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Repression of Phenobarbital-Dependent CYP2B1 mRNA Induction by Reactive Oxygen Species in Primary Rat Hepatocyte Cultures

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ABSTRACT

Xenobiotic-metabolizing cytochrome P-450 (P-450) enzymes not only play a pivotal role in elimination of foreign compounds but also contribute to generation of toxic intermediates, including reactive oxygen species, that may elicit cellular damage if produced excessively. Expression of several xenobiotic-metabolizing P-450 enzymes is induced by phenobarbital (PB). Pronounced induction is observed for the rat CYP2B1 isoform. A primary rat hepatocyte culture system was used to investigate whether reactive oxygen species might modulate PB-dependent CYP2B1 induction. In cells cultivated for 3 days with 1.5 mM PB, substantial CYP2B1 mRNA induction was observed (100%). Addition of H_2O_2 or of the catalase inhibitor 3-amino-1,2,4-triazole (AT) to the medium repressed induction to approximately 30% (at 1 mM H_2O_2 and 2 mM AT, respectively). Accordingly, treatment of hepatocytes with PB and the glutathione precursor *N*-acetylcysteine (NAC) led to enhanced PB-dependent induction (to over 1000% at 10 mM NAC). In primary hepatocyte cultures transfected with a *CYP2B1* promoter-luciferase construct containing approximately 2.7 kilobase pairs of the native *CYP2B1* promoter sequence, PB-dependent reporter gene activation was repressed by AT and stimulated by *N*-acetylcysteine. Furthermore, a 263-base pair *CYP2B1* promoter fragment encompassing the phenobarbital-responsive enhancer module conferred suppression of PB-dependent luciferase expression by AT and activation by NAC in a heterologous *SV40*-promoter construct. In summary, these data demonstrate a regulatory mechanism that is dependent on the cellular redox status, which modulates CYP2B1 mRNA induction by PB on the transcriptional level, thus representing a feedback mechanism preventing further P-450–dependent production of reactive oxygen intermediates under oxidative stress.

The superfamily of cytochrome P-450 (P-450) proteins consists of enzymes involved in metabolism of an array of endogenous and xenobiotic compounds (Nelson et al., 1996). Processes mediated by P-450 enzymes include steroidogenesis and metabolism of cholesterol, vitamin D3, and fatty acids but also biotransformation of innumerable drugs, environmental chemicals, and pollutants. Four of the cytochrome P-450 families (CYP1, CYP2, CYP3, and CYP4) are of relevance in drug and xenobiotic metabolism. Expression of many members of xenobiotic-metabolizing P-450 isoforms is induced by their substrates, thus allowing adaptation of metabolism. The liver, constituting the major site of biotransformation of xenobiotics, exhibits abundant basal expression and induction of xenobiotic-metabolizing cytochrome P-450 isoforms. Phenobarbital (PB) is regarded as the prototype of structurally unrelated inducers that affect expression of a specific spectrum of genes (reviewed by Honkakoski and Negishi, 1998). Although members of several P-450 subfamilies are inducible by PB (CYP2A, CYP2B, CYP2C, CYP3A, CYP4B), phenobarbital-dependent induction of the rat CYP2B1 isoform is most pronounced [up to 100-fold or greater increase of CYP2B1 protein in rat liver microsomes (Waxman and Azaroff, 1992)]. Several laboratories recently demonstrated the pivotal role of a phenobarbital-responsive enhancer found in the distal region of the rat *CYP2B2* promoter in conferring *CYP2B2* gene activation by phenobarbital (Trottier et al., 1995; Park et al., 1996; Stoltz et al., 1998). The relevance of a homologous region in conveying PB-dependent CYP2B induction was later confirmed in the mouse *Cyp2b10* promoter (Honkakoski and Negishi, 1997). The ability to confer induction has recently been localized to a 51-bp sequence in the mouse *Cyp2B10* promoter, termed the phenobarbital-responsive enhancer module (*PBREM*; reviewed by Honkakoski and Negishi, 2000). Sequences highly homologous to the mouse *PBREM* are found in the human *CYP2B6*

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ABBREVIATIONS: P-450, cytochrome P-450; PB, phenobarbital; bp, base pair(s); *PBREM*, phenobarbital-responsive enhancer module; AT, 3-amino-1,2,4-triazole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; mdr, multidrug resistance transporter; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; NF1, nuclear factor 1; *XRE*, xenobiotic-responsive element.

and the rat *CYP2B1/CYP2B2* promoters (Sueyoshi et al., 1999).

In addition to participating in elimination of xenobiotics, P-450 activity may also contribute to generation of potentially toxic products. Many procarcinogens are metabolically activated by P-450 isoforms to ultimate carcinogens. CYP2B isoforms in particular are involved in activation of aflatoxin B1 or cyclophosphamide to genotoxic metabolites (Chang et al., 1993). Furthermore, reactive oxygen species (ROS) are released during the catalytic reaction cycle of P-450 (Heinemeyer et al., 1980). It is assumed that activity of P-450 systems constitutes a major source of intracellular ROS in the liver (Bondy and Naderi, 1994; Puntarulo and Cederbaum, 1998), indicating that excessive P-450 activity resulting from P-450 induction may lead to cellular damage caused by generation of noxious metabolites or ROS. Accordingly, adaptive mechanisms, repressing P-450 induction during cellular oxidative stress and thus lowering P-450-dependent production of active intermediates, would be expected to minimize cellular damage. Indeed, several lines of evidence suggest that ROS might play a negative regulatory role in CYP2B induction by phenobarbital. Induction of CYP2B isoforms by phenobarbital, as well as basal and induced expression of other P-450 isoforms, have been shown to be decreased by inflammatory processes in the liver (reviewed by Morgan, 1997), during which also enhanced liberation of ROS occurs. Several proinflammatory cytokines, including interleukin 6 and tumor necrosis factor-α, and also other cytokines (e.g., epidermal growth factor), that may lead to intracellular ROS production via physiological signaling (Bae et al., 1997; reviewed by Morel and Barouki, 1999), have also been identified as mediators of repression of phenobarbital-dependent CYP2B1 induction in rat hepatocytes (Aubrecht et al., 1995; Clark et al., 1995; Carlson and Billings, 1996).

To investigate whether ROS might affect CYP2B1 induction, we used as a model system primary rat hepatocyte cultures in which inducibility of CYP2B1 expression by phenobarbital was retained. Although treatment of hepatocytes with H_2O_2 and the catalase inhibitor 3-amino-1,2,4-triazole (AT) decreased CYP2B1 mRNA induction by PB, induction was markedly enhanced by the antioxidant *N*-acetylcysteine. In primary hepatocytes transfected with a *CYP2B1* promoter-luciferase construct, promoter activity was significantly repressed by 3-amino-1,2,4-triazole and, conversely, enhanced by *N*-acetylcysteine. These data, therefore, demonstrate for the first time that CYP2B1 mRNA induction by phenobarbital is modulated in a redox-sensitive manner and that transcriptional activation participates in redox-dependent regulation of CYP2B1 mRNA induction.

Experimental Procedures

Materials. All chemicals were of reagent grade and purchased from commercial suppliers. Collagenase was obtained from Biochrom (Berlin, Germany) and fetal calf serum from PAA (Coelbe, Germany). Cell culture dishes were purchased from Nunc (Wiesbaden, Germany). T4 polynucleotide kinase was from Roche Molecular Biochemicals (Mannheim, Germany). Hybond N nylon membrane and [γ-^{32}P]ATP were obtained from Amersham Pharmacia Biotech (Freiburg, Germany). The transfection reagent Effectene was purchased from Qiagen (Hilden, Germany). Firefly and *Renilla* luciferase expression plasmids as well as the Dual Luciferase Reporter Assay kit were obtained from Promega (Mannheim, Germany).

Hepatocyte Culture and Induction Experiments. Primary hepatocytes were isolated from adult male Wistar rats (180–220 g) by collagenase perfusion (Seglen, 1976). Hepatocyte suspensions showed viabilities >90% as determined by trypan blue exclusion. Cells were plated onto culture dishes at a density of 8.6×10^4 cells/cm^2 in MX-82 medium (Hoffmann et al., 1989) supplemented with 10% fetal calf serum. After an initial attachment period of 3 h at 37°C in a humidified atmosphere of 10% CO_2 and 90% air, the medium was replaced with serum-free MX-83 medium (Hoffmann et al., 1989) that lacked arginine, but contained 1 μM insulin and 20 μM hydrocortisone hemisuccinate. The cells were further cultured (37°C, humidified atmosphere of 10% CO_2/90% air) in the absence or presence of 0.75 or 1.5 mM PB, with or without oxidants (0.1–2 mM H_2O_2 or 0.5–5 mM 3-amino-1,2,4-triazole) or antioxidants (1–20 mM *N*-acetylcysteine or 100 μM tocopherol acetate), as indicated, for up to 3 days. Media changes were performed daily.

RNA Isolation and Northern Blot Analysis. Total cellular RNA was isolated by guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) and separated through formaldehyde agarose gels (20 μg/lane). The gels were stained with ethidium bromide to ensure equal loading of lanes, and RNA was subsequently blotted onto Hybond N nylon membranes by capillary transfer. RNA blots were hybridized to the rat *CYP2B1* gene-specific oligonucleotide probe 5′-GGTTGGTAGCCGGTGTGA-3′ (corresponding to bases 66–49 of exon 7 region, GeneBank L00318), which had been end-labeled by T4-polynucleotide kinase using γ-^{32}P-ATP (Omiecinski et al., 1985).

Control hybridizations were performed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide probe (5′-CAGGATGCATTGCTGACAATCTTGA-3′, corresponding to nucleotides 520–496 of the rat *GAPDH* gene; European Molecular Biology Laboratory no. X02231). Mdr1b mRNA was detected by hybridization to a specific oligonucleotide probe as described previously (Ziemann et al., 1999). RNA expression was quantified with the use of the Fujix BAS 1500 Bio-Imaging Analyzer (Fujix, Tokyo, Japan).

Hepatocyte Transfection and Luciferase Reporter Gene Assay. Firefly and *Renilla* luciferase expression plasmids (pGL3-Basic, pGL3-Control, pGL3-Promoter, and pRL-CMV) were purchased from Promega. A fragment representing approximately 2.7 kilobase pairs of the native rat CYP2B1 promoter sequence was amplified by PCR from genomic rat hepatocyte DNA using primers corresponding to sites of the promoter sequence published by Shaw et al., 1996 (bases −2648 to −2623 and 29 to 4, respectively). To facilitate cloning, the primers contained an additional 5′ *Nhe*I recognition site and a three-base overhang: 5′-AAAGCTAGCAGGTTCCCAACCATTTGGCCTACGAA-3′ (forward) and 5′-ATTGCTAGCTCCTGGTGTAACCACGGTAGACTTCA-3′ (reverse).

The obtained PCR fragment was digested with *Nhe*I and ligated into the *Nhe*I site of the pGL3-Basic vector. The sequence of the resulting promoter firefly luciferase construct designated as *pGL3C2B1* was verified by sequence analysis employing standard sequencing primers of the pGL3 plasmid and internal primers of the insert. A 263-bp *CYP2B1* promoter fragment bearing the *PBREM* region was obtained by introduction of an *Eco*RI site into *pGL3C2B1* by PCR and subsequent digestion with *Bam*HI and *Eco*RI. This 263-kilobase-pair fragment, representing the *CYP2B1* promoter section from −2413 to −2151, was treated with T4-DNA polymerase to yield blunt ends and further cloned into the *Sma*I site of the pGL3-Promoter vector containing the *SV40* promoter, yielding the heterologous promoter construct *pGL3CS1*. Sequence and orientation of the insert were confirmed by sequencing using the standard pGL3 primers. Endotoxin-free maxi-prep kits (Macherey-Nagel, Düren, Germany) were employed for plasmid purification following amplification in *Escherichia coli*. Primary adherent rat hepatocyte cultures, plated at a density of 8.6×10^4 cells/cm^2 onto six-well plates (Nunc), were transiently transfected 24 h after seeding with 0.5 μg of one of the firefly luciferase constructs (pGL3-Basic, pGL3-Control, pGL3-Promoter, *pGL3C2B1*, or *pGL3CS1*) and 0.03 μg of the *Renilla* lu-

ciferase construct pRL-CMV using the Effectene reagent (QIAGEN) according to the manufacturer's instructions. Six hours later the medium was replaced with fresh MX-83 medium with or without 1.5 mM phenobarbital or modulators of induction (2 mM 3-amino-1,2,4-triazole, 10 mM *N*-acetylcysteine) as indicated. The medium was exchanged 24 h after transfection; 48 h after transfection, hepatocytes were lysed with 150 μl of lysis buffer (Dual Luciferase Reporter Assay kit, Promega) and firefly and *Renilla* luciferase activity were measured in 20 μl of cell lysate according to the Promega protocol using a Berthold Lumat LB 9501 luminometer (Berthold Technologies, Bad Wildbad, Germany). *Renilla* luciferase activity was used to normalize the transfection efficiency in all culture wells.

Results

In the present study, we addressed whether phenobarbital-dependent CYP2B1 induction might be subject to repression by reactive oxygen species. Experiments were performed with primary rat hepatocyte cultures in which induction of CYP2B1 by phenobarbital, a hepatocyte-specific function lost in hepatoma cell lines, was retained (Aubrecht et al., 1995). Maximal CYP2B1 mRNA induction was observed in hepatocyte cultures exposed to 0.75 or 1.5 mM PB for three days, which is consistent with previous reports (Aubrecht et al., 1995; Kietzmann et al., 1999). Therefore, primary hepatocytes were treated with PB and possible modulators of CYP2B1 mRNA induction for 3 days.

Repression by H_2O_2 of Phenobarbital-Dependent CYP2B1 mRNA Induction. To examine the possible role of oxidants in modulation of CYP2B1 mRNA induction by PB, hepatocyte cultures were treated with H_2O_2, a highly diffusible molecule that easily penetrates through cellular membrane structures. A H_2O_2 concentration of 1 mM was previously shown to up-regulate mRNA expression of antioxidant enzymes (catalase, Mn-superoxide dismutase; Röhrdanz and Kahl et al., 1998) as well as expression of the rat multidrug resistance *mdr1b* gene (Ziemann et al., 1999) in primary rat hepatocyte cultures. Thus, in the present study, initial concentrations of H_2O_2 from 0.1 to 2 mM were added to the medium once daily for 3 days. PB-dependent CYP2B1 mRNA induction was determined in Northern blot analyses. Induction, defined as the difference between PB-dependent CYP2B1 mRNA expression and basal CYP2B1 mRNA levels in the absence of PB, was set to 100% in cultures treated with PB in the absence of H_2O_2. Indeed, H_2O_2 elicited repression of PB-dependent CYP2B1 mRNA induction in a concentration-dependent manner (Fig. 1). This effect reached statistical significance at concentrations of 0.5 mM H_2O_2 and above, amounting to mRNA levels of about 37% at 0.5 mM H_2O_2, 30% at 1 mM H_2O_2, and 12% at 2 mM H_2O_2, respectively (Fig. 1, A and B). Expression of GAPDH mRNA in control hybridizations remained stable under H_2O_2 (Fig. 1B). On the other hand, we observed stimulation of *mdr1b* gene expression by H_2O_2 in the same culture system (shown for 0.5 mM H_2O_2 in Fig. 1C), with maximal stimulation at 0.5 to 1 mM H_2O_2 (Ziemann et al., 1999). Thus, H_2O_2 significantly repressed PB-dependent CYP2B1 mRNA induction at concentrations that did not result in a deleterious general repression of mRNA expression.

In an alternative approach to test the role of ROS in modulation of CYP2B1 induction, the catalase inhibitor AT, which increases intracellular ROS levels by interfering with decomposition of H_2O_2 (Starke and Farber, 1985) was employed. AT, applied to the culture medium at 0.5 to 5 mM concentrations, also led to a concentration-dependent repression of PB-dependent CYP2B1 mRNA induction, amounting to approximately 34% of control induction at 1 mM AT and 30% at 2 mM AT (Fig. 2). On the other hand, up to 5 mM AT

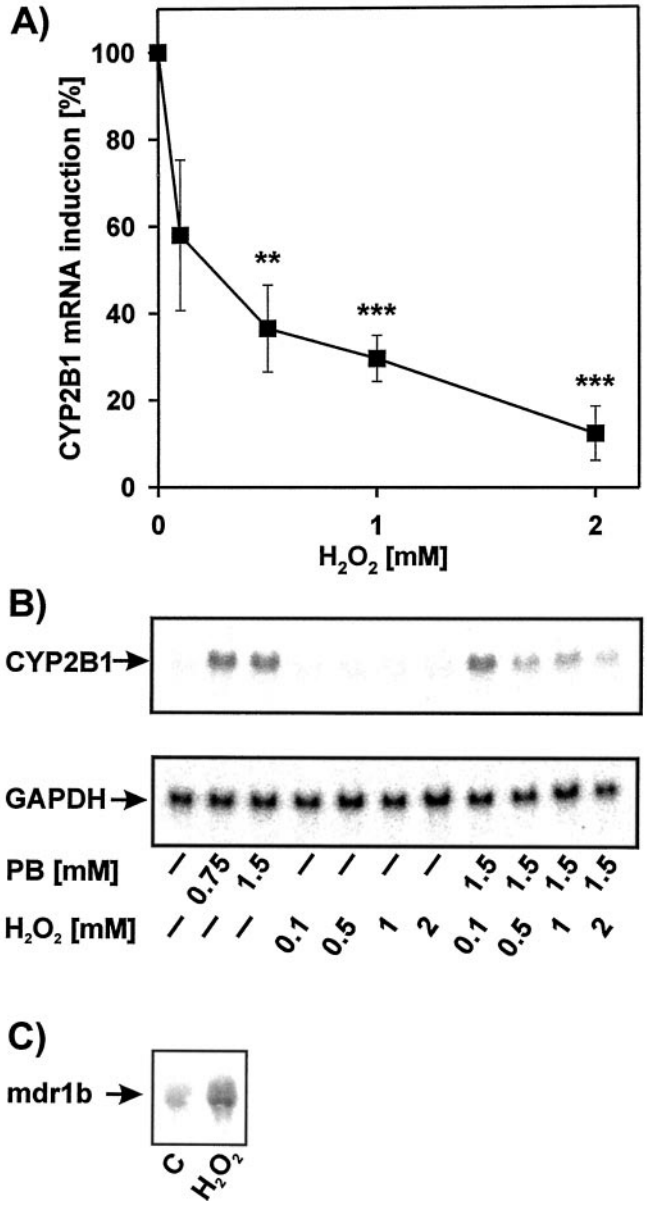

Fig. 1. Repression by H_2O_2 of PB-dependent CYP2B1 mRNA induction in primary rat hepatocyte cultures. Hepatocytes were cultured for 3 days in the presence or absence of PB with or without H_2O_2. H_2O_2 was added to the medium at initial concentrations of 0.1 to 2 mM. Total RNA (20 μg/lane) was subjected to RNA blot analysis. A, CYP2B1 mRNA expression was determined in Northern blots by hybridization to a ^{32}P-labeled CYP2B1-specific oligonucleotide probe as described under *Experimental Procedures*. Hybridization signals were quantified using a Bio-Imaging Analyzer system. Induction was defined as the difference between expression under 1.5 mM PB and expression in the absence of PB. Induction with 1.5 mM PB in the absence of H_2O_2 was set to 100%. Data represent mean values ± S.E.M. of three or five (0 mM and 1 mM H_2O_2) independent experiments. Significant difference between PB and PB+H_2O_2 (**$p < 0.01$; *** $p < 0.001$; Student's t test). B, representative Northern blot. After hybridization to a CYP2B1-specific probe, the blot was rehybridized to a rat GAPDH-specific oligonucleotide probe. C, stimulation of mdr1b mRNA expression as an example of mRNA up-regulation by H_2O_2 (0.5 mM); representative Northern blot. c, control cells without H_2O_2.

stimulated mdr1b mRNA expression (data not shown). Thus, repression of CYP2B1 mRNA induction by the catalase inhibitor AT is consistent with the notion that ROS (H_2O_2) act as regulators of PB-dependent CYP2B1 mRNA induction.

Enhancement by *N*-Acetylcysteine of Phenobarbital-Dependent CYP2B1 mRNA Induction. Because H_2O_2 and 3-amino-1,2,4-triazole repressed PB-dependent CYP2B1 mRNA induction, it was hypothesized that an increase in hepatocyte antioxidant capacity might enhance CYP2B1 mRNA induction or counteract ROS-dependent inhibition of induction. Therefore, the effect of the antioxidant *N*-acetylcysteine (NAC), a precursor of glutathione, on PB-dependent CYP2B1 mRNA induction was examined. In hepatocytes concomitantly treated with PB and 1 to 20 mM NAC for 3 days, a dramatic concentration-dependent enhancement of CYP2B1 mRNA induction was observed that was maximal (approximately 1000%) between 10 and 20 mM NAC (Fig. 3, A and B). The incubation of hepatocytes with PB and a 100 μM concentration of the antioxidant tocopherol acetate (vitamin E), a membrane-bound radical scavenger, also led to a moderate increase (to approximately 200%) of PB-dependent CYP2B1 mRNA induction (Fig. 3C).

The marked enhancement of PB-dependent CYP2B1 mRNA induction by 10 mM NAC was abolished by 1 mM concentrations of the catalase inhibitor AT (Fig. 4), again supporting the conclusion that the extent of CYP2B1 mRNA induction by PB was regulated by the hepatocyte redox status.

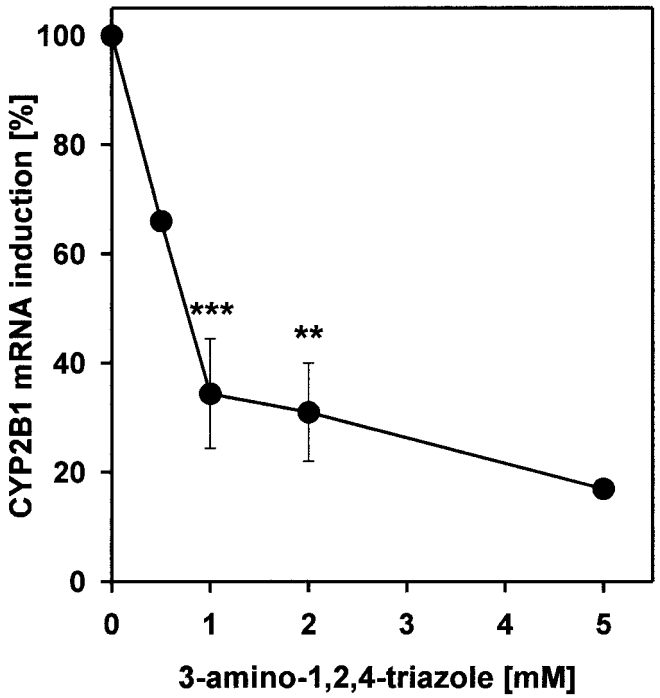

Fig. 2. Repression of PB-dependent CYP2B1 mRNA induction by AT in primary rat hepatocyte cultures. Hepatocytes were cultured for 3 days in the presence of 1.5 mM PB with or without 0.5 to 5 mM AT. CYP2B1 mRNA expression was determined by Northern blot analysis as outlined in Fig. 1. Induction of CYP2B1 mRNA was defined as the difference between CYP2B1 mRNA expression under PB and basal mRNA expression in the absence of PB. Induction without AT was set to 100%. Data represent mean values of two (0.5 mM and 5 mM AT) or of seven (1 mM AT) or three (2 mM AT) independent experiments ± S.E.M. Significant difference between cells treated with PB only and cells treated with PB+AT (**$p < 0.01$; ***$p < 0.001$; Student's t test).

Repression of PB-dependent *CYP2B1* Promoter Activation by Aminotriazole and Enhancement of Promoter Activation by *N*-Acetylcysteine. To investigate whether the modulation of PB-dependent mRNA induction by ROS and *N*-acetylcysteine might be based on regulation of *CYP2B1* transcription, a *CYP2B1* promoter-luciferase re-

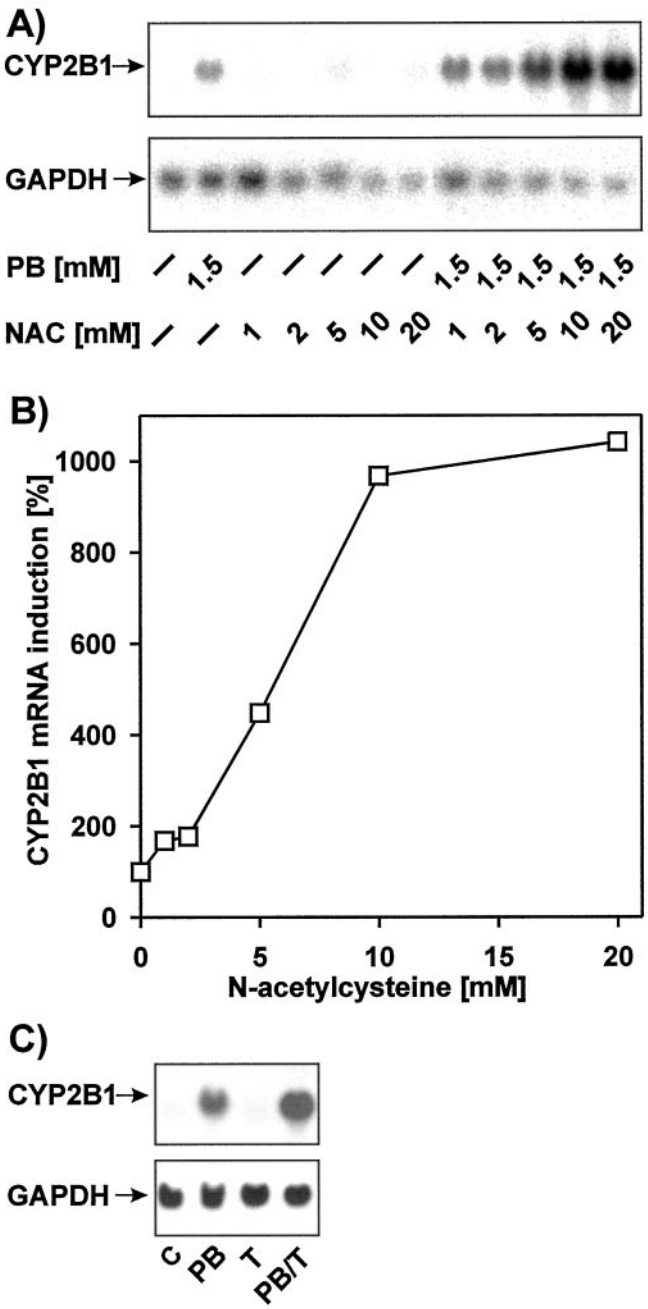

Fig. 3. Enhancement of PB-dependent CYP2B1 mRNA induction by *N*-acetylcysteine or tocopherol acetate in primary rat hepatocyte cultures. Hepatocytes were cultured for 3 days in the presence or absence of PB with or without further addition of NAC (1–20 mM) or tocopherol acetate (T, 100 μM) to the medium. A, enhancement of PB-dependent CYP2B1 mRNA induction by NAC (representative Northern blot analysis). B, CYP2B1 mRNA hybridization signals were quantified by Bio-Imaging Analyzer analysis of blot A. Induction was defined as the difference between CYP2B1 mRNA expression in the presence of PB and mRNA expression in the absence of PB. Induction without NAC was set to 100%. C, enhancement of PB-dependent CYP2B1 mRNA induction by tocopherol acetate (representative Northern blot analysis).

porter gene construct was generated, bearing the native *CYP2B1* promoter sequence from +30 to −2648 (Fig. 5A). Primary rat hepatocyte cultures were transiently transfected with the original luciferase vector without the *CYP2B1* promoter insert (pGL3-Basic) or with the *CYP2B1* promoter-luciferase construct (*pGL3C2B1*) 24 h after seeding of hepatocytes and subsequently treated with 1.5 mM PB or with a combination of PB and AT and/or NAC for 48 h. Firefly luciferase activity was determined in hepatocyte lysates as a measure of promoter activation. Although AT or NAC alone did not influence promoter activation, PB significantly induced reporter gene expression (Fig. 5B). Mean promoter activation by PB alone was set to 100%. In accordance with modulation of PB-dependent CYP2B1 mRNA induction, 2 mM AT repressed PB-dependent promoter activation to approximately 25%, whereas 10 mM NAC enhanced promoter activation to about 900%. In combination with PB and NAC, AT counteracted enhancement by NAC of promoter activation (Fig. 5B). Thus, these results support the conclusion that modulation of CYP2B1 mRNA induction by AT and NAC occurs on the transcriptional level.

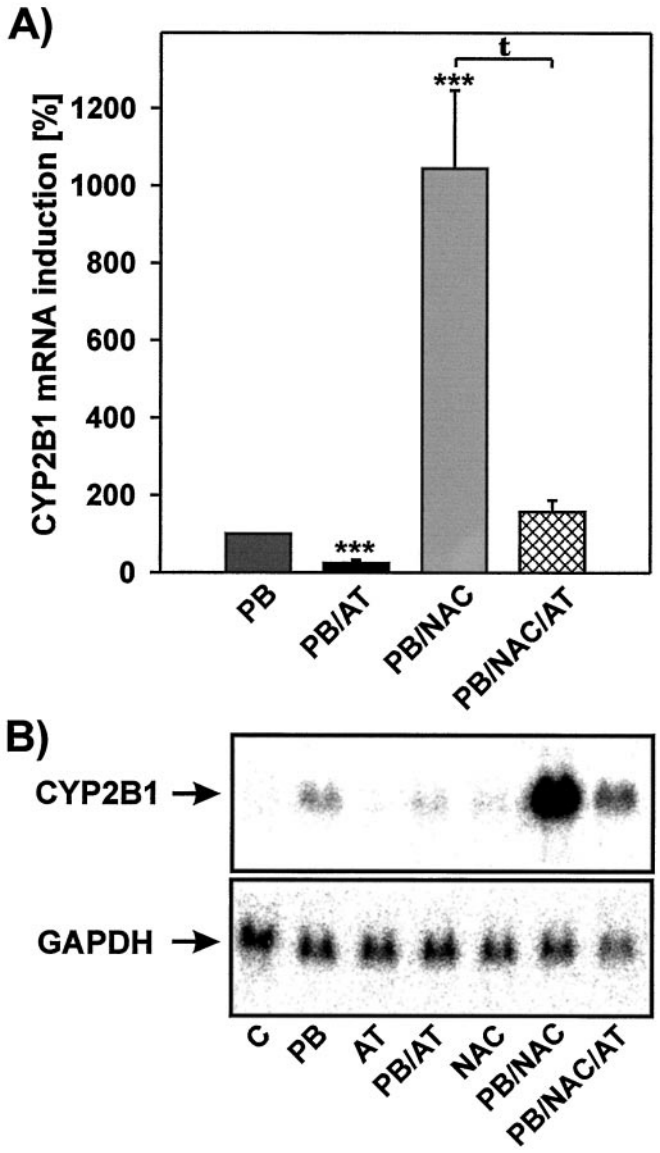

Fig. 4. Aminotriazole-dependent abolishment of enhanced CYP2B1 induction under *N*-acetylcysteine in primary rat hepatocyte cultures. Cells were cultured in the presence or absence of 1.5 mM PB with or without 1 mM AT or 10 mM NAC for 3 days. CYP2B1 mRNA expression was determined by Northern blot analysis. A, hybridization signals were quantified by a Bio-Imaging Analyzer. Induction was defined as the difference between expression under 1.5 mM PB and expression in the absence of PB. Induction with 1.5 mM PB in the absence of AT or NAC was set to 100%. Data represent mean values ± S.E.M. of five (PB/AT and PB/NAC/AT) or 12 (PB and PB/NAC) independent experiments. Significant difference between PB and PB + modulator of induction (***$p < 0.001$). t, significant abolishment by AT of NAC-dependent enhancement of CYP2B1 induction ($p < 0.05$), Student's *t*-test. B, representative Northern blot. After hybridization to a CYP2B1-specific oligonucleotide probe, the blot was rehybridized to a rat GAPDH-specific probe.

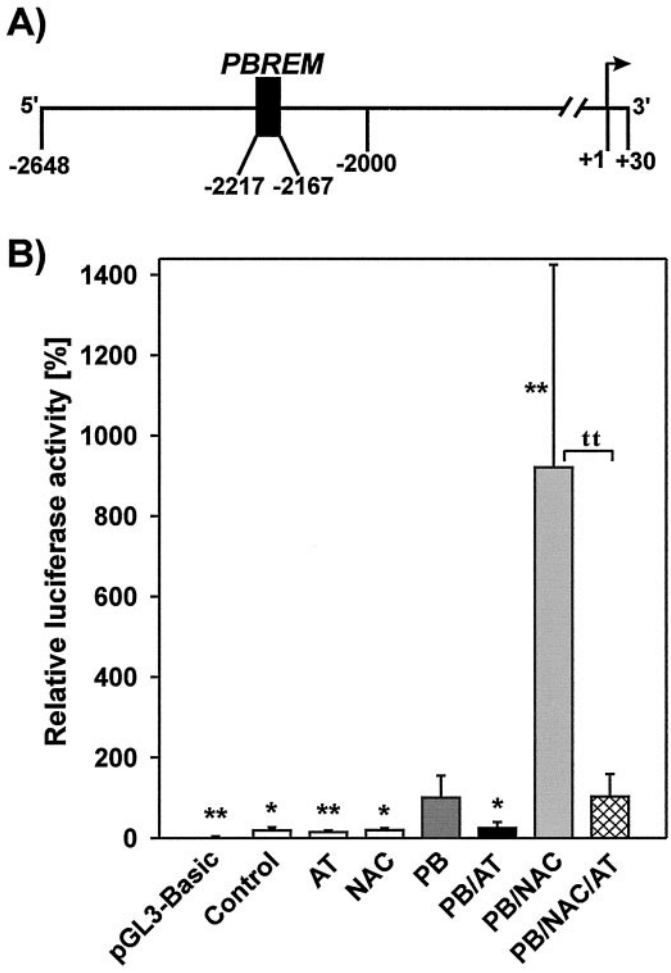

Fig. 5. Modulation of PB-dependent *CYP2B1* promoter activation by 3-amino-1,2,4-triazole and *N*-acetylcysteine. Primary rat hepatocytes cultured for 1 day were transiently cotransfected with the pRL-CMV *Renilla* luciferase vector and with the original pGL3-Basic vector (pGL3-Basic) or with the *pGL3C2B1* construct, in which luciferase transcription was driven by the native *CYP2B1* promoter sequence, respectively. Six hours after transfection, cells were incubated with 1.5 mM PB, 2 mM AT, or 10 mM NAC, as indicated, for an additional 48 h, and firefly luciferase activity was subsequently determined in cell lysates as described under *Experimental Procedures*. A, schematic representation of the *CYP2B1* promoter fragment ligated into the pGL3-Basic luciferase vector, yielding the *pGL3C2B1* construct. *PBREM*, phenobarbital-responsive enhancer module. The arrow indicates the transcription start site. B, relative firefly luciferase activity in lysates of transfected hepatocytes, standardized according to *Renilla* luciferase activity. Control, cells transfected with the *pGL3C2B1* construct, but not treated with a modulator of CYP2B1 expression. Data represent mean values of five parallel culture wells ± S.D. of an experiment representative of three independent hepatocyte preparations. Significant difference from cells treated with PB (*$p < 0.05$;** $p < 0.01$, Student's *t* test). tt, significant abolishment by AT of the NAC-dependent enhancement of gene activation by PB, $p < 0.01$.

Further experiments were conducted to specify the promoter region possibly involved in conveying responsiveness to AT or NAC. The PB-responsive enhancer module (*PBREM*) and related sequences have been identified as the major PB-dependent regulatory regions in the mouse *Cyp2b10* promoter and in *CYP2B* promoters of human and rat (reviewed by Waxman, 1999). A 263-bp fragment of the *CYP2B1* promoter, containing the 51-bp region corresponding to the mouse *PBREM* (Fig. 6A), was linked to a heterologous promoter-luciferase expression plasmid, and its responsiveness to PB and to PB in combination with AT and/or NAC was examined in transiently transfected hepatocytes.

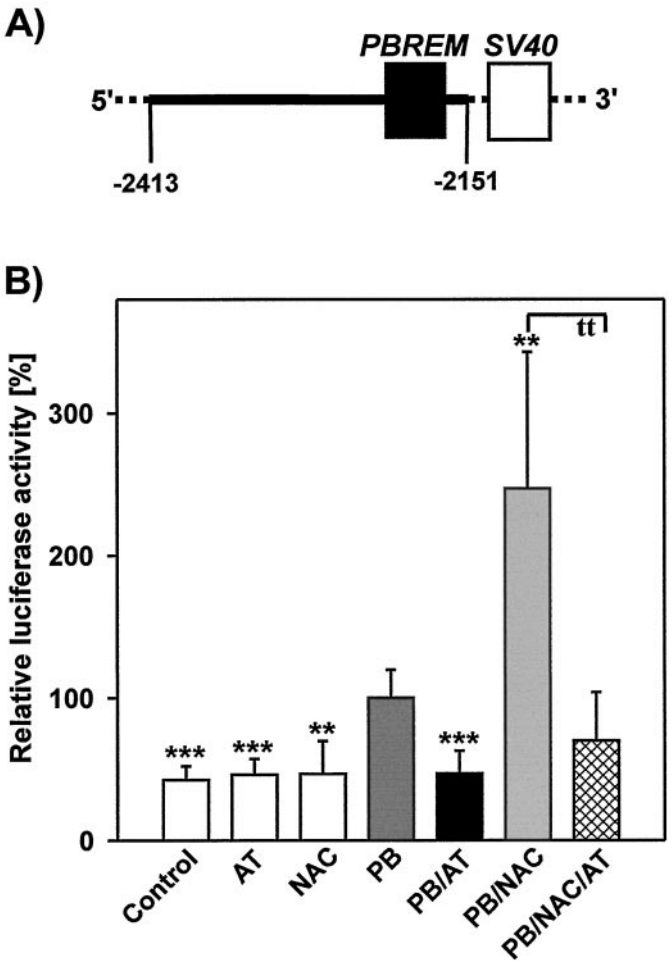

Fig. 6. A 263-bp *CYP2B1* promoter fragment confers inducibility by PB and redox-dependent modulation of promoter activation when linked to the heterologous *SV40* promoter. Primary rat hepatocyte cultures were cotransfected with the *Renilla* luciferase expression vector pRL-CMV and with the *pGL3CS1* construct in which the firefly luciferase was driven by the *SV40* promoter and a 263-bp sequence of the distal *CYP2B1* promoter, indicated under A. Transfected cells were incubated with 1.5 mM PB or modulators of CYP2B1 induction (2 mM AT, 10 mM NAC) for 48 h. A, schematic representation of the enhancer/promoter area of the firefly luciferase expression vector *pGL3CS1*. The *CYP2B1* promoter fragment is indicated by a continuous line, while adjacent sequences of the pGL3-Promoter vector are dotted. *PBREM,* phenobarbital-responsive enhancer module. B, relative firefly luciferase activity, standardized according to *Renilla* luciferase activity in hepatocyte lysates. Control, cells transfected with *pGL3CS1*, but not treated with a modulator of *CYP2B1* gene expression. Data represent mean values of six parallel culture wells ± S.D. of representative experiment. Significant difference from cells treated with PB (**$p < 0.01$; ***$p < 0.001$, Student's t test). tt, significant abolishment by AT of the NAC-dependent enhancement of gene activation by PB, $p < 0.01$.

As expected, promoter activation by PB was retained with the heterologous construct (Fig. 6B), although basal promoter activity (control, without PB) was higher than with the reporter gene construct *pGL3C2B1*. Furthermore, AT elicited repression of PB-dependent activation, whereas NAC markedly enhanced PB-dependent luciferase expression (Fig. 6B). Again, AT significantly counteracted enhancement by NAC of promoter activation (Fig. 6B). Thus, these results demonstrate that the 263-bp *CYP2B1* promoter region that contains the *PBREM* confers responsiveness to redox-dependent modulation of *CYP2B1* gene induction.

Discussion

Extreme exposure to ROS is deleterious to the cell, resulting either in necrosis or apoptosis. A transient or moderate increase in ROS, however, may allow reconstitution of the cellular redox status, either by direct detoxification processes or by long-term adaptive alterations in gene expression (Morel and Barouki, 1999). Thus, ROS-dependent repression of activation of genes encoding enzymes contributing to ROS production, as well as induction of proteins participating in ROS detoxification, may be regarded as part of a general defense response to maintain cellular redox homeostasis. In the present study, H_2O_2 and the catalase inhibitor 3-amino-1,2,4-triazole, which interferes with decomposition of intracellular H_2O_2, repressed PB-dependent induction of CYP2B1 mRNA.

Cytochrome P-450-dependent systems are regarded as a major source of intracellular production of free radicals in hepatocytes, even in the uninduced state (Bondy and Naderi, 1994; Puntarulo and Cederbaum, 1998). Under conditions of enhanced exposure to ROS (e.g., during inflammation), marked induction of P-450 enzymes, resulting in further P-450-dependent ROS production, would be expected to result in oxidant damage to the cell. Therefore, adaptive mechanisms by which ROS might prevent induction of several P-450 isoforms in the liver seem feasible. Indeed, the transcriptional suppression by H_2O_2 of basal expression of the CYP1A1 and CYP1A2 isoforms and of their β-naphthoflavone-dependent induction has been described previously (Barker et al., 1994). The present study demonstrates ROS-dependent repression of induction by a different type of inducer (phenobarbital) of a different P-450 isoform (CYP2B1). At this point, the questions of whether PB-dependent induction of other xenobiotic-metabolizing genes apart from *CYP2B1* might also be subject to regulation by ROS and whether overlapping mechanisms might be crucial in redox-dependent regulation of P-450 induction remain to be resolved.

The xenobiotic-responsive elements (XREs), to which heterodimers of the aryl hydrocarbon receptor and the aryl hydrocarbon receptor nuclear translocator protein bind, play a pivotal role in conferring induction of CYP1A enzymes by aromatic hydrocarbons (Hankinson, 1995). The ability of AT and NAC in the present study to modulate promoter activation in *CYP2B1* promoter-reporter gene constructs transiently transfected into primary hepatocytes supports the conclusion that regulation of CYP2B1 induction by ROS occurs on the transcriptional level. However, in contrast to *CYP1A* gene regulation, XRE regions have not been shown to be of relevance in mediating PB-dependent induction (Wax-

man, 1999); rather, the major PB-responsive region in *CYP2B* promoters is a PB-responsive enhancer (Trottier et al., 1995; Park et al., 1996; Stoltz et al., 1998), which has been delimited to the *PBREM* in the mouse *Cyp2b10* promoter (reviewed by Honkakoski and Negishi, 2000). To further specify the *CYP2B1* promoter region conveying modulation of PB-dependent gene activation by AT and NAC, a promoter construct was employed in which a 263-bp fragment of the *CYP2B1* promoter, encompassing the 51-bp *PBREM*, was placed in front of the heterologous *SV40* promoter (Fig. 6A). Responsiveness not only to PB but also to AT and NAC was retained in hepatocytes transfected with the heterologous promoter construct, which suggests that either the *PBREM* itself or functional synergism between the *PBREM* and a promoter region in the vicinity of the *PBREM* is involved in conferring redox-sensitive regulation of PB-dependent *CYP2B1* promoter activation.

The cellular redox status may affect transcription by different pathways. First, ROS might lead directly to oxidation of sensitive moieties of transcription factors, (e.g., of thiol groups), thus resulting in possible alterations in transcription factor DNA binding, in translocation to the nucleus, or in *trans*-activation (reviewed by Morel and Barouki, 1999). The *PBREM* region contains a nuclear factor 1 (NF1) binding motif, flanked by two nuclear receptor binding sites (reviewed by Honkakoski and Negishi, 2000). Interestingly, studies conducted with hepatoma cells suggest that oxidation of NF1 protein binding to a proximal NF1 site in the *CYP1A1* promoter, which acts in synergy with *XRE* regions, constitutes the basis of suppression of CYP1A induction by ROS: the NF1/CTF *trans*-activating domain was repressed by oxidative stress, mediated by a critical cysteine (Morel et al., 1999). On the other hand, a different study indicates that the H_2O_2-dependent suppression of CYP1A1 induction is linked to the potential of the *XREs* to confer transcriptional activation by aromatic hydrocarbons (Xu and Pasco, 1998). Further examples of redox-sensitive regulation have been provided for members of the nuclear receptor superfamily (Morel and Barouki, 1999). Although oxidation of a cysteine residue in the nuclear localization signal domain NL1 of the glucocorticoid receptor interferes with nuclear translocation (Okamoto et al., 1999), thiol oxidation in the DNA binding domain of the estrogen receptor seems to constitute the major factor contributing to redox-dependent estrogen receptor regulation (Liang et al., 1998).

Alternatively, ROS may indirectly modulate the activity of transcription factors by leading to an alteration in their phosphorylation status. ROS (H_2O_2) have been shown to enhance protein phosphorylation by reducing protein tyrosine phosphatase and serine/threonine phosphatase activities (Whisler et al., 1995).

Hyperphosphorylation of as-yet-unspecified proteins is known to lead to repression of CYP2B1 mRNA induction by phenobarbital: inhibition of serin/threonine phosphatases (e.g., by okadaic acid) or inhibition of phosphotyrosine phosphatases (e.g., by orthovanadate) both result in inhibition of CYP2B induction (Sidhu and Omiecinski, 1997; Honkakoski and Negishi, 1998; Kawamura et al., 1999). Phosphorylation of members of the nuclear receptor family may interfere with nuclear translocation: phosphorylation of the nuclear orphan receptor NGFI-B, which is induced by nerve growth factor, results in redistribution of a NGFI-B-retinoid-X-receptor complex out of the nucleus, reducing transcriptional activation (Katagiri et al., 2000). Nuclear receptor dimers consisting of the constitutive androstane receptor and the retinoid-X-receptor are thought to play an essential role in PB-dependent transcriptional activation of *CYP2B* genes via the *PBREM* (reviewed by Honkakoski and Negishi, 2000). Nuclear translocation of the constitutive androstane receptor protein into the nucleus was shown to be inhibited by the protein phosphatase inhibitor okadaic acid (Kawamoto et al., 1999), indicating that either hyperphosphorylation of the nuclear receptor protein itself or hyperphosphorylation of a different protein essential for the translocation process interferes with nuclear translocation.

Thus, it may be hypothesized that ROS may repress PB-dependent transcriptional activation of *CYP2B1* gene expression directly, by oxidation of redox-sensitive transcription factors, or indirectly, by leading to an alteration in transcription factor phosphorylation status and/or by interfering with translocation of a transcription factor into the nuclear compartment.

In summary, the present study demonstrates the regulation of PB-dependent *CYP2B1* gene activation by the cellular redox status. Repression of CYP2B1 induction under oxidative stress may be interpreted as part of a general defense strategy that is activated under enhanced exposure to ROS or when the antioxidative status of the hepatocyte is compromised.

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