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Differential regulation of multidrug resistance-associated protein 2 (MRP2) and cytochromes P450 2B1/2 and 3A1/2 in phenobarbital-treated hepatocytes

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Abstract

Multidrug resistance-associated protein 2 (MRP2) is a drug efflux pump found at the biliary pole of hepatocytes. In the present study, we have investigated its expression in response to phenobarbital, a liver tumor promoter known to up-regulate hepatic cytochromes P450 (CYPs), such as CYP2B1/2 and CYP3A1/2. MRP2 mRNA and protein levels were found to be markedly increased in both primary rat and human hepatocytes exposed to phenobarbital. However, features of this up-regulation, especially the dose-response, were different from those of the induction of CYP2B1/2 and CYP3A1/2. In addition, hepatic MRP2 expression remained unaltered in rats treated by phenobarbital that, by contrast, increased CYP2B1/2 and CYP3A1/2 gene expression in the liver. Therefore, MRP2 and CYPs appeared differently regulated in response to phenobarbital in both *in vivo* and *in vitro* situations, suggesting that cellular and molecular mechanisms underlying up-regulation of MRP2 are, at least in part, unrelated to those operating for CYPs. Phenobarbital-related MRP2 induction in primary rat hepatocytes was associated with some phenotypic effects of the barbiturate, such as prolonged cell survival and inhibition of cell proliferation. Phenobarbital also inhibited growth of human hepatoma $HepG_2$ cells and increased their level of MRP2 gene expression. Such results may favor a putative relationship between phenobarbital-mediated MRP2 regulation in cultured liver parenchymal cells and alteration of cell cycle and survival. © 2002 Published by Elsevier Science Inc.

Keywords: Cell proliferation; Cytochrome P450; Drug transport; Hepatocyte; Multidrug resistance-associated protein 2; Phenobarbital

1. Introduction

MRP2, previously called canalicular multispecific organic anion transporter (cMOAT), is a 190 kDa plasma membrane phosphoglycoprotein. It is predominantly expressed at the biliary pole of hepatocytes, but has also been found in kidney and intestine [1,2]. MRP2 is thought to mediate the transport of organic anions, including glucuronate, sulphate and glutathione conjugates, across the canalicular hepatocyte membrane [3]. Mutant rat strains lacking MRP2, such as the transport deficient (TR) Wistar rat and the Eisai hyperbilirubinemic rat (EHBR), therefore display low biliary secretion of conjugates of xenobiotics and endogenous compounds such as bilirubin [4]. Similarly, in humans, the Dubin–Johnson syndrome, an autosomal dominant hereditary disease characterized by a chronic hyperbilirubinemia, is caused by lack of MRP2 due to a mutation in the gene [5].

MRP2 belongs to the ATP-binding cassette (ABC) protein superfamily which comprises other drug efflux pumps such as MRP1, MRP3 and P-glycoprotein [6]. P-glycoprotein, also expressed at the biliary pole of hepatocytes, and MRP1 have been demonstrated to lower intracellular levels of unstructurally-related anticancer drugs, thereby conferring multidrug resistance to tumor cells [6,7]. MRP2 also transports some anticancer drugs, such as vinblastine and cisplatin, out of cells and may therefore also participate to cellular drug resistance [8]. MRP3, found at the sinusoidal pole of hepatocytes, has also

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Abbreviations: CAR, constitutive androstane receptor; CYP, cytochrome P450; DMSO, dimethylsulfoxide; MRP, multidrug resistance-associated protein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PXR, pregnenolone X receptor.

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been shown to handle anticancer compounds such as epipodophyllotoxins [9].

Owing to the major role played by MRP2 in biliary elimination of endogenous compounds and xenobiotics, including anticancer drugs, precise knowledge of factors affecting its hepatic expression is required. Recent data from our group and others have indicated that expression of MRP2 can be altered by compounds known for modulating the expression of drug metabolizing enzymes, thus suggesting a coordinate regulation between these detoxifying pathways in the liver. Indeed, dexamethasone, a potent inducer of hepatic cytochrome P450 3A1/2 (CYP3A1/2) in rats, also strongly enhances MRP2 expression [10,11]. In the same way, phenobarbital, a potent non-genotoxic liver tumor promoter in rodents [12], which markedly up-regulates cytochrome P450 2B1/2 (CYP2B1/2) and CYP3A1/2 in hepatocytes [13], has been found to increase MRP2 gene expression in hepatoma cells [14]. Effects of phenobarbital on hepatic functions have been extensively studied. Tumor promotion due to the barbiturate has been linked, at least partly, to inhibition of apoptosis and alteration of the cell cycle [15], whereas its effects towards CYPs involves nuclear receptors such as the constitutive androstane receptor (CAR) [16]. By contrast, hepatic induction of MRP2 by phenobarbital remains poorly characterized. The present study was therefore designed to gain insights into this regulation, especially by comparing features of phenobarbital-induction of MRP2 and CYPs. Our data demonstrate that MRP2 and CYP2B1/2 and CYP3A1/2 are differentially regulated by phenobarbital, in particular in *in vivo* situations, suggesting that cellular and molecular mechanisms underlying up-regulation of MRP2 are, at least partly, unrelated to those operating for CYPs. We also report that MRP2 induction occurring in primary rat hepatocytes exposed to phenobarbital is associated with known phenotypic changes due to the barbiturate such as enhanced cell survival and inhibition of cell proliferation.

2. Materials and methods

2.1. Chemicals

Phenobarbital and dimethylsulfoxide (DMSO) were purchased from Sigma. Methyl-[^{3}H]thymidine (specific activity 25 Ci/mmol) was provided by Amersham.

2.2. Cell isolation and culture

Hepatocytes from male Sprague–Dawley rats weighing 150–200 g were isolated by liver perfusion as previously described except that a liberase RI solution (Roche) corresponding to a blend of highly purified dissociating enzymes was used instead of a collagenase solution [17]. Human hepatocytes from five adult donors undergoing resection for primary and secondary tumors were obtained by perfusion using a collagenase solution [18]. All experimental procedures complied with French laws and regulations and were approved by the National Ethics Committee. Cells were seeded at a density of 10^5 cells/cm^2 in plastic dishes in Williams'E medium supplemented with 0.2 mg/mL bovine serum albumin, 10 μg/mL bovine insulin and 10% (v/v) fetal calf serum. The medium was discarded 4 hr (for rat hepatocytes) or 24 hr (for human hepatocytes) after cell seeding and hepatocytes were thereafter maintained in serum-free medium supplemented with 10^{-7} M dexamethasone. Treatment with phenobarbital was performed in the serum-free medium using a stock solution of the barbiturate dissolved in sterile phosphate buffer saline.

HepG$_2$ human hepatoma cells were cultured in Williams'E medium supplemented with 10% (v/v) fetal calf serum and dexamethasone and were passaged every week using a trypsin solution.

2.3. Animal treatment

Male Sprague–Dawley rats weighing 180–200 g were treated with phenobarbital (80 mg/kg daily, i.p.) dissolved in saline for 2 days. Control rats received only saline. Rats were killed 48 hr after the first administration of the barbiturate. Livers were removed and immediately stored at −80° until use.

2.4. Isolation of total RNAs and Northern blot analysis

Total RNAs were extracted from cells or liver fragments by the guanidinium thiocyanate/cesium chloride method of Chirgwin *et al.* [19]. For Northern blotting, 10 μg total RNAs were subjected to electrophoresis in a denaturing 6% (v/v) formaldehyde–1.2% (w/v) agarose gel and transferred onto Hybond-N+ nylon filters (Amersham). After pre-hybridization, the filters were hybridized with ^{32}P-labelled cDNA probes, washed, dried and autoradiographed at −80°. MRP2 mRNAs were analyzed with rat and human MRP2 cDNA probes generated by reverse transcription-polymerase chain reaction as previously reported [10,20]. CYP2B1/2, CYP3A1/2 and albumin transcripts were detected using CYP2B1/2, CYP3A1/2 and albumin probes [21–23]. Equal gel loading and efficiency of the transfer were checked using an 18S rRNA probe.

2.5. Preparation of crude membranes and Western blotting

Crude membranes and microsomal fractions were prepared from cultured hepatocytes and liver fragments by differential centrifugation as previously described [24]. Proteins were then separated on a 7.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose

sheets. Sheets were blocked by incubation for 2 hr with Tris-buffered saline containing 3% bovine serum albumin and 10% milk. Then the blocked sheets were incubated with the rabbit anti-rat MRP2 polyclonal antibody RM2 [10], or with the mouse anti-human MRP2 monoclonal antibody M2III-6 [1], or with rabbit anti-CYP2B1/2 polyclonal antibody (Valbiotech). A peroxidase-conjugated anti-rabbit or anti-mouse antibody was thereafter used as secondary antibody. After washing, blots were developed by chemiluminescence using the Amersham ECL detection system. Control blot was performed in parallel using the same protocol, except that anti-MRP2 antibody was replaced by non-immune serum.

2.6. Evaluation of cell proliferation and viability

Proliferation of $HepG_2$ cells and viability of primary hepatocytes were evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described [25]. The MTT-derived blue formazan product formed in viable cells was quantified by its absorbance at 540 nm using a Titertek Multiskan MCC/340 spectrophotometer (Flow Laboratories).

Proliferation of primary hepatocytes was assessed through determination of cellular DNA synthesis. Briefly, primary hepatocytes were incubated for 24 hr with 1 μCi/mL methyl-[^{3}H]thymidine. DNA synthesis was then assessed through scintillation countings of methyl-[^{3}H]thymidine incorporated into DNA; data were normalized to total cellular protein content.

2.7. Statistical analysis

Data of cell proliferation and viability were analyzed using the Student's *t*-test. The criterion of significance between the means was $P < 0.05$.

3. Results

Since MRP2 up-regulation by phenobarbital has been previously demonstrated only in hepatoma cells [14], we first investigated whether their normal counterparts, i.e. primary rat and human hepatocytes, also respond to the barbiturate. As indicated in Fig. 1A, rat and human hepatocytes exposed to 3.2 mM phenobarbital were found to markedly overexpress MRP2 mRNAs when compared to untreated cells. Similarly, they displayed enhanced MRP2 protein expression as assessed by Western blotting (Fig. 1B). Primary hepatocyte cultures therefore appeared as a convenient *in vitro* model for studying MRP2 up-regulation in response to phenobarbital and, owing to the limited availability of primary human hepatocytes, rat hepatocytes were retained for most of the further studies.

Dose-response of MRP2 mRNA induction in primary rat hepatocytes exposed to phenobarbital for 48 hr was then

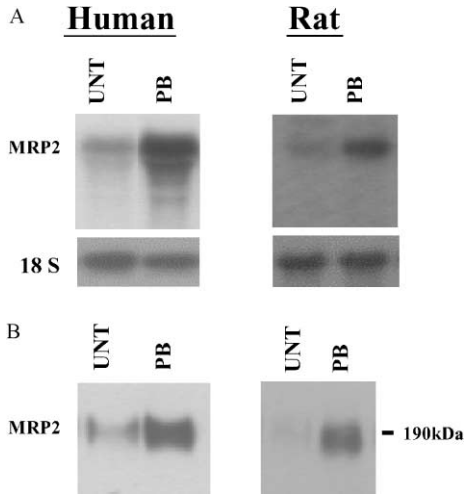

Fig. 1. Up-regulation of MRP2 expression in primary rat and human hepatocytes exposed to phenobarbital. Primary rat and human hepatocytes were cultured in the absence (UNT) or in the presence of phenobarbital (PB). After 48 hr (rat cells) or 96 hr (human cells) treatment, MRP2 mRNA levels (A) and MRP2 protein amounts (B) were analyzed by Northern and Western blotting, respectively, as described in Section 2. Data shown are representative of three independent experiments.

investigated (Fig. 2). Low concentrations of the barbiturate (0.2–0.4 mM) were found to have only marginal, if any, effects on MRP2 mRNA amounts whereas 1.6 mM phenobarbital enhanced MRP2 mRNA levels. A 3.2 mM barbiturate concentration was however required to obtain maximal induction of MRP2 gene expression (Fig. 2). As previously reported [10,26], MRP2 mRNAs of different sizes, especially of 5.5 and 7.5 kb, were evidenced on the blot (Fig. 2); they likely correspond to alternative mRNA splicing variants with different 3′-untranslated region lengths [26] and their relative proportion was not modified in response to phenobarbital whatever concentrations used. When compared to MRP2 mRNAs, CYP2B1/2 mRNAs were maximally induced with a lower concentration of phenobarbital, i.e. 0.2 mM, and the treatment with elevated concentrations resulted in a decrease or a disappearance of the CYP2B1/2 up-regulation (Fig. 2). CYP3A1/2 transcript induction required the use of phenobarbital at 0.8 mM; in contrast to MRP2 induction, higher doses (1.6–3.2 mM) were not more effective. Time-course induction of MRP2 mRNA levels occurring in rat hepatocytes treated with 3.2 mM phenobarbital was determined by Northern blotting and compared to those for CYP2B1/2 and CYP3A1/2 transcripts. As indicated in Fig. 3, MRP2 mRNAs were clearly up-regulated in hepatocytes exposed to the barbiturate for 24 hr; longer exposure (48 hr) did not obviously enhance the induction whereas shorter treatments were uneffective. A similar time-course was found for the phenobarbital-mediated up-regulation of CYP3A1/2 mRNAs. By contrast, an induction of CYP2B1/2 mRNA was detected as early as 8 hr of treatment, whereas a longer exposure (48 hr) markedly down-regulated CYP2B1/2 response (Fig. 3).

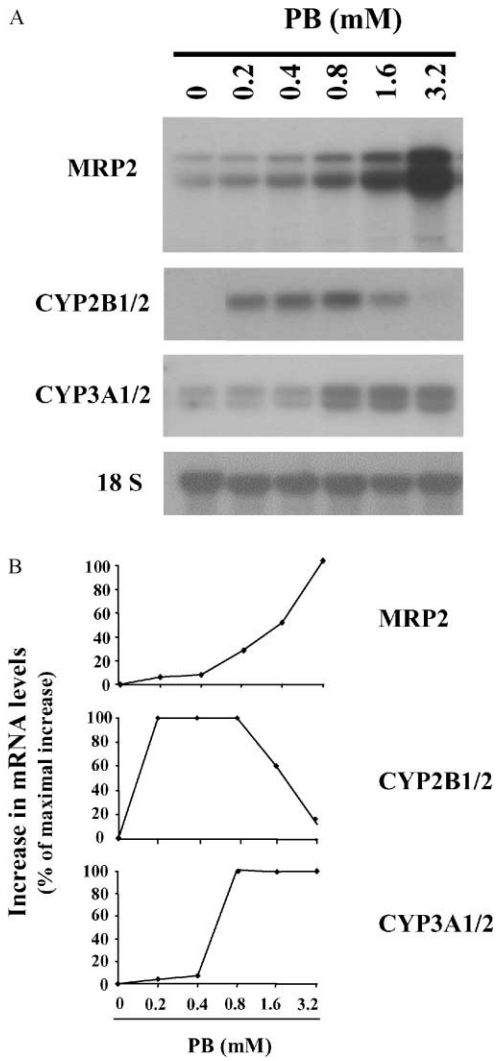

Fig. 2. Concentration-dependent induction of MRP2, CYP2B1/2 and CYP3A1/2 mRNA levels by phenobarbital treatment of primary rat hepatocytes. (A) Each lane contains 10 μg total RNAs isolated from primary rat hepatocytes exposed to various concentrations of phenobarbital (PB) (0–3.2 mM) for 48 hr. RNAs were transferred to Hybond-N+ sheets after electrophoresis and hybridized with MRP2, CYP2B1/2 and CYP3A1/2 and 18S probes. (B) Increases in MRP2, CYP2B1/2 and CYP3A1/2 mRNA levels occurring in response to various concentrations of phenobarbital were quantified by densitometric analysis. Results were expressed as percentage of maximal increase of MRP2, CYP2B1/2 and CYP3A1/2 obtained with 3.2, 0.2 and 0.8 mM phenobarbital, respectively. The data shown are the means of three independent experiments.

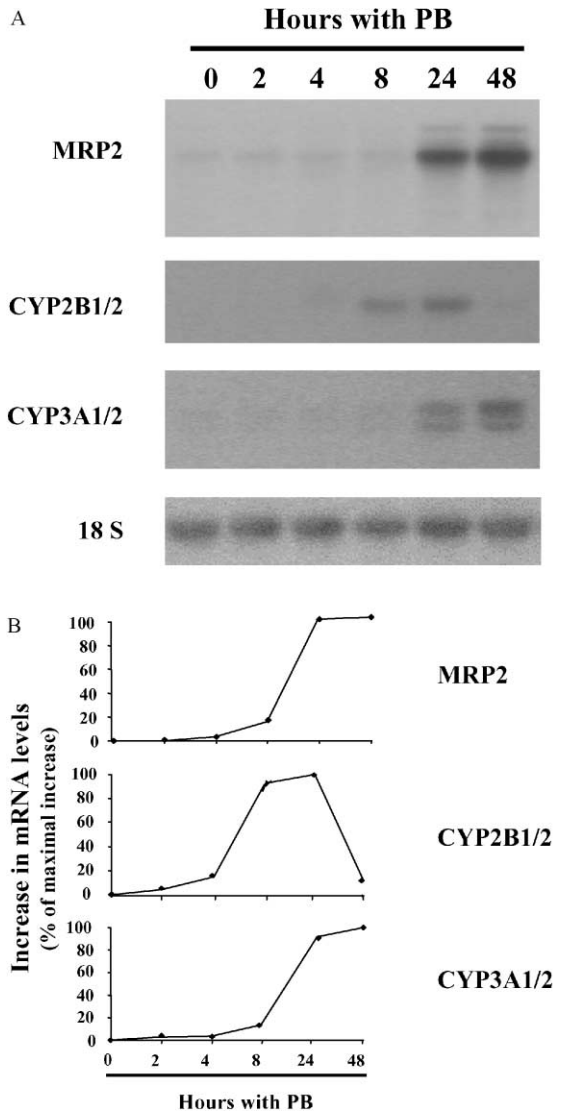

Fig. 3. Time-course of MRP2, CYP2B1/2 and CYP3A1/2 mRNA induction in response to phenobarbital in primary rat hepatocytes. (A) Each lane contains 10 μg total RNAs isolated from primary rat hepatocytes exposed to 3.2 mM phenobarbital (PB) for various periods of time (0–48 hr). RNAs were transferred to Hybond-N+ sheets after electrophoresis and hybridized with MRP2, CYP2B1/2 and CYP3A1/2 and 18S probes. (B) Increases in MRP2, CYP2B1/2 and CYP3A1/2 mRNA levels occurring in response to 3.2 mM phenobarbital for various lengths of time (0–48 hr) were quantified by densitometric analysis. Results were expressed as percentage of maximal increase of MRP2, CYP2B1/2 and CYP3A1/2. The data shown are the means of three independent experiments.

We next determined whether other drug transporters such as P-glycoprotein could be affected by phenobarbital. As shown in Fig. 4, primary rat hepatocytes treated with 3.2 mM phenobarbital for various lengths of times (ranging from 2 to 48 hr) failed to exhibit induction of P-glycoprotein mRNA levels.

Hepatic MRP2 expression was further analyzed both in rats acutely treated with phenobarbital and in their untreated counterparts. Surprisingly, MRP2 mRNA amounts were not increased in the liver of rats treated by the barbiturate for 48 hr whereas, by contrast, CYP2B1/2, CYP3A1/2 and MRP3 mRNA levels were markedly increased (Fig. 5A). Similarly, Western blotting indicated that hepatic MRP2 protein amounts, in contrast to those of CYP2B1/2, were not altered in response to *in vivo* administration of phenobarbital for 48 hr (Fig. 5B). Livers of rats treated with phenobarbital (80 mg/kg daily, i.p.) for 5 days also failed to display enhanced MRP2 gene expression (data not shown).

Since MRP2 expression in primary rat hepatocytes has been linked, at least in part, to phenotypic characteristics of these liver cells, especially the proliferation status [27], and

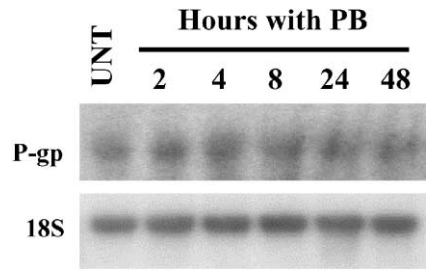

Fig. 4. Effect of phenobarbital on P-glycoprotein mRNA expression in primary rat hepatocytes. Each well contains 10 μg total RNAs isolated from primary rat hepatocytes either untreated (UNT) or exposed to 3.2 mM phenobarbital (PB) for various periods of time (2–48 hr). RNAs were transferred to Hybond-N+ sheets after electrophoresis and hybridized with P-glycoprotein (P-gp) and 18S probes. The data shown are representative of two independent experiments.

since such features have been found to be highly responsive to phenobarbital *in vitro* [28,29], we next investigated whether the MRP2 up-regulation occurring in primary rat hepatocytes exposed to phenobarbital was associated with changes related to differentiation status, cell survival and cell growth. As indicated in Fig. 6, expression of albumin, a liver-specific protein used here as a differentiation marker, was not altered in hepatocytes exposed to 3.2 mM phenobarbital when compared to their untreated counterparts. By contrast, addition of 2% (v/v) DMSO, a

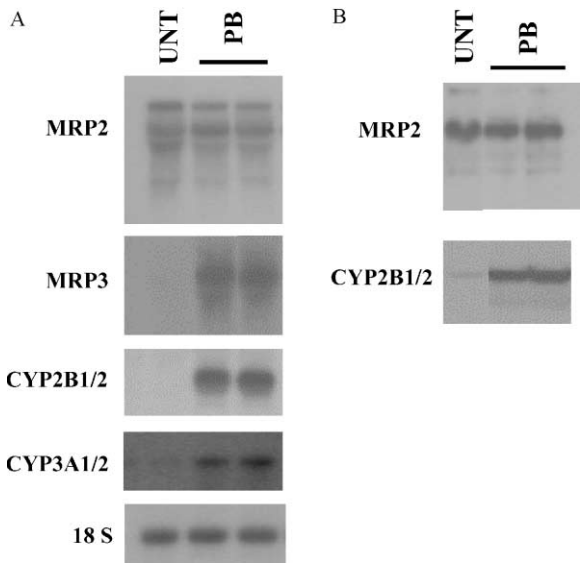

Fig. 5. Effect of phenobarbital treatment on MRP2, MRP3, CYP2B1/2 and CYP3A1/2 expression in rat liver. Rats were either untreated (UNT) or treated with phenobarbital (PB) (80 mg/kg, i.p. daily) for 2 days. Rats were sacrificed 48 hr after the first administration of the barbiturate and the livers were removed. (A) Each lane contains 10 μg total RNAs isolated from the liver of untreated or phenobarbital-treated rats. RNAs were transferred to Hybond-N+ sheets after electrophoresis and hybridized with MRP2, MRP3, CYP2B1/2, CYP3A1/2 and 18S probes. (B) Crude membrane proteins (50 μg per lane) and microsomal proteins (40 μg per lane) isolated from the liver of untreated or phenobarbital-treated rats were analyzed for MRP2 and CYP2B1/2 expression by Western blotting as described in Section 2.

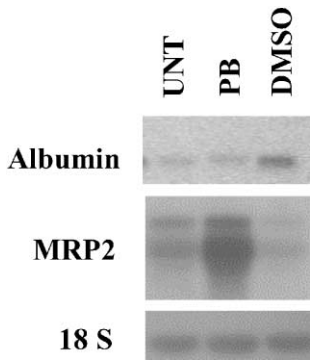

Fig. 6. Expression of the liver differentiation marker albumin in primary rat hepatocytes exposed to phenobarbital. Each lane contains 10 μg total RNAs isolated from primary rat hepatocytes either untreated (UNT) or cultured in the presence of 3.2 mM phenobarbital (PB) or 2 % (v/v) DMSO for 96 hr after cell seeding. RNAs were transferred to Hybond-N+ sheets after electrophoresis and hybridized with albumin, MRP2 and 18S probes. The data shown are representative of two independent experiments.

compound well known to favor maintenance of differentiated liver functions *in vitro* [30], markedly enhanced albumin mRNA levels; DMSO, unlike phenobarbital, however, failed to enhance MRP2 gene expression (Fig. 6). We further studied the effects of phenobarbital on hepatocyte survival using the MTT assay. As shown in Fig. 7A, cell survival was 2-fold more greater in hepatocytes exposed to 3.2 mM phenobarbital for 6 days than that found in their untreated counterparts. This enhanced cell survival with 3.2 mM phenobarbital was associated with up-regulation of MRP2 and CYP3A1/2 mRNAs as demonstrated by Northern blotting (Fig. 7B) whereas the use of 0.4 mM phenobarbital, uneffective on cell survival (Fig. 7A), was required to induce CYP2B1/2 mRNAs. We finally analyzed cell proliferation in primary rat hepatocytes treated by phenobarbital. As shown in Fig. 8, 3.2 mM phenobarbital strongly decreased DNA synthesis during the 48–72 and 72–96 hr culture periods; by contrast, 0.4 mM phenobarbital did not alter thymidine incorporation. This dose-dependent effect of the barbiturate on cell growth was further confirmed using highly proliferating $HepG_2$ cells. Indeed, phenobarbital used at 3.2 mM strongly inhibited proliferation of these human hepatoma cells whereas at 0.4 mM it was totally uneffective (Fig. 9A). In parallel, phenobarbital used at 3.2 mM, unlike the dose of 0.4 mM, markedly enhanced MRP2 mRNA levels in $HepG_2$ cells (Fig. 9B).

4. Discussion

Phenobarbital is a potent liver tumor promoter in rodents which strongly up-regulates expression of drug metabolizing enzymes such as CYP2B1/2 and CYP3A1/2 in normal hepatocytes [12,13]. The present study demonstrates that *in vitro* treatment with this barbiturate also markedly increases MRP2 expression at both mRNA and protein

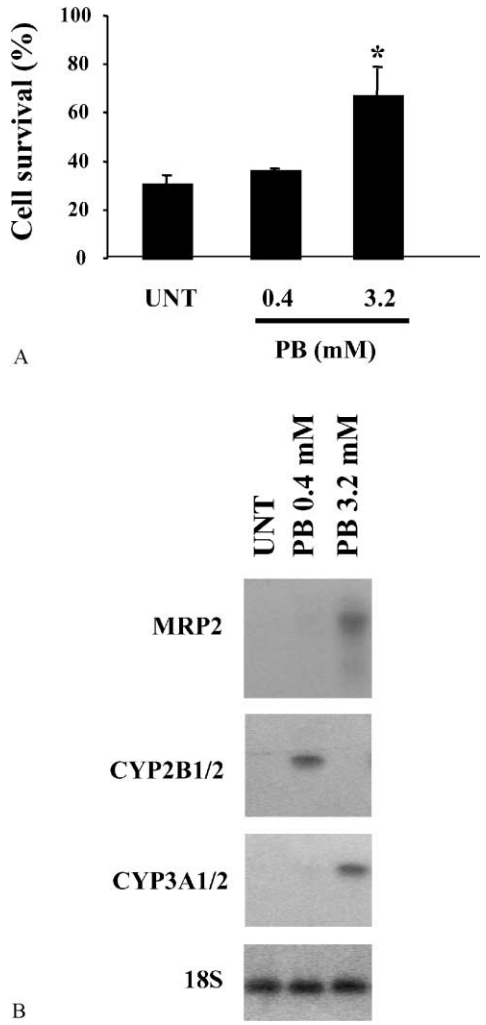

Fig. 7. Effect of phenobarbital on cell survival (A) and MRP2, CYP2B1/2 and CYP3A1/2 mRNA levels (B) in primary rat hepatocytes. Primary rat hepatocytes were cultured in the absence (UNT) or the presence of phenobarbital (PB) used at 0.4 and 3.2 mM for 6 days. (A) Cell survival was estimated using the MTT assay as described in Section 2. It is expressed as % of the initial MTT assay value determined 4 hr after cell seeding. Data shown are the mean ± SD of three independent experiments in duplicate. $^{*}P < 0.05$ when compared to untreated cells. (B) Each lane contains 10 μg total RNAs isolated from hepatocytes either untreated or exposed to 0.4 or 3.2 mM phenobarbital. RNAs were transferred onto Hybond-N+ sheets after electrophoresis and hybridized with MRP2, CYP2B1/2, CYP3A1/2 and 18S probes. The data shown are representative of two independent experiments.

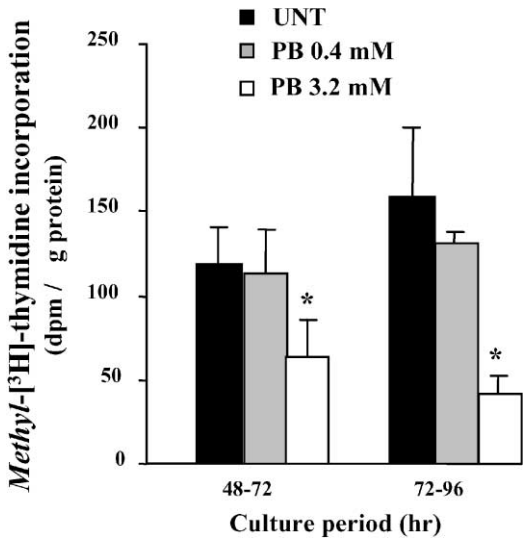

Fig. 8. Effect of phenobarbital on DNA synthesis in primary rat hepatocytes. Primary rat hepatocytes were cultured for 4 days in the absence (UNT) or the presence of phenobarbital (PB) used at 0.4 and 3.2 mM. DNA synthesis was evaluated through measurements of methyl-[^{3}H]thymidine incorporation into DNA during the 48–72 and 72–96 hr culture periods. The results are expressed as dpm/μg protein and are the mean ± SD of three independent experiments in duplicate. $^{*}P < 0.05$ when compared to untreated cells.

levels in human and rat hepatocytes. These data therefore extend the phenobarbital-mediated up-regulation of MRP2 previously reported in cultured rat and human hepatoma cells [14,31] to their normal counterparts and unequivocally include phenobarbital in the growing list of compounds, such as the carcinogen 2-acetylaminofluorene, the chemopreventive agents oltipraz and sulforaphane and the anticancer drug cisplatin, which induce MRP2 expression in cultured hepatocytes [32–34]. However, treatment by phenobarbital does not lead to a general overexpression of biliary drug transporters because P-glycoprotein was not induced in primary rat hepatocytes exposed to the barbiturate. Interestingly, MRP3, a sinusoidal drug transporter known to handle bile acids, was induced in the liver of phenobarbital-treated rats, indicating that this barbiturate can also regulate membrane carrier systems at the vascular pole of hepatocytes. The fact that phenobarbital has been shown to increase uptake of the organic anion sulfobromophtalein into rat hepatocytes supports this conclusion [35].

Comparison of the features of MRP2 induction by phenobarbital with those of CYP up-regulation reveals striking differences. Indeed, analysis of dose-response indicates that low doses (0.2–0.4 mM) of phenobarbital, capable of enhancing CYP2B1/2 expression, failed to enhance MRP2 mRNA levels in primary rat hepatocytes; in the same way, the concentration for which MRP2 was maximally induced, i.e. 3.2 mM, was higher than those required for maximal up-regulation of CYP3A1/2, i.e. 0.8 mM. Analysis of the time-course of the response also indicated a earlier induction for CYP2B1/2, detected 8 hr after the onset of the treatment, than for MRP2, which was clearly observed after 24 hr of exposure to the barbiturate; in addition, longer treatment (48 hr) by the barbiturate resulted in the disappearance of CYP2B1/2 mRNA induction whereas that of MRP2 transcripts was fully preserved. Marked differences were also observed with phenobarbital-treated rat livers since such rats failed to display altered hepatic MRP2 mRNA and protein levels whereas they exhibited up-regulation of CYP2B1/2 and CYP3A1/2. In addition, phenobarbital induced MRP2 in hepatoma $HepG_2$ cells whereas CYP2B6 and CYP3A4, considered as human orthologs of rat CYP2B1/2 and CYP3A1/2, have

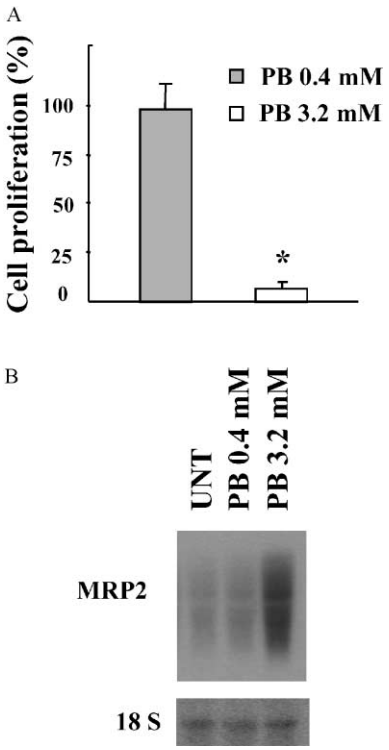

Fig. 9. Effect of phenobarbital on cell growth (A) and MRP2 mRNA levels (B) in human hepatoma $HepG_2$ cells. (A) Cell growth of $HepG_2$ cells untreated or exposed to 0.4 or 3.2 mM phenobarbital (PB) for 96 hr was determined using the MTT assay. The results are expressed as % of cell proliferation of untreated cells and are the mean ± SD of three independent experiments in triplicate. $^{*}P < 0.05$ when compared to untreated cells. (B) Each lane contains 10 μg total RNAs isolated from $HepG_2$ cells either untreated (UNT) or exposed to 0.4 or 3.2 mM PB. RNAs were transferred onto Hybond-N+ sheets after electrophoresis and hybridized with MRP2 and 18S probes. The data shown are representative of two independent experiments.

been shown to be not responsive to the barbiturate in such human cells [36]. Taken together these results suggest that the cellular and molecular mechanisms involved in MRP2 up-regulation in response to phenobarbital are, at least partly, different from those involved in CYP induction. The nuclear receptor CAR, which mediates CYP up-regulation in phenobarbital-treated hepatocytes [16], seem therefore unlikely to play a major role in MRP2 regulation by phenobarbital. The fact that (i) phenobarbital responsive elements known for interacting with CAR and found in the 5′-flanking region of the rat CYP2B1/2 gene have not been functionally reported in the sequence of the rat MRP2 promoter [14] and (ii) the barbiturate was found to increase MRP2 gene expression in hepatoma $HepG_2$ cells although these cells have been presumed to lack CAR-mediated regulations [37], agrees with this conclusion. In addition, the pregnenolone X receptor (PXR), involved in up-regulation of CYP3A1/2 and CYP2B1/2 by some xenobiotics [16,38], is also unlikely to play a role in MRP2 induction by phenobarbital since this barbiturate is not thought to interact with PXR [39].

The absence of MRP2 up-regulation in the liver of phenobarbital-treated rats is rather intriguing. It could be related to inadequate experimental conditions when treating the rats. However, it should be emphasized that our *in vivo* treatment was sufficient to increase expression of CYP2B1/2, CYP3A1/2 and MRP3; in addition, the absence of MRP2 up-regulation in the liver of phenobarbital-treated rats has also been recently reported by others [40]. Nevertheless, we cannot exclude that induction of MRP2, in contrast to that of CYPs, requires particularly high intracellular concentrations of phenobarbital that were not reached with our experimental protocol. The fact that *in vitro* up-regulation of MRP2 was obtained at high concentrations of phenobarbital may favor this hypothesis.

The failure of the barbiturate to increase MRP2 expression *in vivo* may alternatively mean that induction of MRP2 by phenobarbital is restricted to cultured liver cells, thus reflecting specific *in vitro* effects of the barbiturate. In this respect, phenobarbital has been previously thought to favor the expression of differentiated functions in primary rat hepatocytes [29]. However, we found that phenobarbital used at the concentration of 3.2 mM which led to MRP2 induction, did not enhance expression of the liver marker albumin in primary rat hepatocytes, suggesting that the barbiturate did not alter their differentiation status in our culture conditions. MRP2 up-regulation by phenobarbital therefore seems to be unrelated to modulation of the differentiation status of the primary liver cells. The fact that the differentiating agent DMSO, which strongly increased expression of albumin in agreement with previous reports [30,41], failed to enhance MRP2 expression in primary hepatocytes also fully supports this conclusion.

Another known phenotypic effect related to high concentrations of phenobarbital corresponds to enhancement of cell survival [28,42]. Indeed, the barbiturate used at the dose of 3.2 mM, unlike the dose of 0.4 mM, strongly increased hepatocyte survival as indicated by MTT assay. The mechanism involved remains to be determined; it could be linked to inhibition of spontaneous apoptosis occurring in primary hepatocytes [15]. Whether MRP2 up-regulation and prolonged cell survival are related may deserve further studies. It is however noteworthy that MRP2 is thought to efflux oxidized glutathione [43], which may delay hepatocyte death by conferring some protection against oxidative stress occurring during culture processes.

Phenobarbital used at 3.2 mM, unlike the concentration of 0.4 mM, was also demonstrated to inhibit cell proliferation of primary rat hepatocytes in agreement with previous studies [29]. It also blocked growth of highly replicating $HepG_2$ cells. The mechanism of such an effect may involve down-regulation of epidermal growth factor receptors [44]. Interestingly, the dose of phenobarbital down-modulating DNA synthesis also markedly enhanced MRP2 expression in both rat hepatocytes and human liver $HepG_2$ cells. Since MRP2 levels have been found to be up-regulated in

quiescent liver cells when compared to their proliferating counterparts [27], it is tempting to speculate that MRP2 induction in response to phenobarbital may be linked to the inhibition of cell proliferation due to the barbiturate. Additional studies are required to validate this hypothesis.

In summary, the data reported in the present study indicate that phenobarbital strongly increases MRP2 expression in both primary rat and human hepatocytes. The features of this regulation, i.e. dose-response, time-course and *in vivo* responsiveness, however differ from those of phenobarbital-related CYP2B1/2 and CYP3A1/2 up-regulation, suggesting that the mechanisms involved in MRP2 induction are at least in part unrelated to those operating for CYPs. By contrast, MRP2 regulation in primary hepatocytes appears to be associated with other effects of the barbiturate such as prolonged cell survival and inhibition of cell proliferation. The putative relationships between these phenotypic alterations and MRP2 induction remain to be clarified.

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