Heme-Reversible Impairment of CYP2B1/2 Induction in Heme-Depleted Rat Hepatocytes in Primary Culture: Translational Control by a Hepatic α-Subunit of the Eukaryotic Initiation Factor Kinase?

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ABSTRACT

The role of heme in the phenobarbital-mediated induction of CYP2B1/2 was reexamined in rat hepatocytes in monolayer culture, acutely depleted of heme by treatment with either 3,5-dicarboethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP) or N-methylprotoporphyrins (NMPP). The findings revealed that this acute hepatic heme depletion markedly impaired CYP2B1/2 protein induction, an effect that was reversible by heme resupplementation. However, TaqMan analyses of hepatic mRNA isolated from these heme-depleted cells revealed that this impairment was not due to faulty transcriptional activation of either CYP2B1 or CYP2B2 gene expression as previously proposed, thereby confirming literature reports that heme is not a transcriptional regulator of the CYP2B1/2 gene. In contrast, the rate of de novo CYP2B1/2 protein synthesis was found to be dramatically inhibited in both DDEP- and NMPP-treated hepatocytes. Concurrently, a marked (>80%) suppression of de novo hepatocellular protein synthesis was also observed, along with a significantly enhanced phosphorylation of the α-subunit of the eukaryotic initiation factor eIF2α (eIF2α), as monitored by the phosphorylated eIF2α/total eIF2α ratio in these heme-depleted cells. Indeed, the parallel reversal of all these three effects by heme supplementation suggests that this impaired CYP2B1 induction most likely stems from blocked translational initiation resulting from the activation of a heme-sensitive eIF2α kinase. Such global suppression of hepatic protein synthesis may disrupt a myriad of vital cellular functions, thereby contributing to the clinical symptoms of acute hepatic heme-deficient states such as the hepatic porphyrias.

The liver endoplasmic reticulum (ER)-anchored hemoproteins cytochromes P450 (P450s) are enzymes responsible for the metabolism of numerous endogenous and foreign compounds that are often inducible by these substrates. Each P450 is composed of a heme and an apoprotein moiety, whose cellular syntheses are tightly coordinated. Similar tight coordination of synthesis is also observed with other hemoproteins such as yeast mitochondrial isocitrate c and cytochrome c oxidase subunits IV, VI, and Va, hemoglobin, myoglobin (Graber and Woodworth, 1986), neuroglobin (Zhu et al., 2002b), catalase, and liver tryptophan 2,3-dioxygenase (TDO). In some, but not all, of these cases, heme has been established as a transcriptional activator of the relevant genes (for review, see Padmanaban et al., 1989). Although phenobarbital (PB)-mediated induction of P450s 2B unequivocally involves transcriptional activation of both protein and heme synthetic machinery (Waxman and Azaroff, 1992; De Matteis and Marks, 1996, and references therein), the role of hemin and for iron-protoporphyrin IX are used interchangeably throughout the text. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

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ABBREVIATIONS: ER, endoplasmic reticulum; P450, cytochrome P450; TDO, tryptophan 2,3-dioxygenase; PB, phenobarbital; PBGD, porphobilinogen deaminase; ALA, δ-aminolevulinic acid; DDEP, 3,5-dicarboethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine; NMPP, N-methylprotoporphyrins; eIF2, α-subunit of the eukaryotic initiation factor; WME, Williams’ medium E; BSA, bovine serum albumin; PMSF, phenylmethylsulphonyl fluoride; E-64, L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane; PBS, phosphate-buffered saline; PROD, 7-pentoxyresorufin O-dealkylase; PCR, polymerase chain reaction; SUS, β-glucuronidase; bp, base pairs; AK, adenylate kinase; eIF2α, eukaryotic elongation factor 2; eIF2αP, phosphorylated eIF2α; Gsp78, 78-kDa glucose-regulated protein; HRP, horseradish peroxidase; eIF2αP, phosphorylated α-subunit of the eukaryotic initiation factor; HRI, heme-regulated inhibitor; PERK, PKR-like ER kinase.
heme as a specific activator of CYP2B transcription is controversial. Just one group to date has advocated a positive role for heme in CYP2B transcriptional activation (Dwarki et al., 1987; Rangarajan and Padmanaban, 1989). Two other research teams having examined this possibility concluded that heme is not involved in CYP2B transcriptional activation (Hamilton et al., 1988; Srivastava et al., 1989; Sinclair et al., 1990). Similarly, no role for heme in CYP3A11 transcriptional activation was found in mice treated with the heme synthesis inhibitor lead acetate (Jover et al., 1996). On the other hand, a positive role for heme in PB-mediated transcriptional activation of CYP2B5 has been documented in a heme-deficient mouse model generated by genetic disruption of the heme-synthetic enzyme porphobilinogen deaminase (PBGD) (Jover et al., 2000). However, in these mice neither the expression nor function of CYP2B10, the mouse liver CYP2B ortholog was affected by heme deficiency (Jover et al., 2000). Apparently, as monitored by the rate of [14C]-8-aminolevulinic acid (ALA) incorporation into heme, heme synthesis was reduced to only 50% of normal in these mice, which might not have been sufficient to deplete heme to the extent required to verify heme regulation of CYP2B hemoproteins.

Understandably, acute hepatic free heme pool depletion is critical for the documentation of heme-dependent gene regulation in intact animals. In our experience, agents such as allylisopropylacetamide, succinylacetone, aminotriazole, CoCl₂ and Pb-acetate used previously as heme synthesis inhibitors and/or heme depletors were incapable of depleting the hepatic free heme pool (determined by significantly reduced hepatic TDO heme saturation) sufficiently to document the role of heme-mediated transcriptional activation of hepatic TDO in rats (D.A. Litman, H. Liu, and M.A. Correia, unpublished observations). However, such acute hepatic heme depletion was observed after treatment of rats with the P450 suicide inactivator 3,5-dicarboxethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP) (Litman and Correia, 1983, 1985; Ren and Correia, 2000). DDEP irreversibly destroys the heme of hepatic P450s 2C11, 2C6, and 3A, but not CYP2B1 (Lee et al., 1988; Sugiyama et al., 1989). Such CYP2C11 and CYP2C6 destruction through heme pyrrole N-ethylolation generates N-ethylprotoporphyrins, potent inhibitors of the terminal heme synthetic enzyme ferrochelatase. This dual DDEP effect on hepatic P450 heme destruction and inhibition of heme synthesis causes profound hepatic heme depletion in rats, much more intense than that seen in PBGD-deficient mice or after treatment with any other heme inhibitor. DDEP also similarly induces acute hepatic heme depletion in isolated hepatocytes. We have therefore reexamined the controversial role of heme in PB-mediated transcriptional activation of CYP2B1/2 in DDEP-treated rat hepatocytes in primary monolayer culture. Furthermore, to exclude any possible confounding effects elicited by ethyl radicals generated from P450-dependent DDEP metabolism, N-methylprotoporphyrins (NMP), the methyl analogs of N-ethylprotoporphyrins that are even more potent ferrochelatase inhibitors (De Matteis et al., 1980; Ortiz de Montellano et al., 1980, 1981), were used to deplete heme in these cells. Using these models for acute hepatic heme depletion, we have found that heme is definitely required for PB-mediated induction of CYP2B1/2 protein. However, consistent with the previous findings (Srivastava et al., 1989; Sinclair et al., 1990), such regulation is not exerted at the level of CYP2B1/2 mRNA induction, but rather it occurs at a posttranscriptional/translational step. Indeed, our findings in both DDEP- and NMPP-treated hepatocytes of global suppression of de novo hepatic protein synthesis and enhanced phosphorylation of the α-subunit of the eukaryotic initiation factor eIF2α (eIF2α), as well as the reversal of these effects by heme, suggest that this impaired CYP2B1 induction results from blocked translation stemming partly from the activation of a heme-sensitive eIF2α kinase. The implications of these findings in acute hepatic heme deficiency are discussed.

**Materials and Methods**

**Materials.** Williams' medium E (WME), insulin-transferrin-selenium-G (100×), bovine serum albumin (BSA), penicillin/streptomycin, l-glutamine, liver digestion medium, and liver perfusion medium were obtained from Invitrogen (Carlsbad, CA). Phenylmethylsulfonyl fluoride (PMSF), 4-(2-aminoethyl)benzene sulfonyl fluoride, hemin hydrochloride, phenobarbital sodium (PB), and dexa-methasone were purchased from Sigma-Aldrich (St. Louis, MO). DDEP was synthesized as described previously (Ortiz de Montellano et al., 1991). N-Methylprotoporphyrin IX (a mixture of all four isomers) was purchased from Frontier Scientific Inc. (Logan, UT). Methionine/cysteine-free WME was prepared by the University of California San Francisco Cell Culture Facility (San Francisco, CA). Collagen type I was prepared by the University of California San Francisco Liver Center Cell and Tissue Biology Core Facility. Matrigel was obtained from BD Biosciences (Bedford, MA). E-64, leupeptin, and aprotinin were purchased from Roche Diagnostics (Indianapolis, IN). [35S]EXPRESS and ALA were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

**Animals.** Male Sprague-Dawley rats (225–250 g) were purchased from Charles River Laboratories, Inc. (Wilmington, MA), and maintained on a 12-h light/dark cycle in a controlled environment at the University of California San Francisco Animal Care Facility.

**Rat Hepatocyte Isolation.** Hepatocytes were isolated from rats by in situ perfusion of the liver with collagenase using a modification of the technique described by Irving et al. (1984). Briefly, the portal vein was cannulated with a 16-gauge catheter and the inferior vena cava was severed to permit outflow. The liver was then flushed with a calcium-chelating buffer (liver perfusion medium) for 3 to 5 min, followed by perfusion with collagenase (liver digest medium), for an additional 15 min. At the end of the digestion, the liver was removed to a sterile dish and minced thoroughly with scissors. This crude liver cell isolate was suspended in 50 ml of Dulbecco's modified Eagle's medium/Ham's F-12, filtered through sterile gauze, pelleted by centrifugation at 70g for 2 min, and resuspended in Dulbecco's modified Eagle's medium/Ham's F-12. After an additional round of pelleting and resuspension, hepatocytes were purified by centrifugal elutriation (Irving et al., 1984).

**Rat Hepatocyte Culture.** Rat hepatocytes were cultured as described previously (LeCluyse et al., 1999). Briefly, 3 × 10⁶ purified hepatocytes in 3 ml of WME containing insulin-transferrin-sele-nium-G (1×), 0.1 μM dexmethasone, 50 U/ml penicillin/streptomycin, 2 mM l-glutamine, and 0.1% BSA were seeded onto Permanox culture dishes precoated with collagen type I. Hepatocytes were allowed to attach at 37°C for 2 to 3 h in a humidified chamber gassed with 95% air and 5% CO₂. Culture dishes were gently swirled, and the medium containing unattached cells and debris was aspirated. Cultures were then overlaid with WME containing 0.25 mg/ml Matrigel and returned to the humidified chamber. The cells were maintained for 3 days with a daily change of medium before initiation of any treatment.

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Hepatocytes were treated with vehicle (methanol, dimethyl sulfoxide, or saline), 200 μM PB, 10 μM DDEP, or 1 μM NMPP, with or without 20 μM hemin in different combinations and/or times as indicated. Cells from each of the treatment groups were harvested for the isolation of microsomes, total RNA, or preparation of cell lysate.

**[14C]ALA Incorporation into Heme.** To determine the degree to which DDEP or NMPP inhibits heme synthesis, [14C]ALA incorporation into heme was examined in cultured hepatocytes treated with dimethyl sulfoxide (control), PB, DDEP, or NMPP as described above. After 23 h of each treatment, 1 ml of [14C]ALA along with unlabeled ALA (final concentration, 30 μM) was added to the medium. One hour later, the cells were washed thrice with ice-cold phosphate-buffered saline (PBS) before harvesting with 0.4 ml of lysate buffer (see below). The lysate was centrifuged at 10,000 g for 10 min at 4°C. Carrier heme (80 nmol) in the form of rat erythrocyte lysate was added to the supernatants (0.38 ml). Heme was extracted as described previously (Bonkovsky et al., 1985) with freshly prepared acetone/concentrated HCl (20:1 v/v), 3.5 ml. After centrifugation (1000g, 10 min), the supernatant was extracted again with 6 ml of diethyl ether and 2.5 ml of 2 N HCl. The diethyl ether layer was dried by nitrogen and the pH was raised to 7.0 with 0.4 ml of 1 M NaOH. One hour later, the cells were washed thrice with ice-cold 0.1 M PBS, pH 7.0. Homogenates were prepared from each of the treatment groups, with or without the indicated treatments as follows: 1 ml of Tris-HCl, pH 8.4, 50 mM KCl, 7.5 mM MgCl2, 1 mM dATP, 1 mM dTTP, 1 mM dGTP, and 1 mM dCTP, 5 μM random primers, 2.5 U/μl Moloney murine leukemia virus reverse transcriptase, 0.4 U/μl RNase inhibitor, 250 ng of cDNA, and 1 μM pyrrocarbonate-treated water, 10 ng of cDNA (based on the RNA concentration added to reverse transcription reaction). PCR was performed employing an ABI Prism TaqMan 7700 sequence detector system with denaturation at 95°C for 12 min, and 40 cycles with denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min (Bustin, 2000; Ginzinger et al., 2002; Goodsaid et al., 2003). The expression level of each gene was normalized to the endogenous control gene (GUS). Relative gene expression was calculated as 2^ΔΔCt, where ΔΔCt is Ct (treated or untreated) – Ct (GUS), wherein Ct is the cycle number when the fluorescent signal of the PCR reaction reaches the threshold level. Values were expressed as percent of the PB-treated value.

**Validation of the TaqMan Method.** CYP2B1 and CYP2B2 oligos were designed to assess the specificity of the CYP2B1/CYP2B2 primers and probes used in the assay. The sequence of CYP2B1 oligo used was 75 bp in length (TGTTGGCCCAAGCTGAGGAT-TTCTCAATCCGTGTTGAGGACCA-ATCTTACAGG-GAGAGAGAGAGCCCTTCGGCATTCTCC) consisting of CYP2B1 forward primer (250–267 bp), 5 μM dATP, 200 μM dTTP, 200 μM dGTP, 200 μM dCTP, 1.25 U of AmpliTaq Gold DNA polymerase, 200 nM probes, 900 nM primers, diethyl pyrrocarbonate-treated water, 10 ng of cDNA (based on the RNA concentration added to reverse transcription reaction). PCR was carried out under the same conditions as described above except that the oligo input was 10^−15 mol.

**[35S]Methionine/Cysteine Labeling of Cell Protein.** Cells were treated with various chemicals for 23 h as indicated above. The media from individual cell culture treatment groups were harvested as described previously. Cells from each of the treatment groups were treated with various chemicals for 23 h as indicated above. The media from individual cell culture treatment groups were harvested as described previously.

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**TABLE 1**

<table>
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<th>GenBank (Accession No.)</th>
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<tr>
<td></td>
<td>Reverse primer (352–368)</td>
<td>GAGCAATGCCGAGAACCC</td>
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<tr>
<td></td>
<td>Probe (286–313)</td>
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<tr>
<td>CYP2B2 (M34452)</td>
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<tr>
<td></td>
<td>Reverse primer (560–583)</td>
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<tr>
<td></td>
<td>Probe (518–544)</td>
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</tr>
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<td></td>
<td>Reverse primer (1810–1831)</td>
<td>TCTGGCGGATTTGGAGCAGGAGAATCTTACAGG-GHQQ-3’</td>
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</tbody>
</table>

FAM, 5-carboxyfluorescein fluorescent reporter dye; BHQ1, black hole quencher 1.
Impaired CYP2B Translation in Heme-Depleted Hepatocytes

Lyase was sedimented at 10,000g at 4°C for 10 min, and the supernatant was used for CYP2B immunoprecipitation.

**CYP2B Immunoprecipitation.** Lysate protein (60 μg) was adjusted to 2% SDS, boiled for 5 min, and diluted 1:4 (v/v) with 50 mM Tris-HCl, pH 7.4, 2.5% Triton X-100 (v/v), 190 mM NaCl, and 6 mM EDTA. Rabbit anti-rat CYP2B IgGs (0.5 mg) were added along with protein A-Sepharose slurry (50 μl). The mixture was then incubated at 4°C overnight with end-to-end rotation. The antigen/protein complex was collected by centrifugation and washed five times with PBS containing 0.1% SDS and 0.5% Nonidet P-40. The antigen was eluted by heating the complex for 5 min in the presence of a sample loading buffer (70 μl, 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 10% SDS, 5% β-mercaptoethanol, and 0.01% bromphenol blue). A 50-μl aliquot of the eluate was subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel, and the radioactivity of another 10-μl aliquot was monitored in 4 ml of Ecolume by liquid scintillation spectrometry using a Beckman LS3801 liquid scintillation counter.

**Total Protein Incorporation.** Cold rat microsomal protein (6–7 mg) was added as a protein carrier to 35S-labeled cell lysate protein (1 mg). The protein was precipitated with 5% H2SO4 in (6–7 mg) was added as a protein carrier to 35S-labeled cell lysate prepared as described above and also immunoblotted in parallel against rabbit anti-human eEF2 IgGs [1:1000 (v/v)] (Cell Signaling Technology Inc., Beverly, MA) as primary antibody and a secondary anti-rabbit HRP-coupled antibody [1:3000 (v/v)] (BioSource International), and the secondary antibody was a goat anti-mouse HRP-coupled antibody [1:3000 (v/v)]. Whereas the anti-human eEF2α IgGs recognize both phosphorylated and nonphosphorylated species of the protein, anti-human eEF2αP IgGs recognize only the phosphorylated species. For eukaryotic elongation factor 2 (eEF2), eEF2P, and Grp78 analyses, aliquots of cell lysates prepared as described above were also immunoblotted in parallel against rabbit anti-human eEF2P IgGs [1:1000 (v/v)] (Cell Signaling Technology Inc.) as primary antibody and a secondary anti-rabbit HRP-coupled antibody [1:10,000 (v/v)]; or rabbit anti-rat Grp78 IgGs [1:2000 (v/v)] (StressGen Biotechnologies, Victoria, ON, Canada) as primary antibody and a secondary goat anti-rabbit HRP-coupled antibody [1:5000 (v/v)]. The immunoblots were in all cases developed with a SuperSignal West Pico chemiluminescent substrate detection system (Pierce Chemical). The immunoblots were quantitated densitometrically by the Fluorchem IS-5500 system equipped with AlphaEase PC Stand Alone Software (Alpha Innotech, San Leandro, CA).

**Statistical Analyses.** After statistical consultation with Prof. P. Bacchetti (University of California San Francisco), we did not adjust for multiple comparisons, as these methods are controversial (Rothman, 1990; Savitz and Olshan, 1995; Bacchetti, 2002) because they require that the results of each analysis detract from the other. That is, each analysis is considered to be less believable than if it had been the only analysis. Furthermore, the results presented herein fit into a coherent biological framework and therefore reinforced each other. Since there are really only two primary comparisons of interest for either heme depletor [PB versus PB + DDEP (or NMPP) ± heme], the extent of the multiplicity is relatively small. Data were therefore analyzed for statistically significant differences between control and treated rat hepatocyte cultures by the Student’s t test at the 5% level of significance.

**Results**

**PB-Mediated CYP2B Induction in Primary Cultured Hepatocytes.** Most constitutive hepatic P450 isoforms were lost during the first 72 h of primary culture. At this stage, when the hepatocytes were treated with 25 to 400 μM PB for 24 h, a dramatic concentration-dependent induction of CYP2B protein was detected by Western immunoblotting analysis (data not shown). This induction was paralleled by a concentration-dependent stimulation of PROD, a relatively selective CYP2B functional probe (data not shown). The functional stimulation of CYP2B observed in cultured hepatocytes at 200 μM PB for 24 h was comparable with that of hepatic CYP2B-dependent PROD activity observed in rats at 18 h after a single intraperitoneal dose of 80 mg/kg PB (228 ± 80.0 versus 224 ± 24.1 pmol resorufin/mg microsomal protein/min). For this reason, 200 μM PB was used to induce CYP2B in all subsequent studies with primary rat hepatocyte cultures.

**Effect of DDEP or NMPP Treatment on [14C]ALA Incorporation into Heme.** In preliminary studies, the effectiveness of DDEP and NMPP as inhibitors of hepatic heme synthesis was assessed by monitoring the rate of [14C]ALA incorporation into heme after treatment with either agent (Fig. 1). After DDEP treatment, the rate of heme synthesis was dramatically reduced to 25% of that in PB-treated cells within 4 h (data not shown), remaining at that level for over
24 h, irrespective of the presence or absence of PB (Fig. 1). NMPP was slightly more effective, reducing the rate of heme formation to 15% of that in PB-treated cells within 1 h of treatment (data not shown) and persisting at that level for 24 h, also irrespective of the presence or absence of PB in the cultures (Fig. 1). These findings thus indicated that both agents were excellent inhibitors of hepatic heme formation in cultured hepatocytes.

**Effect of DDEP or NMPP Treatment on PB-Induced CYP2B Protein.** Immunoblotting analyses of microsomes isolated from hepatocytes cultured in the presence of 200 μM PB for 24, 48 or 72 h revealed a time-dependent induction of CYP2B (Fig. 2, A and B). To examine the role of heme in PB-mediated CYP2B induction, hepatocyte cultures were first treated with either 10 μM DDEP or 1 μM NMPP for 1 h to deplete the heme, before PB was added to the cultures. Corresponding microsomal immunoblotting analyses revealed that DDEP treatment of cultures completely blocked PB-mediated induction of CYP2B protein, and this block was significantly reversed at 24 h by the addition of 20 μM exogenous heme (Fig. 2). This heme-mediated reversal, detectable at a 5 μM concentration, progressed over the 3-day culture. Heme added to PB-treated cultures in the absence of DDEP treatment had little effect on PB-mediated induction of CYP2B protein. Although NMPP by itself had little effect on constitutive CYP2B (CYP2B2) synthesis, when added to the cultures 1 h before PB, NMPP almost completely blocked PB-induced CYP2B protein induction (Fig. 2). This effect, as after DDEP, was also reversed by exogenous heme addition (Fig. 2), thereby indicating that the blockade of PB-mediated CYP2B induction was indeed due to heme depletion and that heme is required for CYP2B induction. Furthermore, the similar impairment obtained with NMPP, which depletes hepatocellular heme solely by inhibiting heme synthesis, also excludes any DDEP-elicited effects unrelated to its heme depletion.

**Effect of DDEP- or NMPP-Elicited Heme Depletion on PB-Induced CYP2B mRNA by TaqMan Analyses.** To examine whether either DDEP- or NMPP-elicited heme depletion inhibited PB-mediated CYP2B induction by blocking transcriptional activation of the CYP2B gene, CYP2B1 and CYP2B2 mRNA expression was quantitated in treated hepatocytes by quantitative PCR analysis. In preliminary studies, all TaqMan assays were optimized to yield greater than 90% PCR efficiency, and oligos for each amplicon were used to determine the specificity of each assay. Table 2 verifies that the CYP2B1 and CYP2B2 probes and primers were specific for each mRNA. That is, although a Ct of 25.05 ± 0.18 was detected with the CYP2B1 oligo when CYP2B1 primers and probes were subjected to 40 PCR cycles, no corresponding Ct signal with the CYP2B2 oligo was detected within this set limit. Conversely, CYP2B2 primers and probes yielded no detectable signal with the CYP2B1 oligo. These results thus exclude any cross-reactivity between these assays, in spite of the high level of sequence conservation between CYP2B1 and CYP2B2.

As expected, PB induced both CYP2B1 and CYP2B2 mRNA expression 50-fold over control levels in hepatocyte cultures within 24 h of treatment (Table 3). This induction was not affected by the exogenous heme concentration used in these studies. Furthermore, neither DDEP- nor NMPP-elicited heme depletion had any statistically significant effect (p > 0.05) on PB-induced CYP2B1 or CYP2B2 mRNA expression at 24 h (Table 3), a time point at which significant CYP2B protein induction (~10-fold) is detected (Fig. 2). Not surprisingly, 20 μM heme treatment had little additional effect on these values. Collectively, these findings indicate that the impaired CYP2B protein induction in PB-treated hepatocytes after acute heme depletion is not due to faulty PB-mediated transcription of CYP2B mRNA. Furthermore, parallel Northern blot analyses of hepatic RNA from PB- and PB/DDEP-treated rats not only yielded similar results to that of cultured hepatocytes but also revealed that the CYP2B mRNA transcript was comparable in size after both treatments. This excluded the possibility that heme depletion affects CYP2B mRNA elongation. A similar inference was drawn from profiles of these mRNA samples in a microarray analysis with a CYP2B10 probe that recognizes the CYP2B1/2 nucleotide sequence beyond the 3'-coding region (K. Hayes, C. Bradfield, X.-M. Han, M. Liao, and M. A. Correia, unpublished observations).

**Effect of DDEP- or NMPP-Mediated Heme Depletion on PB-Induction of CYP2B and Total Hepatocellular Protein Synthesis.** Since heme depletion distinctly impaired PB-mediated CYP2B1 protein induction without appreciably altering CYP2B mRNA expression, the possibility existed that heme regulates CYP2B protein induction at a posttranscriptional level. In principle, such impairment could be exerted through inhibition of the initiation/elongation of CYP2B translation and/or decreased stability of the CYP2B protein. To determine the effect of heme depletion on PB-mediated CYP2B synthesis, the rate of [35S]Met/Cys incorporation into immunoprecipitable CYP2B protein was monitored in DDEP- and NMPP-treated hepatocytes in the presence or absence of PB or PB/heme (20 μM). As expected, PB increased the rate of [35S]Met/Cys incorporation into immunoprecipitable CYP2B protein 5-fold (Fig. 3, open columns). Notably, consistent with the marked decrease in total CYP2B immunoreactive protein, the rate of [35S]Met-incorporation into immunoprecipitable CYP2B protein was markedly decreased in PB/DDEP- or PB/NMPP-treated cells, and heme repletion significantly reversed this effect. However, this effect was not unique to CYP2B hemoproteins, because...
the rate of [35S]Met incorporation into total hepatocellular protein was also markedly reduced in these heme-depleted cells, and heme supplementation similarly blocked this effect (Fig. 3, cross-hatched columns). Indeed, comparable reduction in [35S]Met incorporation into total hepatocellular protein from control levels after treatment of cells with either DDEP or NMPP, revealed that the heme depletion resulting from inhibited heme synthesis and/or enhanced P450 heme destruction was responsible for the global reduction in protein synthesis. In spite of this suppression of de novo protein synthesis, the assay of total lysate protein revealed no significant differences in total hepatocellular protein content between the various treatment groups (data not shown).

Because this global inhibition of de novo protein synthesis could result from DDEP- or NMPP-induced cytotoxicity, the integrity of the cultured hepatocytes was monitored by the ToxiLight assay. As can be seen, minimal (<3.5% of cellular AK complement) toxicity was detected in the medium at 24 h, irrespective of treatment (Fig. 4), and remained so even after 3 days of culture (data not shown). These findings, coupled with the lack of any appreciable effect on either CYP2B1/2 mRNA levels (Table 3) or total hepatocellular protein content (data not shown), strongly exclude cytotoxicity as a possible cause for the marked reduction in de novo hepatocellular protein synthesis.
Effect of DDEP or NMPP on the PB-induced CYP2B1 and CYP2B2 mRNA content in primary cultured rat hepatocytes

For experimental details of cell treatments, see Fig. 2. Cells were harvested at 24 h after PB treatment for the isolation of mRNA. Values (mean ± S.D.; n = 3) are expressed as the percentage of corresponding PB values (59,956 ± 11,704 and 9215 ± 2648 for CYP2B1 and CYP2B2, respectively) at 24 h.

<table>
<thead>
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<th>Treatment</th>
<th>CYP2B1 mRNA</th>
<th>CYP2B2 mRNA</th>
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<tr>
<td>Control</td>
<td>1.70 ± 0.80</td>
<td>0.50 ± 0.40</td>
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<td>PB</td>
<td>100\textsuperscript{*}</td>
<td>100\textsuperscript{*}</td>
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<tr>
<td>PB/heme</td>
<td>122 ± 1.80\textsuperscript{a,d}</td>
<td>118 ± 3.40\textsuperscript{a,d}</td>
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<td>74.6 ± 16.8\textsuperscript{a,b,c,d}</td>
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<td>DDEP/PB/heme</td>
<td>106 ± 22.3\textsuperscript{a,d}</td>
<td>80.6 ± 15.8\textsuperscript{a,b,c,d}</td>
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<tr>
<td>NMPP</td>
<td>1.50 ± 0.30</td>
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<td>NMPP/PB</td>
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<td>NMPP/PB/heme</td>
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<td>125 ± 27.1\textsuperscript{b,d}</td>
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</table>

\(a p < 0.01 \) and \(b p < 0.05\) compared with the corresponding control group; \(c p < 0.05\) compared with the corresponding PB/heme group; and \(d \) not statistically different from the corresponding PB group.

Effect of DDEP or NMPP on CYP2B2 syntheses in primary rat hepatocyte cultures. For experimental details of cell treatment, see Fig. 2. Cells were harvested 24 h after PB treatment for the preparation of lysates. Values (mean ± S.D.; n = 3) are expressed as the percentage of the corresponding PB value for \(^{35}\)S incorporation (cpm per milligram of protein per hour) into total cellular protein (cross-hatched columns) or CYP2B2 immunoprecipitates (open columns) at 24 h as described under Materials and Methods. Statistically significant differences between values at (a) \(p < 0.01\) and (b) \(p < 0.05\) compared with the corresponding PB group (PB alone); (c) \(p < 0.01\) and (d) \(p < 0.05\) compared with corresponding PB/heme group (PB/heme alone); and (e) \(p < 0.01\) and (f) \(p < 0.05\) compared with corresponding groups without heme treatment.

**Fig. 3.** Effect of DDEP and NMPP on total protein and PB-induced CYP2B2 syntheses as determined by the radiolabel incorporation assay. Protein synthesis is expressed as a percentage of the corresponding PB value. Values (mean ± S.D.; n = 3) are expressed as the percentage of the corresponding PB value for \(^{35}\)S incorporation (cpm per milligram of protein per hour) into total cellular protein (cross-hatched columns) or CYP2B2 immunoprecipitates (open columns) at 24 h as described under Materials and Methods.

**Fig. 4.** Assessment of relative cytotoxicity of DDEP or NMPP treatments of primary cultured rat hepatocytes. For experimental details of cell treatment, see Fig. 2. At the time corresponding to 24 h after PB treatment, media from the variously treated hepatocyte cultures were collected and assayed for AK activity by the ToxiLight assay procedure (see Materials and Methods). Values were expressed as relative luminescence units (RLU; mean ± S.D.; n = 3) and compared with a positive control consisting of AK released by cultured hepatocytes upon damage by repeated freeze thawing.

**Effect of DDEP or NMPP on eIF2\(\alpha\) Phosphorylation.** Rapid control of gene expression is frequently exerted at the initiation of translation via eIF2\(\alpha\) phosphorylation (eIF2\(\alpha\)P) by one of the several now known active Ser/Thr eIF2\(\alpha\) kinases, including a heme-sensitive kinase (heme-regulated inhibitor; HRI). Such eIF2\(\alpha\) phosphorylation is known to virtually shut down translational initiation (Chen and London, 1995; Kaufman, 1999; Ron, 2002; Harding et al., 2003, and references therein; see Discussion). The resulting suppression of global protein synthesis is reversible by inhibition of the relevant eIF2\(\alpha\) kinase and/or eIF2\(\alpha\) dephosphorylation. To determine whether the observed reduction of hepatic protein synthesis and hence of CYP2B induction were triggered by eIF2\(\alpha\) phosphorylation, the relative levels of eIF2\(\alpha\) and eIF2\(\alpha\)P were determined at various times after DDEP and NMPP treatment of hepatocytes (Fig. 5). As illustrated by the relative ratio of eIF2\(\alpha\)P/eIF2\(\alpha\) at 0 h (Fig. 5), some basal eIF2\(\alpha\) phosphorylation apparently exists in cultured hepatocytes. As expected, thapsigargin, a well known ER-stress inducer used as a positive control, increased this level. This basal hepatic eIF2\(\alpha\) phosphorylation was also further increased 30 min after DDEP treatment (data not shown), and remained significantly elevated over the entire 24-h treatment period (Fig. 5). Like DDEP, NMPP also increased eIF2\(\alpha\) phosphorylation at 3 to 6 h, but this effect declined at 8 h (data not shown) and returned to normal at 24 h. Most relevantly, both effects were significantly prevented by inclusion of as low as a 5 \(\mu\)M concentration of exogenous heme (Fig. 5).

Effect of DDEP or NMPP on Grp78 Levels. Induction of ER stress activates the ER-resident eIF2\(\alpha\) kinase PERK, and this in turn induces the expression of ER-chaperones such as Grp78 (Bip) (Ron, 2002; see Discussion). To determine whether the eIF2\(\alpha\) phosphorylation observed after either DDEP or NMPP treatment was due to PERK activation, the relative content of Grp78 was assayed as an ER stress marker. As illustrated (Fig. 7), no appreciable increase in Grp78 content was detected after treatment of hepatocytes with either DDEP or NMPP.

**Discussion**

The findings described above reveal that DDEP- or NMPP-induced depletion of heme from hepatocytes markedly impairs PB-mediated induction of immunoreactive and func-
tional CYP2B protein, and this effect is reversible by heme supplementation. However, consistent with previous findings (Srivastava et al., 1989; Sinclair et al., 1990), this impairment was not due to the inhibition of PB-induced transcriptional activation of the CYP2B gene but rather due to inhibition of CYP2B translation. A similar impairment of PB-mediated induction of CYP2B1/2 protein has been observed in succinylacetone-treated rat hepatocytes and is proposed to reflect a plausible heme requirement for P450 mRNA translation (Sinclair et al., 1990). Jover et al. (2000) reported that PB-mediated transcriptional and translational activation of CYP2A5 but not CYP2B10 was impaired in a PBGD-knockout mouse model whose hepatic heme synthesis was reduced by 50% as monitored by the rate of [14C]ALA incorporation into heme. We have found that DDEP or NMPP treatment of hepatocytes caused a substantially more profound (>75%) inhibition of hepatic heme synthesis that was sufficiently severe to inhibit de novo rat hepatic CYP2B2 and total protein syntheses. Yet, even this profound heme depletion had no effect on PB-mediated transcriptional activation of rat CYP2B1 and CYP2B2 genes. Our studies, which used a model of an even more severe hepatic heme pool depletion and highly sensitive quantitative PCR analyses that could distinguish between the highly homologous CYP2B1 and CYP2B2 genes, support previous conclusions that heme is not required for PB-mediated transcriptional activation of either P450 (Srivastava et al., 1989; Sinclair et al., 1990; Jover et al., 2000), as proposed previously (Dwarki et al., 1987; Rangarajan and Padmanaban, 1989).

Our findings also revealed that DDEP- and NMPP-elicited heme depletion of hepatocytes was associated with marked suppression of their basal de novo protein synthesis. Thus,
the observed impairment of PB-induced CYP2B translation was most likely due to global suppression of hepatic protein translation, rather than a selective/direct effect of either DDEP or NMPP on CYP2B induction. More relevantly, both these effects were heme-reversible. Indeed, the rapid and significantly enhanced phosphorylation of hepatic eIF2α, a hallmark of attenuated protein synthesis in eukaryotic cells (Chen and London, 1995; Kaufman, 1999; Harding et al., 2003, and references therein), after either DDEP- or NMPP-mediated heme depletion, and its blockade by heme supplementation, suggest that heme may also control hepatic protein translation.

Translational control leading to the suppression of global protein synthesis is an effective way to preserve cellular energy and nutrients. Moreover, unlike the transcriptional control of gene expression that can take hours to manifest, it provides a rapid and reversible mechanism to control gene expression. Thus, it plays a critical role in the regulation of various vital cellular processes, including growth stimulation, cell cycle progression, differentiation, hypoxia, ER stress, and heme deficiency (Chen and London, 1995; Kaufman, 1999; Harding et al., 2003, and references therein). In all these instances, this control is exerted at the initiation of translation through phosphorylation of eIF2αSer51. For productive translational initiation, GTP-bound eIF2α has to bind initiator Met-tRNA before transferring Met-tRNA to the 40S ribosomal subunit to form the 43S preinitiation complex. GTP hydrolysis before elongation releases GDP-bound eIF2α. eIF2α-phosphorylation for fresh initiation cycles requires GDP exchange with GTP, a reaction catalyzed by the relatively less abundant, rate-limiting regulatory subcomplex eIF2B. eIF2α phosphorylation stabilizes the GDP-eIF2α-eIF2B complex, thereby sequestering eIF2B, and preventing eIF2B-catalyzed GTP-GDP exchange and subsequent regeneration of GTP-eIF2 for fresh initiation cycles.

eIF2α phosphorylation is catalyzed by four independent mammalian eIF2α kinases (GCN2, mammalian interferon inducible RNA-dependent kinase, PERK, and HRI) that are activated by various cellular stresses (Chen and London, 1995; Kaufman, 1999; Harding et al., 2003, and references therein). Of these, GCN2 and mammalian interferon inducible RNA-dependent kinase can be excluded a priori as relevant as neither contains heme regulatory motifs nor is heme dependent. Of direct interest is HRI, the eIF2α kinase regulated by heme through two heme-binding domains and activated by heme-deficiency (Chen and London, 1995; Rafie-Kolpin et al., 2000). Erythroid HRI has been extensively characterized both structurally and functionally, and a HRI-knockout mouse model confirms the phenotype (Lu et al., 2001). Limiting heme availability in erythroid cells shuts off globin synthesis and hence hemoglobin formation. In addition to heme-deficiency, arsenite, heat shock, and osmotic stress, but not ER stress or nutrient deprivation, activate HRI in reticulocytes and fetal liver nucleated erythroid progenitors (Lu et al., 2001). A mouse liver ortholog has been purified and shown to be hemin-sensitive. Cloning of this enzyme has revealed a 69.7-kDa protein with two heme regulatory motifs, exhibiting 94% amino acid sequence identity to rat brain and rabbit reticulocyte HRIα, respectively (Berlanga et al., 1998). HRI mRNA has been documented in fetal rat liver erythroid cells and adult rat hepatocytes (Mellor et al., 1994; Lu et al., 2001). Thus, it is plausible that a hepatic HRI or HRI-like eIF2α kinase activated by DDEP or NMPP-induced heme depletion of hepatocytes is responsible for the translational control. Accordingly, heme supplementation blocks its activity, thereby normalizing protein synthesis. Collaborative studies with Dr. J.-J. Chen (Massachusetts Institute of Technology, Cambridge, MA) are currently underway to explore this particular possibility.

PERK, the ER-resident eIF2α kinase activated by protein misfolding induced by ER stress, unfolded protein response, and hypoxia (Kaufman, 1999; Ron, 2002), could also be involved in the translational control of CYP2B in hepatocytes. PERK most likely contributes to the basal, heme-insensitive eIF2α phosphorylation observed in untreated hepatocytes, possibly triggered by "cell culture" stresses. DDEP treatment could further enhance this hepatic eIF2α phosphorylation through heme destruction of several P450s with consequent structural damage to the ER-bound proteins sustained over 24 h due to both heme stripping and DDEP-derived ethyl radicals. In contrast, the case for PERK activation by NMPP is less persuasive, because NMPP inhibits heme synthesis without directly destroying P450 heme or protein. Thus, its potential for ER-stress induced PERK activation would be considerably lower than that of DDEP. This consideration together with the comparable, heme-reversible suppression of hepatic protein translation observed with both heme depletors, argues against PERK participation in these events. Furthermore, PERK activation is usually associated with marked induction of the ER-chaperone Grp78 (Bip) (Ron, 2002). However, although abundant basal Grp78 content is immunochemically detectable in untreated hepatocytes, it is not detectably increased during the course of DDEP- or NMPP-induced heme depletion as expected after PERK activation. Examination of additional ER stress markers may be required to determine whether PERK is responsible for such eIF2α phosphorylation.

The specific kinase(s) responsible for eIF2α phosphorylation in heme-depleted hepatocytes remain(s) to be identified. We find it intriguing that each temporal eIF2α phosphorylation profile, albeit exhibiting a relatively rapid onset, is very different: being sustained over 24 h after DDEP, but transient (declining after 6–8 h; data not shown) after NMPP. In spite of these differences in eIF2α phosphorylation profiles, the corresponding extent and persistence of hepatic protein translational suppression and its heme-reversibility profile are comparable after each treatment. Particularly noteworthy is that the suppression of protein translation in NMPP-treated hepatocytes persists for 24 h, long after eIF2α phosphorylation is restored to basal levels. Similar temporal dissociation of eIF2α phosphorylation and suppression of protein synthesis has also been reported in transient mouse brain ischemia associated with middle cerebral artery occlusion (Althausen et al., 2001).

Alternatively, such prolonged suppression of protein synthesis in heme-depleted hepatocytes could also reflect the lack or inactivation of additional heme-dependent translational factors, such as eEF2, which catalyzes the translocation of peptidyl-tRNA from the A to P site on the ribosome during nascent peptide chain elongation (Carlberg et al., 1990; Ryazanov et al., 1991). Phosphorylation of eEF2-Thr56 blocks this function and arrests protein elongation. However, in contrast to the marked eIF2α phosphorylation, eEF2 phos-
phosphorylation was not altered in either DDEP- or MNPP-treated hepatocytes. These findings tend to exclude hepatic eEF2 as a plausible factor in the translational control of protein synthesis after acute heme depletion.

Conceivably, enhanced CYP2B proteolytic degradation could also contribute to the impaired CYP2B protein expression in DDEP or MNPP heme-depleted hepatocytes. Accordingly, an appreciable fraction of CYP2B protein generated under these heme-deficient conditions, being deprived of heme for hemoprotein assembly, could succumb to proteolytic degradation. However, the marked suppression of de novo hepatic protein synthesis after acute hepatic heme depletion, precluded accurate assessment of the relative extent of CYP2B protein degradation through pulse-chase turnover analyses.

Collectively, our findings indicate that acute heme depletion of hepatocytes results in suppression of de novo protein synthesis and consequent impairment of CYP2B1/2 induction, most likely via eIF2α phosphorylation-mediated shutdown of translational initiation. These effects are heme-reversible but the eIF2α kinase responsible for this effect remains to be identified. Clinically, acute hepatic heme deficiency is a hallmark of the genetic diseases known as the hepatic porphyrias that are characterized by acute abdominal and neurological symptoms that are heme-reversible. Acute heme deficiency in neuronal cells is proposed to disrupt neuronal signaling pathways that may contribute to the neurological symptoms (Zhu et al., 2002a). We have previously documented that acute hepatic heme depletion in rats also alters the serotonergic tone by impairing transcriptional and translational regulation of hepatic TDO, the rate-limiting enzyme in tryptophan catabolism (Litman and Correia, 1983, 1985; Ren and Correia, 2000). Our current findings indicate that translational suppression of other short-lived hepatic proteins may similarly affect other key homeostatic functions and/or alter the flux of additional neuroactive amino acids, thereby also contributing to the clinical symptoms of these acute heme-deficient states.

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