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Zone-specific inducibility of cytochrome *P*450 2B1/2 is retained in isolated perivenous hepatocytes

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The expression and induction of the cytochrome *P*450 2B1/2 isoenzyme is heterogeneous, exhibiting a regional pattern in the intact liver and a varied response to phenobarbital in isolated cultured hepatocytes. We report that *P*450 2B1/2 immunostaining of hepatocytes isolated from the perivenous liver region and cultured in the presence of phenobarbital is much stronger than that of cells identically treated but isolated from the periportal region. *P*450 2B1 mRNA, quantified by a sensitive and specific RNAase protection assay, is also preferentially induced in perivenous hepatocytes, demonstrating that the difference in induced expression is at the pretranslational level. Our results suggest that perivenous and periportal hepatocytes are differentially imprinted to retain regiospecific factors governing their inducibility after isolation.

INTRODUCTION

There is accumulating evidence that the level of expression of many proteins in hepatocytes differs depending on their location within the liver acinus, the functional unit of the liver [1,2]. Members of the cytochrome *P*450 gene superfamily appear to be particularly unevenly expressed, and are usually constitutively distributed and induced preferentially in the perivenous (zone 3) region [3–9]. The mechanisms underlying these regional differences in constitutive and induced *P*450 gene expression are currently unknown. The unidirectional perfusion of cords of hepatocytes in the acinus leads to gradients of oxygen, nutrient and hormone concentrations along the sinusoid. It has been suggested that these gradients create differences in hepatocyte phenotype within the acinus [10]. An alternative hypothesis is that hepatocytes acquire different phenotypes in different regions of the liver acinus as a consequence of liver maturation.

The cytochromes *P*450 2B1 and 2B2 are characterized by their high inducibility by phenobarbital and related compounds [11]. These closely related proteins are preferentially distributed and induced in the perivenous region of the acinus [4,9,12–14]. Thus it is intriguing that, when hepatocytes isolated from the whole liver have been exposed to phenobarbital, a heterogeneous *P*450 2B induction response among the cells has been described in both transplanted [15] and cultured [13] hepatocytes. However, the acinar origin of the variously responding cells has not been investigated. In this study we present evidence that induction-responsive cells originate from the perivenous region, by demonstrating that their high inducibility *in vivo* is retained after isolation and during culture.

MATERIALS AND METHODS

Male rats of the Alko mixed strain weighing 150–200 g and fed on a standard laboratory diet (Astra-Ewos Ab, Södertälje, Sweden) were used. Animals were anaesthetized with pentobarbital and the liver perfused *in situ*. Hepatocytes were isolated from either the periportal (zone 1) or the perivenous (zone 3) region of the liver acinus by digitonin/collagenase perfusion as described previously [16,17]. Briefly, hepatocytes from the undesired region were lysed by infusion of digitonin, and intact cells from the unaffected zone were isolated by conventional collagenase perfusion. The marker enzymes alanine aminotransferase (EC 2.6.1.2) and glutamine synthetase (EC 6.3.1.2) were analysed as in [18].

Hepatocytes were plated as a monolayer at a density of 1.4×10^5 cells/cm^2 on plastic dishes (Nunc, Roskilde, Denmark) using minimal essential medium (Gibco, Paisley, Scotland) supplemented with nystatin (1 μg/ml), gentamicin (50 μg/ml), insulin (80 ng/ml), glucagon (1.8 ng/ml) and dexamethasone (0.1 μM), as in [19]. Fetal calf serum (5%) and newborn calf serum (5%) were always present during the first 4 h after plating. The medium was changed 4 and 28 h after plating. Phenobarbital, dissolved in culture medium, was added 4 h after plating and when the medium was changed. For immunocytochemistry, cells were fixed in methanol, and for Western immunoblotting, hepatocyte sonicates were separated by SDS/PAGE and transferred electrophoretically to a nitrocellulose filter [13]. *P*450 2B1/2 was detected by staining with a polyclonal antibody recognizing *P*450 2B1/2, as described [13].

The cDNAs for *P*450 2B1 (pB7) [20] and 2B2 (pR17) [21] used for the RNAase protection assay were generously provided by Dr. A. Anderson, Université de Laval, Quebec, Canada, and Dr. M. Adesnik, New York University, New York, NY, U.S.A., respectively. Plasmid DNA was prepared by an alkaline lysis miniprep. Crude oligonucleotides were used for PCR (polymerase chain reaction) on the plasmid DNAs: TAATACGACTCACTAT*AGATCGATCAGGTGATCGGCTCACAC* (T7 oligo) and AATTAGGTGACACTATAGAA*TCTTGGGAAGCAGGTACCCTCGGA* (SP6 oligo). Italicized bases denote the CYP2B1/2 sequence from 967 to 1138 bp. The PCR cycle was 94 °C for 1 min, 60 °C for 2 min and 72 °C for 1 min, and 15 cycles were performed. Both products produce a fragment of 208 bp. The PCR product was treated with proteinase K/SDS at 65 °C for 15 min; then it was phenol/chloroform (1:1, v/v)-extracted, ethanol-precipitated and resuspended in water. RNA was synthesized essentially as described [7,22]; both T7 and SP6 polymerase produced full-length transcripts. The RNA probes were incubated with 30 μg of RNA throughout the RNAase protection procedure as described [7,22], with the exception that

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hybridization was at 45 °C. Autoradiographs were quantified with laser densitometry of exposures in the linear region of the film response.

RNA was isolated from hepatocytes by the guanidium thiocyanate/LiCl method as previously described [7], and RNA samples were normalized by hybridization in triplicate to oligo-$(dT)_{18}$ [23].

RESULTS

Separation of periportal and perivenous hepatocytes

As described in detail earlier [16,18], a high yield of intact hepatocytes originating mainly from the periportal or the perivenous region is obtained with the digitonin/collagenase perfusion technique. In this study the periportal/perivenous activity ratios of alanine aminotransferase (a periportal marker) and glutamine synthetase (a perivenous marker) were 2.8 ± 0.9 and 0.013 ± 0.007 respectively (means $\pm$ S.D., $n = 7$), demonstrating the different acinar origins of the cell preparations.

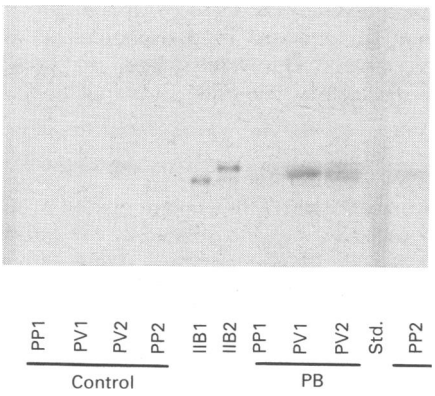

Fig. 1. Western blot analysis of *P*450 2B1/2 in periportal and perivenous hepatocytes exposed to phenobarbital

Isolated periportal and perivenous hepatocyte populations were cultured for 48 h and exposed (PB) or not (control) to 0.2 mM-phenobarbital. Cell sonicates (50 μg of total cell protein) were subjected to Western blot analysis using an anti-(*P*450 2B1/2) polyclonal antibody. Results comparing two periportal (PP1/2) and two perivenous (PV1/2) cell preparations are shown. IIB1 and IIB2 are purified *P*450 2B1 and 2B2 proteins (0.8 pmol/lane). 'Std.' contains Rainbow Molecular Weight Markers (10 μl).

Western blotting and immunocytochemistry

In hepatocytes isolated from either the periportal or the perivenous region and cultured for 48 h under the conditions described, *P*450 2B1/2 was hardly detectable in the absence of phenobarbital (Fig. 1). In the presence of either 0.2 mM-phenobarbital (Fig. 1) or 2 mM-phenobarbital (results not shown) the response of periportal hepatocytes varied, with some preparations showing almost no sign of induction, whereas in others a weak or intermediate signal was detected. In contrast, preparations of perivenous hepatocytes invariably exhibited a clear induction in response to phenobarbital. The intensity of the band was stronger after exposure to 0.2 mM- than to 2 mM-phenobarbital. This was probably due to slight cytotoxicity caused by the 2 mM-phenobarbital, as suggested from microscopic examination of the cells.

The difference in responsiveness between the hepatocyte subpopulations was studied by immunocytochemistry. In the absence of phenobarbital, no *P*450-2B1/2-positive cells could be detected after 48 h in either population (Fig. 2). In the presence of phenobarbital, about 5% of the periportal hepatocytes were stained for *P*450 2B1/2. In contrast, 30–70% of the perivenous hepatocytes showed positive staining after exposure to either 0.2 or 2 mM-phenobarbital. Interestingly, the induction response varied, with some hepatocytes staining more strongly than others. The difference in induction response was independent of the presence or the absence of serum.

Induction of *P*450 2B1 mRNA *in vitro*

An RNAase protection assay was evaluated for discrimination between *P*450 2B1 and *P*450 2B2 transcripts. The hypervariable region of the *P*450 2B family coding region, from 967 to 1138 bp, has 13 mismatches between 2B1 and 2B2 out of 172 bases, or

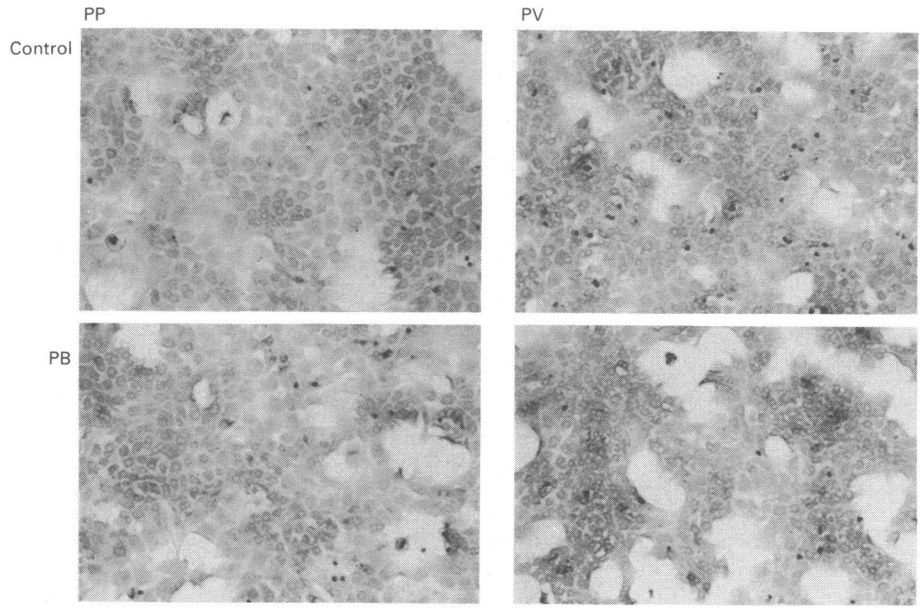

Fig. 2. Comparison of hepatocyte *P*450 2B1/2 immunostaining after phenobarbital induction of cultured periportal and perivenous hepatocytes

Periportal (PP) and perivenous (PV) hepatocytes were cultured for 48 h in the absence (control) or in the presence (PB) of 0.2 mM-phenobarbital.

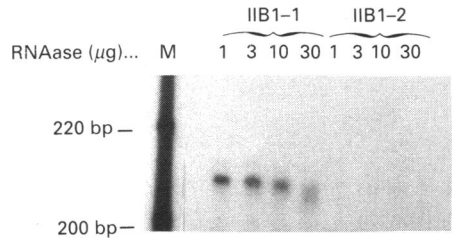

Fig. 3. Specificity of the RNAase protection assay for *P*450 2B1 mRNA

SP6 (antisense) transcripts from the 2B1 PCR template were hybridized with T7 (sense) transcripts from the 2B1 PCR template (IIB1-1) or the 2B2 PCR template (IIB1-2) and digested with 1, 3, 10 or 30 μg of RNAase A. Markers (M) are 1 kb (BRL).

7.5% divergence [20,21]. This region of the 2B1 and 2B2 cDNAs was inserted downstream of the T7 and SP6 polymerase promoters by means of PCR, and the PCR product was used for the production of sense and antisense probes. As shown in Fig. 3, *P*450 2B1 antisense transcripts protect *P*450 2B1 sense transcripts over the range 1–10 μg of RNAase A, but fail to protect 2B2 transcripts over the same range of RNAase concentrations. Thus the RNAase protection assay discriminates between *P*450 2B1 and *P*450 2B2 mRNAs.

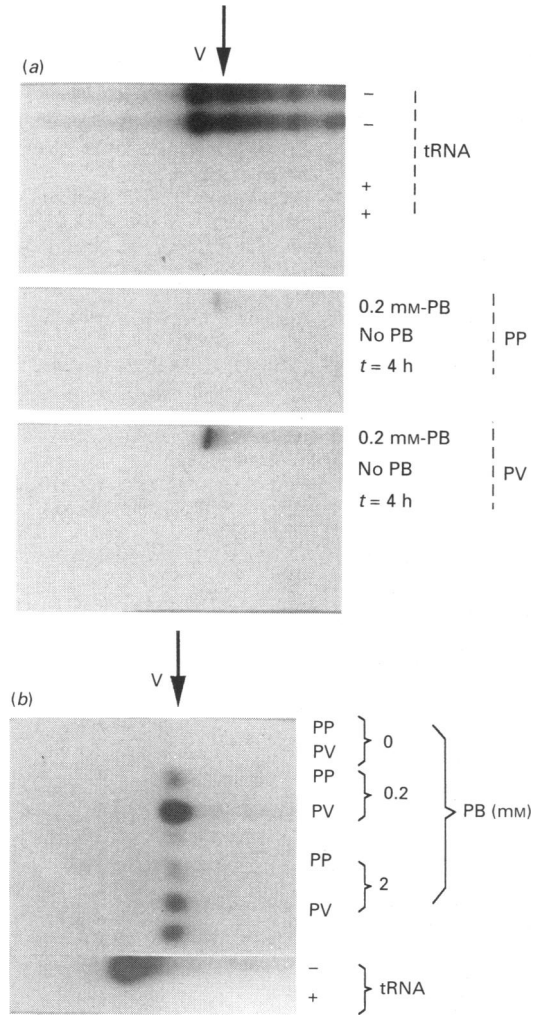

Fig. 4. Induction of *P*450 2B1 mRNAs by phenobarbital in periportal and perivenous hepatocytes

Perivenous (PV) and periportal (PP) hepatocytes were cultured in the presence (*a*) or absence (*b*) of serum. RNA was isolated at 4 h after attachment ($t = 4$ *h*, *a*) and exposure for 48 h to 0 or 0.2 mM-(*a*) and 0, 0.2 or 2 mM-phenobarbital (*b*). A 30 μg sample of RNA was analysed by a RNAase protection assay. The protected fragment is indicated by an arrow, and the higher molecular mass undigested probe is indicated by V. A RNA probe incubated with yeast tRNA with (+) or without (−) RNAase treatment is shown.

The amounts of *P*450 2B1 mRNA in hepatocytes 4 h after collagenase perfusion, or in hepatocytes cultured in the presence of serum for 48 h, were very low, but detectable on extended autoradiography (results not shown). In contrast, considerable amounts of 2B1 mRNA were detected in cells cultured in the presence of 0.2 mM-phenobarbital, and the signal was much more marked in perivenous hepatocytes than in periportal cells (Fig. 4*a*). Quantification by laser densitometry demonstrated that induction of 2B1 mRNA in cultures of perivenous hepatocytes was typically 200-fold.

Hepatocytes exposed to 0.2 mM-phenobarbital for 48 h in the absence of serum also exhibited a dramatic induction of *P*450 2B1 mRNA (Fig. 4*b*). In this situation also the induction was more marked in perivenous compared with periportal hepatocytes. The greater induction by phenobarbital of *P*450 2B1 mRNA in perivenous compared with periportal hepatocytes occurs both in the presence and in the absence of serum in the culture medium.

DISCUSSION

The present study demonstrates that hepatocytes isolated from the perivenous region respond to phenobarbital exposure *in vitro* by a much stronger induction of cytochrome *P*450 2E than in correspondingly treated periportal cells. This is in line with a previous study in which mixed-function oxidases were induced to a greater extent in perivenous than in periportal hepatocytes [19]. A heterogeneous response among hepatocytes to phenobarbital induction has been reported both in cells transplanted into the spleen of syngenic rats [15] and in primary culture [13]. The present data strongly suggest that the heterogeneous response was associated with the acinar origin of the cells, with the more responsive hepatocytes being from the perivenous region. This finding suggests that intrinsic cellular factors governing inducibility of individual hepatocytes remain functional after cell isolation.

A heterogeneous zonated response after induction of several different cytochrome *P*450 isoenzymes *in vivo* has been observed repeatedly [4,7,9,12,13,15,24]. Retention of this heterogeneous response in isolated hepatocytes demonstrates that the zonated response is indeed independent of the acinar organization, as has been previously suggested [15,19,25]. Several previous studies *in vivo* suggested that the zonation of inducibility of a particular *P*450 form is independent of the inducer [9,13,15], and is rather a consequence of the acinar location of the hepatocytes, with cells in zone 3 exhibiting the highest inducibility. The present studies provide a model system for examining the specificity of inducers on various *P*450 isoenzymes.

Although a decline in the expression of *P*450 genes under most culture conditions is perceived as a restriction to studies of *P*450 gene expression *in vitro*, induction of *P*450 genes during short-term primary culture has repeatedly been demonstrated [13,19,26–28]. The present results show that the phenobarbital-induced increase in the *P*450 2B apoprotein is associated with a corresponding increase in the level of mRNA for *P*450 2B1. This suggests that phenobarbital-responsive elements regulating *CYP-2B1* gene expression remain active in cells cultured under these conditions for at least 48 h. The dramatic induction of *P*450 2B1 mRNA seen in perivenous hepatocytes cultured in the presence or in the absence of serum (Fig. 4) compares well with previous studies of induction *in vitro* [26,27] and *in vivo* [11], and reaffirms

the validity of primary cultures of hepatocytes for studying the mechanism of phenobarbital induction. However, while Waxman *et al.* [27] reported that induction by phenobarbital of *P*450 2B proteins was extinguished by serum, we observed induction of *P*450 2B apoprotein (Fig. 1) and of *P*450 2B1 mRNA (Fig. 4) in both the presence and the absence of serum. This discrepancy could be explained by differences in the culture conditions.

In situ hybridization studies have demonstrated a regiospecific pattern of *CYP2B* mRNA expression similar to that of the apoprotein, with the strongest staining occurring around the terminal central veins [14]. Our RNAase protection data confirm the increased sensitivity of perivenous cells to phenobarbital, and suggest that elements responsible for the higher transcriptional activation in the perivenous cells are retained during culture. Such elements could involve inducer-responsive receptor mechanisms or zonation of humoral factors, such as growth hormone and pituitary hormones, which have been shown to influence the expression of *P*450 isoenzymes. Alternatively, liver-specific regulatory proteins acting upstream of the promoter region could act in concert, via a zonated expression, on groups of related genes. Indeed, apparently constitutive and induced co-expression of several *P*450 isoenzymes in the same centrilobular layer of hepatocytes was recently demonstrated [9].

In conclusion, we demonstrate here that elements involved in the activation of *CYP2B1/2* genes show different levels of expression in hepatocytes from different parts of the liver acinus, and that this difference persists *in vitro*. Further studies *in vitro* of hepatocyte populations isolated from different regions of the acinus should help to clarify the nature of these regulatory elements. Since many hepatotoxins elicit a regiospecific pattern of damage, such work may help in elucidating mechanisms of damage involving a regional imbalance in constitutive and induced expression of enzymes responsible for the metabolic activation and detoxification of such substances.

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