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Mimicry in Primary Rat Hepatocyte Cultures of the In Vivo Perivenous Induction by Phenobarbital of Cytochrome P-450 2B1 mRNA: Role of Epidermal Growth Factor and Perivenous Oxygen Tension

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ABSTRACT

Treatment of male rats with phenobarbital (PB) results in a perivenous and mid-zonal pattern of cytochrome P-450 (CYP)2B1 mRNA expression within the liver acinus. The mechanism of this zonated induction is still poorly understood. In this study sinusoidal gradients of oxygen and epidermal growth factor (EGF) besides those of the pituitary-dependent hormones growth hormone (GH), thyroxine (T4), and triiodothyronine (T3) were considered to be possible determinants for the zonated induction of the *CYP2B1* gene in liver. Moreover, heme proteins seem to play a key role in oxygen sensing. Therefore, the influence of arterial (16% O_2) and venous (8% O_2) oxygen tension (pO_2), and of the heme synthesis inhibitors $CoCl_2$ and desferrioxamine (DSF) on PB-dependent CYP2B1 mRNA induction as well as the repression by EGF and, for comparison, by GH, T4, and T3, of the induction under arterial and venous pO_2 were investigated in primary rat hepatocytes. Within 3 days, phenobarbital induced CYP2B1 mRNA to maximal levels under arterial pO_2 and to about 40% of maximal levels under venous pO_2. $CoCl_2$ annihilated induction by PB under both oxygen tensions, whereas desferrioxamine and heme abolished the positive modulation by O_2, suggesting that heme is a necessary component for O_2 sensing. EGF suppressed CYP2B1 mRNA induction by PB only under arterial but not under venous pO_2, whereas GH, T4, and T3 inhibited induction under both arterial and venous pO_2. Thus, in hepatocyte cultures, an O_2 gradient in conjunction with EGF mimicked the perivenous induction by PB of the *CYP2B1* gene observed in the liver in vivo.

Hepatocytes from the periportal and perivenous zones of the liver parenchyma exhibit different metabolic capacities due to differences in their content of key enzymes (Jungermann and Katz, 1989; Gebhardt, 1992; Jungermann and Kietzmann, 1996; Lindros, 1997). The expression of several cytochrome P-450 (CYP) enzymes involved in the metabolism of xenobiotics such as the dioxin-inducible CYP1A1/2, the phenobarbital (PB)-inducible CYP2B1/2, and the ethanol-inducible CYP2E1 is greater in the perivenous area. Therefore, CYP-dependent conversion of xenobiotics, part of which are metabolized to hepatotoxic or carcinogenic compounds, occurs predominantly in the perivenous zone (Jungermann and Katz, 1989; Jungermann and Kietzmann, 1996; Oinonen and Lindros, 1998).

Members of the CYP2B subfamily are involved in the biotransformation of an array of xenobiotics. Metabolic activation of several procarcinogens is mediated by CYP2B isoforms, e.g., conversion of aflatoxin B1 to the ultimate mutagen aflatoxin B1–2,3-oxide by rat CYP2B1 and its human ortholog CYP2B6 or *N*-hydroxylation of acetylaminofluorene by rat CYP2B1 (Soucek and Gut, 1992). CYP2B1 is barely detectable in normal rat liver, yet it is markedly induced by treatment of animals with PB (Waxman and Azaroff, 1992). In rats injected with PB, a predominant perivenous and mid-zonal pattern of CYP2B mRNA (Hassett et al., 1989; Waxman and Azaroff, 1992) and CYP2B protein (Baron et al., 1978; 1981) expression was observed, whereas hepatocytes within the proximal periportal area appeared refractory to induction by PB.

Sinusoidal gradients in oxygen, substrates, and hormones as well as gradients in cell and tissue structures (hormone receptors, extracellular matrix) are thought to contribute to zonated gene expression (Jungermann and Katz, 1989; Gebhardt, 1992; Jungermann and Kietzmann, 1996). Heme proteins appear to play a key role in oxygen sensing (Bunn and Poyton, 1996).

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ABBREVIATIONS: CYP, cytochrome P-450; DSF, desferrioxamine; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; GH, growth hormone; HO-1, heme oxygenase 1; PB, phenobarbital; pO_2, oxygen tension; T3, triiodothyronine; T4, thyroxine.

Inducibility of CYP2B1 mRNA by PB was found to be retained in hepatocytes cultured with serum-free media (Waxman and Azaroff, 1992). Pituitary-dependent or -derived hormones such as triiodothyronine (T3) and thyroxine (T4) or growth hormone (GH) and the hepatotrophic epidermal growth factor (EGF) have been shown to repress PB-dependent CYP2B1 mRNA induction (Schuetz et al., 1990; Murayama et al., 1991; Aubrecht et al., 1995). Yet the mechanism of the perivenous induction by PB of CYP2B1 as well as expression of CYP1A1/2, CYP2E1, or CYP3A1/2 is still poorly understood (Lindros, 1997). In vivo experiments with hypophysectomized rats treated with pituitary-dependent hormones have shown that GH and T3 appear to suppress the expression of some CYP forms strongly, e.g., 2B1/2 and 3A1/2, and other forms moderately, e.g., 1A2 and 2E1. If periportal > perivenous hormone gradients were established during passage of the blood through the liver, as may be the case for GH but not for T3, the inhibitory hormone action would be predominant in the periportal zone, which could explain at least in part the mainly perivenous induction of most CYP forms (Oinonen et al., 1996; Lindros, 1997). The possible role of EGF in conjunction with oxygen in the zonated induction of CYP isoenzymes has so far not been investigated.

Therefore, it was the goal of the present study to examine the role of oxygen and the growth factor EGF in comparison to GH, T3, and T4 in the regulation of the zonated *CYP2B1* gene induction by PB in primary hepatocyte cultures. It was found that PB-dependent CYP2B1 induction was maximal under arterial pO_2 and only less than half-maximal under venous pO_2. Repression of induction by EGF was prevented under venous pO_2 whereas that by GH, T3, and T4 was not modulated by oxygen. Thus, in vitro, an oxygen gradient in conjunction with EGF mimicked the perivenous induction of CYP2B1 by PB observed in the liver in vivo.

Experimental Procedures

Materials. All chemicals were of reagent grade and purchased from commercial suppliers. Collagenase, T4-polynucleotide kinase, digoxigenin-UTP, the digoxigenin nucleic acid detection kit, T3-RNA polymerase, fetal calf serum, and murine EGF were obtained from Boehringer (Mannheim, Germany). Hormones were purchased from Serva (Heidelberg, Germany) with the exception of human recombinant GH, which was obtained from Bachem (Heidelberg, Germany). Hybond-N nylon membrane was supplied by Amersham (Braunschweig, Germany) and [γ-^{32}P]ATP was obtained from DuPont-NEN (Bad Homburg, Germany). The polyclonal antihuman EGF receptor (EGF-R) antibody was obtained from Upstate Biotechnology (Lake Placid, NY).

Hepatocyte Culture and Induction Experiments. Primary hepatocytes were isolated from adult male Wistar rats by collagenase perfusion and plated with a density of 3×10^6 onto 94-mm culture dishes in M199 medium supplemented with 1 nM insulin, 100 nM dexamethasone, and 4% fetal calf serum (Kietzmann et al., 1995). After 4 h cells were cultured in serum-free medium containing 1 nM insulin and 100 nM dexamethasone either with or without 0.75 mM PB for up to 3 days under arterial (16% O_2/79% N_2/5% CO_2) and venous (8% O_2/87% N_2/5% CO_2) pO_2 with daily changes of media. The O_2 values take into account the O_2 diffusion gradient from the media surface to the cells (Nauck et al., 1981). Cell viability and energy content was shown to be the same under long-term influence of arterial and venous pO_2 (Nauck et al., 1981; Wölfle and Jungermann, 1985). Hepatocytes cultured under arterial pO_2 resembled periportal hepatocytes whereas cells cultured under venous pO_2 resembled the perivenous hepatocytes (Wölfle and Jungermann, 1985). Treatment of hepatocytes for 3 days with 50 μM $CoCl_2$ (Kietzmann et al., 1992) or 130 μM desferrioxamine (DSF; Kietzmann et al., 1998) did not impair cell viability within 24 h; to ensure viability for 3 days in the presence of $CoCl_2$ and DSF the concentrations were reduced to 25 and 80 μM, respectively. Heme/BSA was prepared as described (Bissell and Guzelian, 1980) and applied to the cells in a concentration of 10 μM for 3 days. With these concentrations, cell viability controlled by trypan blue exclusion and light microscopy was the same between untreated and treated cells. When indicated, cells were additionally incubated with inhibitors of CYP2B1 mRNA induction: murine (0.16 or 1.6 nM) EGF, 10 μM T3 or T4, respectively, or 2.4 to 12 nM GH. In the presence of EGF cell density did not increase under either of the O_2 tensions.

RNA Preparation and Northern Blot Analysis. Total RNA was isolated from hepatocyte cultures by guanidinium thiocyanate-phenol extraction (Chomczynski and Sacchi, 1987), subjected to electrophoresis on formaldehyde-agarose gels, and subsequently transferred to Hybond-N nylon membranes as described previously (Aubrecht et al., 1993). RNA blots were hybridized to the oligonucleotide probe 5′-ggttggtagccggtgtga-3′ specific for the rat *CYP2B1* gene (bases 49–66 of exon 7 region; Genebank L00318), which had been 5′end-labeled by T4-polynucleotide kinase utilizing [γ-^{32}P]ATP (Omiecinski et al., 1985). RNA expression was quantified by a phosphorimaging system (Raytest, Straubenhardt, Germany).

Control hybridizations were performed using β-actin and heme oxygenase 1 (HO-1) antisense RNA probes. The RNA probes, generated by in vitro transcription, were labeled by incorporation of digoxygenin-11-UTP (Boehringer, Mannheim, Germany) into the transcript. A 550-bp β-actin cDNA (HSA1007, 69-618) and a 800-bp HO-1 cDNA fragment, respectively, cloned into the PBS/Bluescript plasmid (Stratagene, Heidelberg, Germany), served as templates. Hybridizations were performed at 68°C with 40 ng probe/ml for 12 h.

Immunoblot Analysis. Plasma membrane fractions were isolated from hepatocytes by differential centrifugation according to Simpson et al. (1983), except that the buffers used for homogenization and centrifugation contained 1 mM phenylmethylsulfonyl fluoride. Membrane protein (20 μg) were subjected to electrophoresis through SDS-polyacrylamide gels (7.5%) and were then transferred to polyvinylidene difluoride membranes by semidry blotting using a continuous transfer buffer [48 mM Tris, pH 9.0, 39 mM glycine, 0.038% (w/v) SDS, and 15% (v/v) methanol]. Immunodetection of the EGF-R was performed by Western blot analysis using a primary sheep polyclonal antibody against the human EGF-R, which is cross-reactive with the rat EGF-R, at a dilution of 2 μg/ml. A secondary peroxidase-conjugated antibody against sheep IgG (Sigma, Munich, Germany) was used for visualization of immunoreactive protein in conjunction with the enhanced chemiluminescence kit (Amersham, Braunschweig, Germany).

Results

In the present study the induction of CYP2B1 mRNA expression by PB was studied in primary rat hepatocytes under arterial and venous pO_2 in serum-free medium. The involvement of heme as a component of the O_2-sensing system was tested by inhibition of heme synthesis with $CoCl_2$ and DSF. Moreover, the repression by EGF or by the pituitary-dependent hormones GH, T4, and T3 of PB-dependent CYP2B1 mRNA induction was investigated under arterial and venous pO_2. The oligonucleotide probe used (cf. *Experimental Procedures*) for hybridization of Northern blots enabled highly specific detection of CYP2B1 mRNA and ruled out cross-hybridization to the closely related CYP2B2 mRNA.

Positive Modulation by Arterial pO_2 of PB-Dependent CYP2B1 mRNA Induction. In primary rat hepato-

cyte cultures 0.75 mM PB elicited induction of CYP2B1 mRNA, which was linear with time but started with a lag of 1 day under arterial (16%) and of almost 2 days under venous (8%) pO_2 (Fig. 1). The level of CYP2B1 mRNA induction under arterial pO_2 after 3 days was set to 100%. Under venous pO_2, CYP2B1 mRNA induction reached only approximately 40%. Induction was defined as the difference between expression in the presence and absence of PB (Figs. 1 and 2). In the absence of PB, CYP2B1 mRNA was barely detectable.

To ensure specificity, control hybridizations with an HO-1 probe and a β-actin probe were performed. In contrast to the observed expression pattern of CYP2B1 mRNA, HO-1 mRNA was induced to maximal levels by venous pO_2. Furthermore, PB did not modulate HO-1 mRNA expression (Fig. 2). Levels of β-actin mRNA were not influenced by either PB or different O_2 tensions (Fig. 2). Thus, induction by PB of CYP2B1 mRNA in vitro was positively modulated by physiological oxygen tensions, so that higher levels of induction were reached under arterial pO_2.

Inhibition by $CoCl_2$ DSF, and Heme of PB-Dependent CYP2B1 mRNA Induction. To investigate the role of heme proteins in the modulation by oxygen of PB-dependent CYP2B1 mRNA induction, primary hepatocytes were cultured for 3 days under arterial or venous pO_2 with 25 μM $CoCl_2$ or 80 μM DSF, both inhibitors of heme biosynthesis. $CoCl_2$ almost completely repressed induction by PB of CYP2B1 mRNA under both oxygen tensions (Fig. 2). This was not an unspecific deleterious effect, because HO-1 mRNA was strongly induced by $CoCl_2$ to about 400% irrespective of the pO_2, and β-actin mRNA remained stably expressed (Fig. 2).

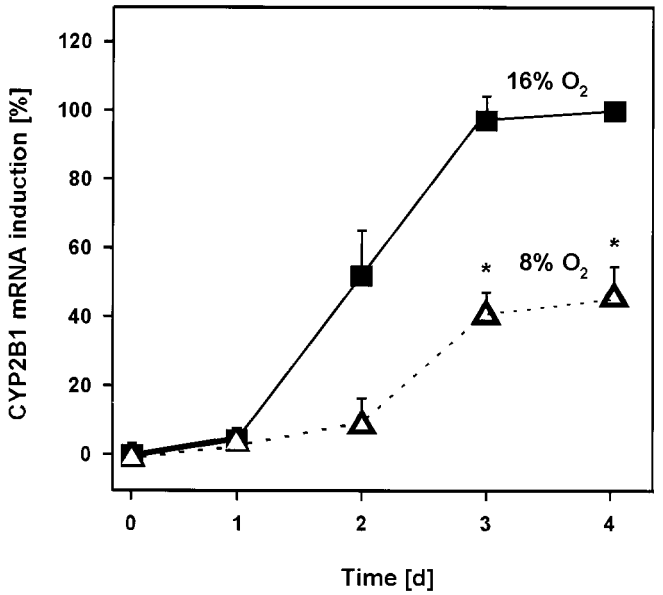

Fig. 1. Time course of CYP2B1 mRNA induction by PB under arterial and venous pO_2 in primary rat hepatocyte cultures. Hepatocytes were cultured under arterial (16%) or venous (8%) pO_2 in the presence of 0.75 mM PB for 1 to 3 days. RNA (total 20 μg) was used for Northern blot analysis. Hybridization signals were quantified by phosphorimaging analysis. Induction is defined as the difference between expression under PB and expression in absence of the inducer. Induction after 3 days under arterial pO_2 was set to 100%. Data represent mean values S.E.M. of three or seven (day 3) independent experiments. *significant difference between arterial and venous pO_2 ($p \leq .05$, Student's t test).

The iron chelator DSF reduced PB-dependent CYP2B1 mRNA expression under arterial pO_2 to about 70%, whereas it had no significant effect under venous pO_2. Apparently, modulation by oxygen of PB-dependent CYP2B1 mRNA induction was nearly lost in the presence of DSF. Induction of HO-1 mRNA by venous pO_2 was reduced by DSF to about 50%, whereas β-actin mRNA expression again remained unchanged in the presence of DSF (Fig. 2).

Because cobalt chloride and DSF were used to deplete the heme content in the cells, it might be possible to reverse the effects of DSF and $CoCl_2$ by exogenous heme. Therefore, hepatocytes were cultured for 3 days with combinations of PB + $CoCl_2$ + heme/BSA, PB + DSF + heme/BSA, or PB + heme/BSA. The presence of heme/BSA did not prevent the complete repression by $CoCl_2$ of the PB-dependent CYP2B1 mRNA induction under both oxygen tensions (Table 1). The combination of heme/BSA with PB and DSF resulted in a further decrease of the DSF-mediated reduction of the PB-dependent CYP2B1 mRNA induction; CYP2B1 mRNA was

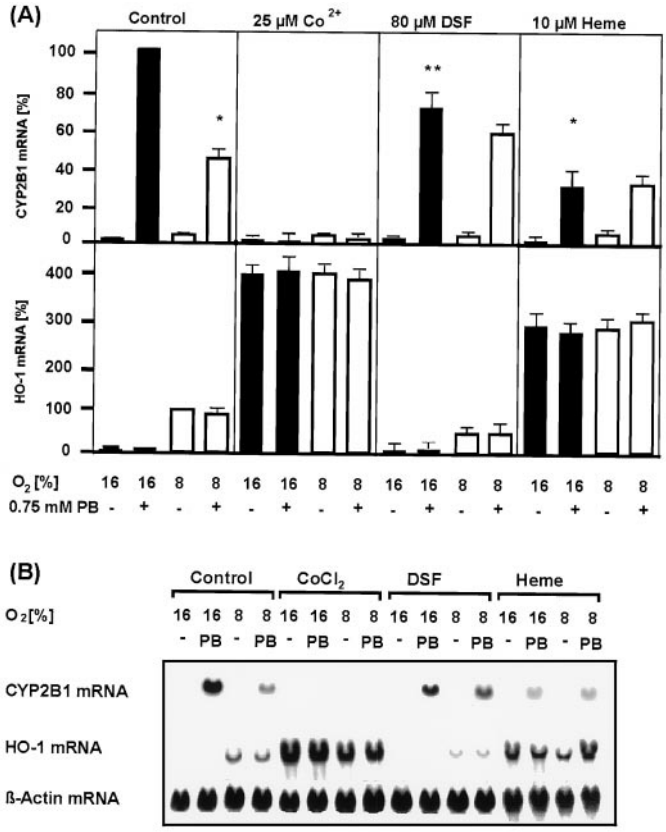

Fig. 2. Modulation by oxygen, Co^{2+}, and DSF of PB-dependent CYP2B1 induction and of HO-1 mRNA expression in primary rat hepatocyte cultures. Hepatocytes were cultured for 3 days under arterial (16%) or venous (8%) pO_2 in the presence or absence of 0.75 mM PB. When indicated, cultures were incubated with 25 μM $CoCl_2$ or 80 μM DSF. Culture media were exchanged every 24 h. mRNA levels were determined by Northern blot analysis as in Fig. 1. A, maximal expression of CYP2B1 mRNA under arterial pO_2 was set to 100%; HO-1 mRNA expression under venous pO_2 in the absence of inhibitors of heme synthesis was set equal to 100%. Values represent means ± S.E.M. of seven (control) or three (Co^{2+}, DSF, Heme/BSA) independent experiments. Statistics, Student's t test: *significant difference between 16% O_2 and 8% O_2, **significant difference between PB + 16% O_2 versus DSF + PB + 16%O_2 and PB + 16%O_2 versus Heme + PB + 16%O_2; $p \leq .05$. B, representative Northern blot. RNA (total 20 μg) were hybridized to a CYP2B1-specific oligonucleotide probe and rehybridized to HO-1 and β-actin antisense RNA probes as described in *Experimental Procedures*.

induced only to less than 20% under arterial and venous pO_2 (Table 1).

Hepatocytes cultured with 10 μM heme/BSA alone exhibited a reduced induction of CYP2B1 mRNA by PB under both oxygen tensions, resulting in about 30% of maximal CYP2B1 mRNA expression (Fig. 2 and Table 1). Heme/BSA acted positively in respect to expression of HO-1 mRNA, which was induced to about 300% under arterial and venous pO_2 (Fig. 2).

Repression by EGF of PB-Dependent CYP2B1 Induction under Arterial but not Venous pO_2. To investigate the role of O_2 tensions in the repression by EGF of PB-dependent CYP2B1 mRNA induction, primary hepatocytes were cultured for 3 days in the presence of the inhibitory growth factor under arterial and venous pO_2. In the absence of EGF, PB induced CYP2B1 mRNA under arterial pO_2 to maximal values. In the presence of 0.16 nM EGF, PB-dependent expression of CYP2B1 mRNA under arterial pO_2 was reduced to approximately 35%. A 10-fold higher concentration of 1.6 nM EGF repressed CYP2B1 mRNA induction by PB nearly completely to about 15% (Fig. 3). Therefore, the lower EGF concentration of 0.16 nM was chosen for the further investigations under venous pO_2.

Surprisingly, under the lower oxygen tension, EGF no longer repressed the PB-dependent CYP2B1 mRNA induction (Fig. 4). The pattern of β-actin expression or of enhancement of HO-1 mRNA expression under venous pO_2 was not altered by EGF (Fig. 4). Thus, the repression by EGF of the PB-dependent CYP2B1 mRNA induction observed under arterial pO_2 was lost under venous pO_2. The pattern of modulation by O_2 of the PB-elicited CYP2B1 induction was reversed in the presence of EGF.

Repression by GH, T4, and T3 of PB-Dependent CYP2B1 mRNA Induction under Arterial and Venous pO_2. To also investigate the role of O_2 tensions in the repression by GH, T4, and T3 of the PB-dependent CYP2B1 mRNA induction, primary hepatocytes were again cultured for 3 days in the presence of the hormones GH, T3, and T4 under arterial and venous pO_2. PB-dependent CYP2B1 mRNA induction was repressed by GH concentrations in the physiological serum concentration range of 4.8 or 12 nM GH under arterial (Fig. 3) and venous (Fig. 5) oxygen tensions. CYP2B1 mRNA induction under venous pO_2 amounted to approximately half of the values that were obtained under arterial pO_2 (Fig. 5). Thus the pattern of modulation by O_2 of PB-dependent CYP2B1 mRNA induction was retained under the inhibitory action of GH, so that higher levels were reached under arterial pO_2.

The thyroid hormones T4 and T3, both applied at a concentration of 10 μM (Rosa et al., 1988), also led to a general repression of PB-dependent CYP2B1 mRNA induction under both oxygen tensions, resulting in an induction of approximately 40% (T4) and 25% (T3) under arterial pO_2 and of about 12% (T4) and 10% (T3) under venous pO_2, respectively (Fig. 5). HO-1 or β-actin mRNA expression were not affected by T4 or by T3. Thus, again the modulation by oxygen of PB-dependent CYP2B1 mRNA induction was conserved in the presence of T4 and T3, so that higher levels of expression were reached under arterial pO_2.

O_2-Independent Expression of EGF-R Proteins in Hepatocyte Plasma Membranes. The finding that EGF but not GH, T3, and T4 changed the pattern of the modulation by O_2 of the PB-elicited CYP2B1 mRNA induction in rat hepatocyte cultures is difficult to understand. The lack of the inhibitory action of EGF on the PB-dependent CYP2B1 mRNA induction under venous pO_2 might be due to an insufficient or absent expression of the EGF-R. Therefore, plasma membranes from primary rat hepatocytes cultured under arterial and venous pO_2 for up to 3 days in the presence of 0.16 nM EGF were analyzed for their contents in EGF-R proteins in the cytoplasmic membrane fraction by Western blotting.

It was found that in the presence of EGF the amount of EGF-R protein decreased within 3 days due to the down-regulation of the receptor by EGF. But at variance with the working hypothesis the EGF-R protein content was always about the same in the membranes prepared from hepatocytes

TABLE 1

Inhibition of the PB-dependent CYP2B1 mRNA induction by $CoCl_2$, DSF, and heme under arterial and venous pO_2 in primary rat hepatocytes

Primary rat hepatocytes were cultured for 3 days under arterial (16% O_2) and venous (8% O_2) pO_2 with serum-free medium in the presence of 0.75 mM PB, 25 μM $CoCl_2$, 80 μM DSF, or 10 μM heme/BSA either alone or in combinations as indicated. Total RNA was isolated as described in *Experimental Procedures* and subjected to Northern blot analysis. In each experiment the maximal CYP2B1 mRNA expression obtained only with PB under arterial pO_2 was set equal to 100%. Numbers show the relative CYP2B1 mRNA levels. Values are means ± S.E.M. of three independent culture experiments (PB, $n = 7$).

Treatment	CYP2B1 mRNA Level	
	16% O_2	8% O_2
	%	
Control	5 ± 2	6 ± 3
PB	100	44 ± 5**
$CoCl_2$	4 ± 3	4 ± 2
DSF	6 ± 3	5 ± 4
PB + $CoCl_2$	5 ± 8*	4 ± 3*
PB + DSF	70 ± 10*	60 ± 6*
Heme/BSA	5 ± 4	8 ± 5
PB + $CoCl_2$ + heme/BSA	5 ± 9*	4 ± 7*
PB + DSF + heme/BSA	18 ± 5***	11 ± 10***
PB + heme/BSA	27 ± 10*	21 ± 6*

Statistics Student's t test for paired values: * significant differences PB versus PB + $CoCl_2$, PB versus PB + DSF, PB versus PB + heme/BSA, PB versus PB + $CoCl_2$ + heme/BSA, PB versus PB + DSF + heme/BSA, $p \leq .05$; ** significant differences 16% O_2 versus 8% O_2, $p \leq .05$; *** significant differences PB + DSF versus PB + DSF + heme/BSA.

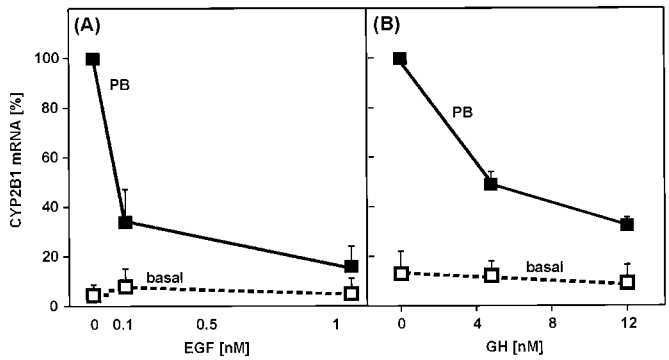

Fig. 3. Repression by EGF and GH of PB-dependent CYP2B1 mRNA induction in primary rat hepatocyte cultures under arterial pO_2. Hepatocytes were cultured for 3 days in the presence of 0.75 mM PB with or without EGF (A) or with and without GH (B) under arterial pO_2 (16% O_2). mRNA expression was determined by Northern blot analysis as in Fig. 1. CYP2B1 mRNA expression in the absence of PB is designated as basal expression. Maximal CYP2B1 mRNA expression obtained without EGF or GH was set to 100%. Values represent means ± S.E.M. of three independent experiments.

cultured under arterial or venous pO_2 (Fig. 6). This finding suggests that the lack of the repressive action of EGF under venous pO_2 could only be due to an impaired function rather than expression of the EGF-R or to alterations in downstream signal transduction at low pO_2.

Discussion

Modulation by Oxygen of PB-Dependent CYP2B1 mRNA Induction In Vitro

The present study demonstrated that PB-dependent induction of CYP2B1 mRNA in primary hepatocytes was modulated by pO_2. Maximal induction occurred under arterial pO_2 whereas only about 40% levels were reached under venous pO_2 (Figs. 2, 4, and 5). The kinetics of induction (Fig. 1) were consistent with a previous study (Aubrecht et al., 1993) according to which maximal values were observed within 3 days of incubation with 0.75 mM PB. Oxygen has been shown previously to modulate gene expression; examples are the genes encoding the glycolytic enzymes aldolase A (Semenza et al., 1994) and lactate dehydrogenase (Ebert et al., 1996) in hepatoma cells and the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in primary hepatocytes (Kietzmann et al., 1996). The modulation by O_2 of the expression of these genes seems to be mediated by a heme protein acting as an O_2 sensor.

Heme Proteins as Oxygen Sensors and Regulators of CYP Expression

Heme Proteins as O_2 Sensors. The suggestion that heme proteins could act as O_2 sensors was based on the observation that in hepatoma cells the "iron analog" Co^{2+} and the iron chelator DSF mimicked hypoxia in the activa-

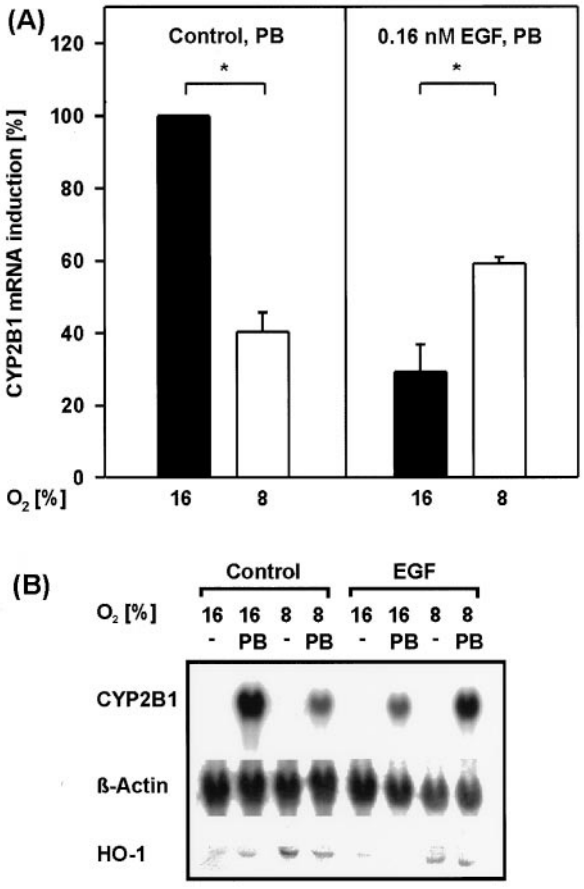

Fig. 4. Loss of repression by EGF of PB-dependent CYP2B1 mRNA induction under venous pO_2 in primary rat hepatocyte cultures. Hepatocytes were cultured for 3 days under arterial (16%) or venous (8%) pO_2 in the presence of 0.75 mM PB. When indicated, the medium contained 0.16 nM EGF. Expression of CYP2B1, β-actin and HO-1 mRNA was determined by Northern blot analysis as in Figs. 1 and 2. A, induction of CYP2B1 mRNA is the difference between CYP2B1 mRNA expression under PB and basal mRNA expression under arterial pO_2. Maximal induction was set equal to 100%. Data represent means ± S.E.M. of seven (control) or three (EGF) independent experiments. Student's t test: *significant difference between 16% and 8% O_2, $p \leq .05$. B, representative Northern blot. RNA (total 20 μg) was analyzed as outlined in Fig. 2.

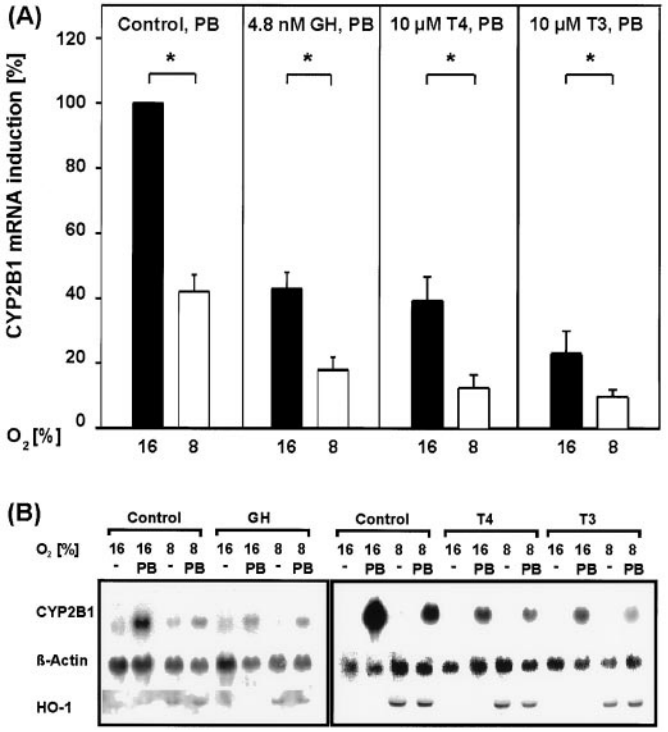

Fig. 5. Repression by GH, T4, and T3 of PB-dependent CYP2B1 mRNA induction in primary rat hepatocyte cultures under arterial and venous pO_2. Hepatocytes were cultured for 3 days under arterial (16% O_2) or venous (8% O_2) pO_2. CYP2B1 mRNA expression was induced with 0.75 mM PB. When indicated, 4.8 nM GH (=100 ng/ml), 10 μM T4, or 10 μM T3 were used to inhibit CYP2B1 mRNA induction. Expression of CYP2B1, β-actin, and HO-1 mRNA was determined by Northern blot analysis as in Figs. 1 and 2. A, induction of CYP2B1 mRNA is the difference between maximal expression under PB and basal mRNA expression. Data represent means ± S.E.M. of seven (control) or three (GH, T4, or T3) independent experiments. Student's t test: *significant difference between 16% and 8% pO_2, $p \leq .05$. B, representative Northern blot. RNA (total 20 μg) was analyzed as in Fig. 2.

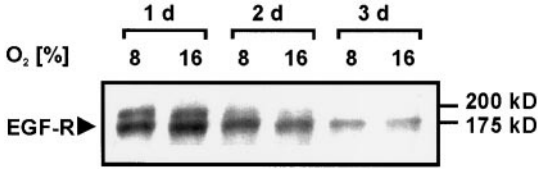

Fig. 6. O_2-independent expression of EGF-R protein in hepatocyte plasma membranes. Plasma membranes from hepatocytes cultured for up to 3 days in the presence of 0.16 nM EGF under arterial (16% O_2) or venous (8% O_2) pO_2 were prepared and analyzed in a Western blot with a polyclonal antibody against the human EGF-R cross-reactive with the rat EGF-R. The 175-kD band represents the EGF-R and the 200-kD band represents a glycoprotein that has been shown to cross-react with several antibodies against the EGF-R (Panneerselvam et al., 1995).

tion of the genes encoding erythropoietin (Goldberg et al., 1988), vascular endothelial growth factor (Goldberg and Schneider, 1994), and the glycolytic enzymes (Semenza et al., 1994). Both Co^{2+} and DSF inhibit heme synthesis; cobalt ions were incorporated by ferrochelatase into the porphyrin moiety, resulting in Co^{2+} protoporphyrin IX, which inhibited 5-aminolaevulinate synthase (Sinclair et al., 1979, 1982). DSF inhibited the incorporation of radiolabeled 5-aminolaevulinate into heme (Shedlofsky et al., 1987). A substantiation of the proposal that the heme proteins act as O_2 sensors came from the findings that the O_2-competitive heme ligand carbon monoxide prevented the induction by low pO_2 of the erythropoietin gene in Hep3B cells (Goldberg et al., 1988; Goldberg and Schneider, 1994) and caused a loss of the modulation by O_2 of the glucagon-dependent induction of the phosphoenolpyruvate carboxykinase gene in primary rat hepatocytes (Kietzmann et al., 1993).

In the present study long-term treatment of hepatocytes with $CoCl_2$ completely blocked CYP2B1 mRNA induction by PB under both venous and arterial oxygen tensions (Fig. 2 and Table 1). The same treatment with DSF reduced the difference in the PB-dependent induction of CYP2B1 mRNA between the two oxygen tensions (Fig. 2). The addition of heme, which contains Fe^{3+} (hemin) to the cultures, reduced the PB-dependent induction of CYP2B1 mRNA under arterial pO_2 to the lower venous values, resulting in abolishment of the modulation by O_2. Thus hemin could simulate venous pO_2 by either increasing the Fe^{3+} content or by repressing the heme synthesis via a negative feedback. Therefore, these findings are in line with the proposal that heme proteins are involved in the modulation of gene expression by O_2.

Heme as a Regulator of PB-Dependent CYP Gene Activation. Heme regulates the activity of heme proteins as a prosthetic group and is also involved in regulation of biosynthesis of several heme proteins such as cytochromes (Padmanaban et al., 1989). Complete repression of CYP2B1 mRNA induction under arterial and venous pO_2 by $CoCl_2$ suggested that heme is also a necessary factor for induction by PB. PB is known to induce δ-aminolevulinate synthase (Schuetz et al., 1990), thus activating heme synthesis. The repression of PB-dependent induction of CYP2B1 and the total abolishment of O_2-dependent modulation by heme itself further support the notion that a heme protein might be involved in the regulation of CYP2B1 mRNA expression; repression by heme may suggest a negative feedback. Thus, CYP gene activation appears to represent a special case in which heme or heme proteins might be involved in a more direct manner by regulating the PB-dependent induction of CYP2B1 mRNA or in a more indirect way as an O_2 sensor in the modulation by O_2 of the PB-dependent induction. Whether or not the heme protein involved is the same in both cases is not known yet.

Responsiveness to O_2 and EGF as Possible Determinants of Zonated CYP2B1 mRNA Expression

Positive modulation of the PB-dependent induction of CYP2B1 mRNA by arterial pO_2 (Figs. 2, 4, and 5) was discrepant from the pattern of CYP2B1 mRNA induction in rat liver. Induction in vivo is observed only throughout the perivenous and mid-zonal regions (Hassett et al., 1989), where the pO_2 is low compared with the periportal region. The results of this study are in line with the finding in primary rat hepatocytes cultured for 3 days under periportal and perivenous pO_2 that the maximal PB-dependent induction of immunoreactive CYP2B protein and of CYP2B-associated testosterone 16β-hydroxylation was found under periportal pO_2 whereas only about half-maximal values were measured under venous pO_2 (Saad et al., 1994). However, a discrimination between CYP2B1 and CYP2B2 isoforms was not performed and expression on the mRNA level was not examined. It was hypothesized that the insulin-glucagon ratio might contribute to the pattern of zonal CYP2B enzyme expression. Because the insulin-glucagon ratio is higher in the perivenous cells, one would expect that insulin would have a positive effect on PB-dependent CYP2B induction under perivenous pO_2. Indeed, in primary rat hepatocytes increasing insulin concentrations from 1 to 100 nM decreased the PB-dependent CYP2B-associated testosterone 16β-hydroxylation under periportal and perivenous pO_2 within 3 days whereas higher levels were still obtained under periportal pO_2 (Saad et al., 1994). This is in line with the finding that in rats with streptozotocin- or alloxan-induced diabetes the periportal to perivenous activity ratio for phosphoenolpyruvate carboxykinase remained unaltered (Mietke et al., 1985). Thus it appeared that the insulin-glucagon ratio was not the major determinant for the zonated CYP2B expression.

Role of Gradients of Pituitary-Derived or -Dependent Hormones in Induction of CYP2B1 mRNA by PB and Its Modulation by O_2. The predominant induction of CYP2B1 in the low pO_2 zone in vivo requires a mechanism that inhibits the induction mainly in the high pO_2 zone. In the present study the pituitary-derived GH and the pituitary-dependent T3 and thyroxin (T4) inhibited the PB-dependent induction of CYP2B1 mRNA both at arterial and venous pO_2 (Fig. 5). This is in line with previous findings that GH, T3, and T4 repressed the PB-dependent induction of CYP2B1 protein (Schuetz et al., 1990; Murayama et al., 1991). If these hormones were degraded during the passage of blood through the liver, their inhibitory effect would be stronger in the periportal region. This periportal inhibition could cause the observed predominant perivenous induction of CYP2B1 by PB only, if it were strong enough to offset the positive modulation by periportal pO_2. Whether a periportal-to-perivenous GH gradient exists, is not known yet. Due to deiodination the level of T4 decreases by about 40% and the level of T3 increases by about 50% from the periportal to the perivenous area (Jungermann and Katz, 1989). Thus the inhibition of PB-dependent CYP2B1 induction by T4 and possibly GH, but not by T3, could contribute to the prevalent perivenous expression of the enzyme after PB. Similarly, a low level expression of receptors for GH, T4, or T3 in the perivenous zone would explain the zonated induction of CYP2B1 by PB. However, the zonal gradient of the GH receptor is very shallow and the zonal distribution of the T4/T3 receptors is not known (Oinonen et al., 1996).

GH and T4/T3 have been proposed to mediate zone-specific basal (uninduced by PB) CYP2B1/2 gene expression in liver. In hypophysectomized rats, the original differences between periportal and perivenous expression were abolished, leading to an overall increase in expression; infusion of GH largely restored the zonated CYP2B1/2 mRNA expression in females but not in males, whereas infusion of T3 failed to do so (Oinonen et al., 1993; Oinonen and Lindros, 1995; for reviews

see Oinonen et al., 1996; Oinonen and Lindros, 1998). Thus these in vivo studies show in part how GH could contribute to the basal mainly perivenous expression of CYP2B1 but they fail to explain the role of thyroid hormones.

Role of Gradients of Growth Factors in Induction of CYP2B1 mRNA by PB and Its Modulation by O_2. In the present study EGF, at a concentration of 0.16 nM, which is in the vicinity of the physiological concentration in the systemic circulation (Wollenberg et al., 1989), substantially repressed the PB-dependent CYP2B1 mRNA induction under arterial but not under venous pO_2 (Fig. 4). The inhibitory effect of EGF under high pO_2 is in line with previous studies (Aubrecht et al., 1995). Thus treatment of hepatocytes with EGF in conjunction with O_2 mimicked the zonal pattern of PB-elicited CYP2B1 mRNA expression in vivo.

The lack of inhibition by EGF of PB-dependent CYP2B1 mRNA induction under perivenous pO_2 indicates a differential responsiveness of hepatocytes toward EGF depending on the pO_2. Factors contributing to a loss of inhibition by EGF of CYP2B1 induction under perivenous pO_2 might be a reduction of the EGF-R protein or a modification of the protein leading to a decreased affinity. Additionally, downstream steps in intracellular signal transduction may also be altered under perivenous pO_2.

In the present study conducted with primary rat hepatocytes, levels of immunodetectable EGF-R protein in cytoplasmic membrane fractions did not differ between cells incubated under periportal or perivenous pO_2. These results are in line with a study (Hirose et al., 1997) in which primary rat hepatocytes cultured under hypoxic conditions for only 3 h exhibited a reduction in EGF binding to receptors, although immunodetectable EGF-R protein levels in cell lysates were not lower than under normoxic conditions. Because the decreased EGF binding was reversible after reoxygenation and the recovery of EGF binding was independent of protein synthesis, it was concluded that a transient modification of EGF-Rs by hypoxia, e.g., receptor phosphorylation status, might lead to desensitization of the receptor (Hirose et al., 1997). Thus the differential responsiveness of hepatocyte cultures to EGF under perivenous and periportal pO_2 was not due to alterations in plasma membrane EGF-R levels, but in receptor affinity.

In the liver, gradients across the acinus in immunoreactive EGF-R protein and also in receptor affinity have been observed (Marti and Gebhardt, 1991). Immunostaining of liver sections revealed predominant EGF-R density in periportal hepatocytes. In addition, high-affinity receptors were restricted to the periportal hepatocytes, whereas low-affinity binding sites were found across the entire acinus decreasing 3- to 4-fold from the periportal to the perivenous region. This distribution pattern would lead to a steep gradient in EGF action in the liver parenchyma (Marti and Gebhardt, 1991). It is thus conceivable that in vivo repression of CYP2B1 induction by EGF occurs primarily in the proximal periportal regions; it could be strong enough to more than offset the positive modulation by O_2 and thus cause the predominance of CYP2B1 mRNA induction in the perivenous and mid-zonal regions.

In conclusion, oxygen and the growth factor EGF are major antagonistic determinants of the differential CYP2B1 mRNA induction by PB; they have a major role in the zonated expression of the gene in the liver.

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