterivex-GSR filter (Millipore), and all steps subsequent to the perfusion were performed in a laminar flow cabinet, whenever possible.

The isolated cells were washed two to three times by centrifugation (90 sec, 15 *g*) in a medium with 2% albumin, and were then transferred to suspension buffer [19] with 2 mM pyruvate, 100 mM Hepes, but no phosphate, and 50 μg/ml amphotericin B (Fungizone®) and 10 μg/ml gentamycin. The suspension, containing 1.5–6 × 10^6 cells/ml, was stored at 0° with occasional stirring.

Culture of cells. The cells were plated on cell culture dishes (8.5 cm dia., Nunc) coated with gels of rat tail collagen [20], 4–6 hr after isolation, at a density of 0.6 × 10^6 cells/ml medium. The medium was changed 4 hr after inoculation, and thereafter every 24 hr. The medium was a 1:1 mixture of Waymouth MB 752/1 and Ham F-12, supplemented with 20 mM Hepes, 5 mM $NaHCO_3$, 10 mM Tricine, 1 μM dexamethasone, 1 I.U./l insulin, 50 μg/l glucagon, 5% fetal calf serum and 5% newborn calf serum and 10 μg/ml gentamycin. When ECDE was to be measured metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone, 0.5 mM) was added to the medium for better preservation of cytochrome P-450 [21].

For induction studies 2 mM phenobarbital or 50 μM β-naphtoflavone was added on day 1 or day 6 and the cells harvested 72 hr later. The time for induction was chosen on the basis of earlier studies with conventional hepatocyte cultures [22]. Optimal induction occurred on days 3 to 5 also in separately cultured periportal and perivenous cells. The cells were harvested with collagenase at indicated times, washed with phosphate-buffered saline and stored frozen until enzyme assays.

Enzyme assays. Freshly isolated cells were immediately frozen in liquid nitrogen and kept at −70° until assayed. Protein, γ-GT, ALAT and GLDH were assayed as cited previously [17]. For drug-metabolizing enzymes the cells were homogenized in 0.15 M KCl, and analysed for ECDE activity [23], UDPGT activity with both *p*NP and HBP as substrates in the absence and presence of optimal amounts (0.01–0.02%) of Brij 58 [24], and GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate [25]. DNA was assayed according to Ref. 26. The capacity of freshly isolated whole cells for glucuronidation and sulfation of 4-methylumbelliferone was measured in the presence of 10 mM K_2SO_4, as described earlier [27], except that both the incubation time and the cell concentration were halved when 10 and 25 μM MU concentrations were used.

Chemicals. Culture media, sera and Fungizone® were from Gibco, (Paisley, Scotland). Insulin (bovine pancreas), dexamethasone, glucagon, gentamycin, metyrapone, Hepes, Tricine, ethoxycoumarine, NADP, isocitrate dehydrogenase and UDP-glucuronic acid were from Sigma Chemical Co. (St. Louis, MO). Digitonin was from BDH Chemicals (Poole, Dorset) and collagenase from Boehringer (Mannheim, F.R.G.).

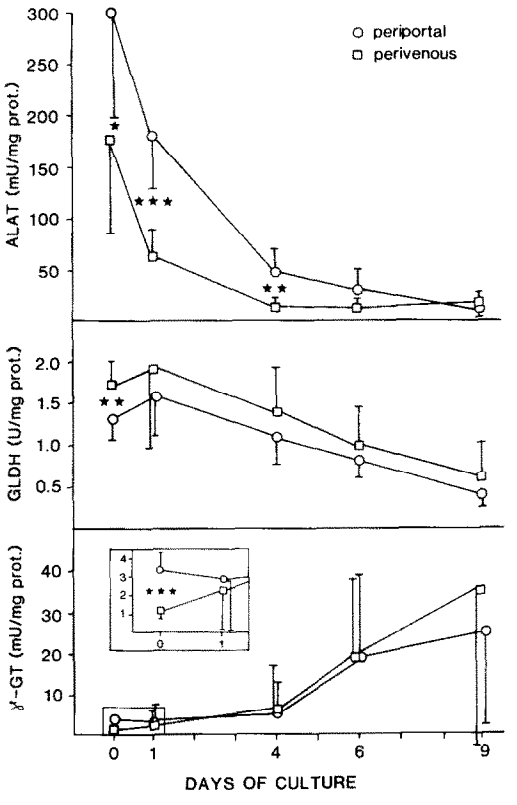

Fig. 1. Changes in marker enzyme activities during culture of periportal and perivenous hepatocytes. Samples were harvested on the indicated days, stored at −70° and assayed as described in Materials and Methods. Means ± SD of 6–9 experiments are given.

RESULTS

Marker enzymes

The average yield of cells, with a viability exceeding 90% (eosin exclusion), was 1.0 g and 1.6 g, for perivenous and periportal preparations, respectively. The pp:pv ratios of the periportal marker enzymes ALAT and γ-GT were 1.61 and 2.83, respectively, and the corresponding ratio for the perivenous marker, GLDH, was 0.76.

The ALAT activity remained significantly higher in pp cells for at least 4 days, but there was a gradual decrease in both cell types, so that the activity after 9 days was only 10% of the original (Fig. 1). GLDH was initially more stable, but the activity also slowly declined after the first two days. The pp–pv difference remained statistically significant during the whole culture time ($P < 0.05$, $F(1,4) = 10.3$, ANOVA). In contrast, the initial difference in γ-GT was lost during the first day, due to its rapid increase in pv cells. Subsequently, the γ-GT activity increased in both cell types.

7-Ethoxycoumarin O-deethylase. The activity of ECDE in freshly isolated pv cells was more than twice that in pp cells (18.3 ± 5.3 vs 8.3 ± 2.3 nmol/min/mg DNA, $P < 0.001$). During culture the activity declined (Fig. 2), but more rapidly in pv cells, so that after a week the pp–pv difference had

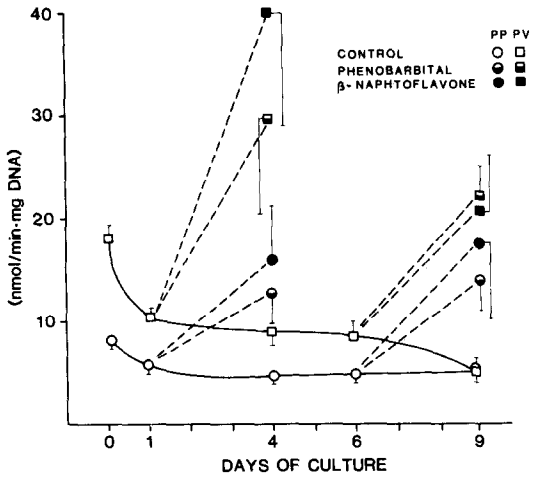

Fig. 2. 7-Ethoxycoumarin *O*-deethylase activity in cultured periportal and perivenous hepatocytes. Phenobarbital or β-naphthoflavone was present on days 1–4 or 6–9 (dashed lines). Samples were stored at −70° and assayed for 7-ethoxycoumarin *O*-deethylase activity and DNA as described in Materials and Methods. Means ± SE of 4–9 experiments are given.

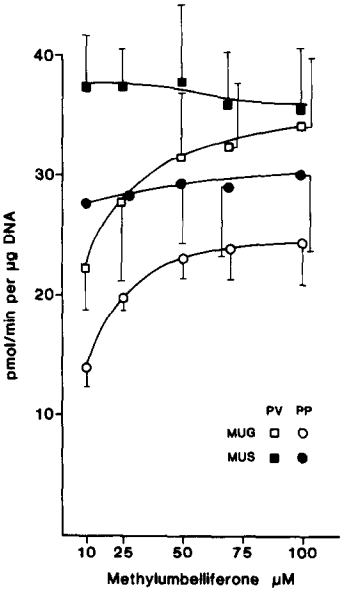

Fig. 3. Glucuronidation and sulfation of methylumbelliferone in pp and pv cells. The concentration of methylumbelliferone was varied between 10–100 μM. Means ± SD of six preparations.

disappeared. The response of the pp and pv cell preparations to *in vitro* induction was compared both during the early phase (day 1–day 4) and during days 6–9. By day 4 βNF had caused a 4-fold increase in pv cells and a 3.2-fold increase in pp cells. The induction by PB was somewhat less, 2.8- and 2.2-fold, respectively. Thus induction by both βNF and PB increased the pp–pv difference. On day 9 there was no difference in either the basal or the PB- and βNF-induced activities between the two cell types.

Conjugation of methylumbelliferone by whole cells. The conjugation of MU to MUG and MUS as a function of substrate concentration was first studied in freshly isolated pp and pv cells (Fig. 3). At all substrate concentrations both glucuronidation and sulfation of MU (expressed per cell DNA) were higher in pv cells, but the MUS/MUG conjugation ratio was slightly higher in pp cells. During culture the cell types exhibited similar changes in their conjugation capacity. During the first 24 hr of culture the sulfation activity rapidly declined whereas glucuronidation of MU remained at the initial level or even increased during one week of culture (results not shown).

UDP-glucuronyltransferase (UDPGT). No significant difference in UDPGT activity between freshly isolated pp and pv cells was found with either *p*NP or HBP as substrate (Table 1). However, after 4 days of culture the basal activity with both substrates was somewhat higher in pv cells. Induction by either βNF or PB was modest (1.3–2.1 fold) in both cell types. Again, induced activities with both substrates was somewhat higher in pv cells. During prolonged culture the differences in both the basal and the induced activities between the cell types vanished (results not shown).

Glutathione S-transferase (GST). The perivenous cell preparations exhibited significantly higher GST activity with CDNB as substrate as compared to pp cells (Table 2). During culture the GST activity remained fairly stable in both cell types, so that the pv > pp difference remained for six days. Induction during culture by either βNF or PB was modest and similar in both cell preparations. Consequently, induced activities were higher in pv than in pp cells.

DISCUSSION

Although the heterogeneous acinar expression of enzymes involved in activation and detoxication of xenobiotics has been established, the mechanisms underlying this heterogeneity are unclear. The present study demonstrates that these questions can be approached by culturing hepatocyte populations isolated from distinct acinar regions. The regioselectivity obtained by isolating cells from the periportal or perivenous region with digitonin–collagenase perfusion has been discussed previously [17, 18, 28]. The pp : pv ratios of the marker enzymes in this study are similar to those obtained previously, demonstrating reasonably good separation. This and previous studies [29, 30] also show that these cells attach to the substratum and survive equally well as conventionally isolated hepatocytes. However, during culture, the cells slowly dedifferentiate, as indicated from the pronounced decreases in ALAT and GLDH activities and from the expression of the γ-GT activity. Similar changes occur in conventional hepatocytes grown under similar conditions [31, 32, 33]. Both cell types behaved in a similar manner, indicating that there is no difference in the rate of dedifferentiation between periportal and perivenous hepatocytes.

A major limitation adherent to hepatocyte cultures

Table 1. UDP-glucuronyltransferase activity in freshly isolated and in cultured (4 days) pp and pv cells

Days of culture	Substrate	pp Cells			pv Cells		
		—	βNF	PB	—	βNF	PB
0	pNP	671 ± 149			685 ± 160		
	HBP	521 ± 53			590 ± 88		
4	pNP	566 ± 141	1170 ± 323† (2.1 ± 0.6)	841 ± 223* (1.6 ± 0.6)	762 ± 208	1253 ± 410* (1.7 ± 0.6)	1095 ± 180* (1.6 ± 0.6)
	HBP	342 ± 77	490 ± 84† (1.3 ± 0.2)	636 ± 298‡ (1.8 ± 0.6)	471 ± 188	630 ± 356 (1.3 ± 0.2)	800 ± 413 (1.9 ± 0.7)

The activities have been measured in the presence of 0.01–0.02% Brij. 58. The values are expressed as pmol/min mg DNA and are the means ± SD of six separate cultures. βNF and PB are inducers and the induction factor is given in parentheses.

*, † and ‡ $P < 0.05$, 0.01 and 0.001, respectively for difference between corresponding uninduced and induced activities.

Table 2. Glutathione *S*-transferase activity in freshly isolated and cultured pp and pv cells

Days of culture	pp Cells			pv Cells		
	—	βNF	PB	—	βNF	PB
0	24.7 ± 8.7			40.7 ± 7.8†		
1	23.4 ± 13.6			31.6 ± 12.1		
4	17.7 ± 2.2	21.4 ± 4.3	25.2 ± 12.1	30.6 ± 7.8†	33.0 ± 14.3	38.9 ± 12.3
6	18.2 ± 4.7			30.5 ± 12.8*		

The values are expressed as pmol/min mg DNA and are the means ± SD of six separate cultures. βNF and PB are inducers.

* $P < 0.05$ and † $P < 0.01$ for pp–pv difference.

has been the gradual loss of the cytochrome P-450 mediated mixed function oxidase activities [20, 21, 34, 35]. Although in the present experiments the composition of the medium was designed to minimize this loss, a relative decrease in the content of cytochrome P-450 nevertheless was indicated by the decline in the ECDE activity during culture. In this study the activity of ECDE was taken to represent P-450-associated activities. The initially higher pv activity is in good agreement with the generally accepted view that most P-450-mediated processes are more active in the perivenous region [6, 7, 11, 12, 36]. They also agree with perfusion studies on the *O*-deethylation of 7-ethoxycoumarin [37] and with immunohistochemical data [5]. Induction of cytochrome P-450 is generally reported to occur preferentially in the perivenous region [7, 11, 12, 38], but there are also reports on greater induction by both 3MC (3-methylcholanthrene) and PB-type inducers in the pp region [5, 39, 40]. These seemingly conflicting results may partly result from the P-450 isozyme organ distribution and inducer specificity. Cytochrome P-450 activity with ECDE as substrate is induced *in vitro* by both PB and MC, although more so by the latter. The PB-inducible form has been shown to be labile during culture [41]. In the present study both βNF and PB caused more induction in the pv cells. This suggests that the greater induction in pv cells cannot be due to different rates of isozyme loss during culture.

Compared to the MFO activities the zonation of conjugation reactions has received less attention. Our data on freshly isolated cells indicate that glutathione conjugation, glucuronidation and sulfation all are more active in the perivenous region. The pv dominance of GSH-*S*-transferase is in agreement with our previous data on total activity in isolated cells [28, 39] and with immunohistochemical data on several isozymes [5]. It is notable that compared to the perivenous dominance of GSH conjugation, the GSH peroxidase activity is higher in periportal cells [28].

In freshly isolated pv cells both the glucuronidation and sulfation of methylumbelliferone was found to be more efficient than in pp cells. Sulfation capacity was maximal, and clearly exceeded glucuronidation at the lowest substrate concentration tested (10 μM). Glucuronidation was faster at higher concentrations of MU (50–100 μM). These effects were similar in pp and pv cells. The finding of similar sulfate to glucuronide conjugation ratio in pp and pv cells is contrary to a study by Conway *et al.* [42], who found sulfation at low substrate concentration to dominate over glucuronidation in the pp region but not in the pv region. A study by Pang and Terrell comparing acetaminophen sulfation during ante- and retrograde liver perfusion also suggested sulfation to be more active in the periportal region [13]. Araya *et al.* made a similar observation based on cell populations separated according to density with Percoll [16] but

the cells assumed to represent pp cells also exhibited higher rates of glucuronidation of acetaminophen and *p*-nitrophenol. Future studies will clarify to what extent the substrate and the experimental model influences the results on the acinar distribution of sulfation.

The rate of glucuronidation by intact cells is not directly comparable to UDPGT activities assayed from detergent-treated cell homogenates. Although in the present study the UDPGT activities in pv cell homogenates were not significantly higher than in pp cells, several previous studies indicate that the amount of specific immunoreactive isozymes [43, 44] is higher in the pv region. Thus both the 3MC-inducible isozyme [43] and the *p*NP conjugating isozyme [44] stained more intensely in the zone III region. Other isozymes may, however, be more evenly distributed in the liver lobule [44], thus explaining why both a previous [39] and the present study based on total UDPGT activity indicate only a slight pv dominance. Different isozymes also are induced to a different extent by various inducers. Thus in one study [43] 3MC caused a pv induction, while after PB treatment staining was more intense in the pp region. In the present study *in vitro* induction by either βNF or PB was modest and there was no clear response difference between pp and pv cells. It is possible that both hepatocyte types undergo adaptive changes in membrane composition that could attenuate activation of UDPGT.

These data suggest that hepatocytes from the perivenous region or zone III have a greater capacity for both oxidation and conjugation of xenobiotics as compared to periportal cells. The persistence of these differences for several days under identical culture conditions demonstrates that the acinar heterogeneity prevailing *in vivo* is little influenced by acute changes in acinar substrate, hormone or oxygen availability. The cultured perivenous cells also seemed to maintain a stronger response to phenobarbital or β-naphthoflavone induction of ethoxycoumarin oxidation as compared to periportal cells. While the present data do not delineate the basis for these acinar differences, they suggest the usefulness of culturing hepatocyte populations with initially different activities of phase I and phase II enzymes to investigate basic mechanisms governing the expression of drug metabolizing enzymes.

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