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P-Glycoprotein Induction in Rat Liver Epithelial Cells in Response to Acute 3-Methylcholanthrene Treatment

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ABSTRACT. Expression of P-glycoprotein (P-gp), a plasma membrane glycoprotein involved in multidrug resistance and encoded by *mdr* genes, was investigated in nonparenchymal rat liver epithelial (RLE) cells in response to acute exposure to carcinogenic polycyclic aromatic hydrocarbons (PAHs). High levels of *mdr* mRNAs were evidenced by Northern blotting in two independent RLE cell lines after treatment by either 3-methylcholanthrene (MC) or benzo-(a)pyrene. MC-mediated *mdr* mRNA induction was demonstrated to be dose-dependent; it occurred through enhanced expression of the *mdr* 1 gene, as indicated by reverse transcriptase-polymerase chain reaction analysis using rat *mdr* gene-specific primers and paralleled an induction of a 140 kDa P-gp as demonstrated by Western blotting. In addition, MC-induced P-gp appeared to be fully functional because RLE cells exposed to MC displayed enhanced cellular efflux of rhodamine 123, a known P-gp substrate, compared to their untreated counterparts. Analysis of time-course induction revealed that *mdr* mRNA levels were maximally increased when RLE cells were treated for 48 to 96 hr and returned to low levels after the PAH was removed. In contrast to P-gp, both cytochrome P-450 1A1 and cytochrome P-450 1A2 were not detected after exposure to MC, thus indicating that these liver detoxification pathways are not coordinately regulated with P-gp in RLE cells. In addition, MC-mediated P-gp regulation was not associated with major cellular disturbances such as alteration of protein synthesis and, thereby, differed from the known *mdr* mRNA induction occurring in response to cycloheximide. Moreover, cotreatment with MC and cycloheximide led to a superinduction of *mdr* mRNAs, thus suggesting that the effects of the two xenobiotics were, at least partly, additive. In contrast to MC and benzo(a)pyrene, 2,3,7,8-tetrachlorodibenzo-p-dioxin and benzo-(e)pyrene were unable to increase P-gp expression. These results indicate that some PAHs can act as potent inducers of P-gp in RLE cells and may be interpreted as an adaptive reaction of these cells in lowering cellular accumulation of toxic drugs, including carcinogens transported by P-gp and, therefore, conferring protection on these compounds. BIOCHEM PHARMACOL 51;11:1427–1436, 1996.

KEY WORDS. cytochromes P-450; *mdr* genes; 3-methylcholanthrene; multidrug resistance; P-glycoprotein; rat liver epithelial cells

Drug-resistant tumor cell lines frequently display overexpression of a plasma membrane phosphoglycoprotein termed P-gp§ [1]. P-gp is thought to act as an energy-dependent efflux pump that lowers the intracellular accumulation of structurally and functionally unrelated antineoplastic agents, such as anthracyclines, vinca alkaloids, and taxol, and, thus, confers multidrug resistance [2–4]. P-gp is encoded by *mdr* genes, which constitute a small gene family comprising two members in humans (*mdr*1 and *mdr*2) and three members in rodents (*mdr*1, *mdr*2, and *mdr*3) [3]. Only *mdr*1 in humans and *mdr*1 and *mdr*3 (also known as *mdr*1b and *mdr*1a, respectively) in rodents are involved in drug resistance as assessed by experiments of *mdr* cDNA transfections [5, 6].

P-gp expression has also been evidenced in many human tumor samples and has been correlated in some cases with failure of chemotherapeutic treatment [7, 8]. In particular, high levels of *mdr*1 mRNAs have been found in human hepatocarcinomas [9], and similar results have been described in chemically-induced liver tumors in both rats and mice [10–13]. In addition, P-gp expression in the liver has been postulated to be directly regulated in response to carcinogens, as shown for various drug metabolizing enzymes such as CYPs [14]. Indeed, administration of 2-acetylaminofluorene or TCDD to rats or mice has been demonstrated to result in a strong increase in both *mdr* and CYP 1A

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§*Abbreviations:* AH, aromatic hydrocarbon; AHR, AH-receptor; ARNT, AH-receptor nuclear translocator; CYP, cytochrome P-450; MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; P-gp, P-glycoprotein; Rh 123, rhodamine 123; RLE cells, rat liver epithelial cells; RT-PCR, reverse transcriptase-polymerase chain reaction; TCDD, 2,3,-7,8-tetrachlorodibenzo-*p*-dioxin.

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mRNA levels, thus supporting the view that xenobiotic-mediated P-gp regulation may be linked to the AH-receptor (AHR) and the AH-receptor nuclear translocator (ARNT) involved in CYP 1A induction [14]. P-gp also augmented in primary cultured rat hepatocytes exposed to PAHs such as MC [15]. However, these data have been questioned by several authors. Both Teeter *et al.* [16] and Russel *et al.* [17] failed to evidence any enhanced P-gp expression in the liver of mice exposed to various carcinogens, including TCDD and 2-acetylaminofluorene. Similarly, Chieli *et al.* [18] did not observe any increase in P-gp activity in cultured rat hepatocytes treated by MC. The basis for these discrepancies is unclear. It could be linked, for *in vitro* studies, to the use of primary rat hepatocyte cultures that spontaneously displayed a marked overexpression of P-gp [19], raising caution in interpreting data on the effects of xenobiotics on multidrug resistance in this *in vitro* model, as recently underlined by Lee *et al.* [20]. The use of another liver cell system could, therefore, help to clarify and characterize *in vitro* regulation of P-gp by carcinogenic compounds. In this work, we analysed *mdr* gene expression in response to MC treatment in nonparenchymal RLE cells. These liver cells have been shown to display no detectable P-gp activity [21]; they can be targets of carcinogens [22] and have been used to study some liver detoxification pathways, such as conjugating enzymes [23]. Our results demonstrate that acute exposure to MC strongly enhanced functional P-gp levels in RLE cells through a specific increased expression of the *mdr*1 gene, without concomitant induction of CYP 1A mRNA levels.

MATERIALS AND METHODS

Chemicals

MC, benzo(a)pyrene, benzo(e)pyrene, rhodamine 123 (Rh 123), and cycloheximide were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Verapamil was purchased from Biosedra Laboratories (Levallois-Perret, France). TCDD was kindly provided by Dr. T. Cresteil (Inserm U 75, Paris, France).

Cell Culture

Two RLE cell lines (SDVI, FIII), established from normal liver of 10-day-old Sprague-Dawley or Fisher rats according to the procedures of Williams *et al.* [24–26], were used between passages 10 and 30. RLE cells were maintained in monolayer culture in Williams' medium supplemented with 10% fetal calf serum and were passaged every week using 0.1% trypsin solution. These nonparenchymal cells most likely originate from bile ductules [26] and constantly fail to exhibit hepatocyte-specific markers, such as tyrosine aminotransferase activity and albumin production [25, 27]. They are not tumorigenic when injected into syngenic newborn rats [25]. For xenobiotic treatment, confluent RLE cells were exposed to PAHs or TCDD dissolved in DMSO before addition to the culture medium. Final concentrations of solvent did not exceed 0.2% (v/v) in either treated or control cultures.

Freshly isolated normal rat hepatocytes were prepared by the two-step collagenase perfusion method and were cultured in a medium containing 75% minimal essential medium and 25% medium 199, supplemented with 0.2 mg/mL bovine serum albumin, 10 μg/mL bovine insulin and 0.1 μM dexamethasone, as described elsewhere [19].

Isolation of RNA and Northern Blot Analysis

Total RNAs were extracted from RLE cells by the guanidinium thiocyanate/cesium chloride method of Chirgwin *et al.* [28]. For Northern blot analysis, 10 μg of total RNAs were subjected to electrophoresis in a denaturing formaldehyde/agarose gel and transferred onto Hybond-N sheets (Amersham, Bucks, U.K.). The sheets were prehybridized and then hybridized with [^{32}P]-labeled probes. *Mdr* mRNAs were detected with a hamster pCHP1 probe obtained from the American Type culture collection (Rockville, MD, U.S.A.) [29]; the pCHP1 sequence has been shown to be within highly conserved regions of *mdr* cDNAs [30] and, therefore, does not discriminate between the different *mdr* gene transcripts [31]. CYPs 1A and albumin mRNAs were analysed with a rat CYP 1A probe [32] and a rat albumin probe, respectively [33]. Equal gel loading and efficiency of transfer were demonstrated by hybridization with an 18S rRNA probe [34]. After hybridization, sheets were washed, dried and autoradiographed at −80°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA (0.5 μg) was reverse transcribed using 200 units of Moloney murine leukaemia virus reverse transcriptase (Clontech, Palo Alto, CA, U.S.A.) and 20 pmols of random hexanucleotide primers in a solution containing 500 μM each dNTP, 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$ and 0.5 unit of RNase inhibitor (Clontech). An amount of cDNA representing 25 ng of RNA was then subjected to PCR for 25 to 32 cycles in a final volume of 50 μL using 2.5 units of Taq polymerase (Eurobio, Les Ulis, France) and 250 ng of each primer. Following an initial denaturation of 2 min at 94°C, each cycle consisted of 30 sec at 94°C, 30 sec at 55°C, and 60 sec at 72°C. The rat *mdr* gene-specific primers used were: *mdr*1 forward primer, 5′-AGTGACACTGGTGCCTCTGA-3′ (nucleotides 1929–1950); *mdr*1 reverse primer, 5′-CAAACACTGGTTGTATGCAC-3′ (nucleotides 2179–2161); *mdr*2 forward primer, 5′-AAGGCTGCTGGAGGTGTGGC-3′ (nucleotides 1939-1959); *mdr*2 reverse primer, 5′-CAGGATGATGGAGAATGCCG-3′ (nucleotides 2190–2170); *mdr*3 forward primer, 5′-GATGGAATTGATAATGTGGAC-3′ (nucleotides 1929–1950); and *mdr*3 reverse primer, 5′-TGCTGTTCTGCCGCTGGAT-3′ (nucleotides 2253–2233). These primers were chosen within highly divergent nucleotide sequences of the three rat *mdr* genes corresponding

to the region linking the two putative domains of P-gp [35] to avoid any coamplification of different *mdr* cDNAs. The rat AHR and ARNT primers used were: AHR forward primer, 5′-ACATAACAGACGAAATCCTGA-3′ (nucleotides 1715–1736); AHR reverse primer, 5′-CGGACTCTGAAACTTGCTTAG-3′ (nucleotides 2436–2415); ARNT forward primer, 5′-AATGGTTTGGGAGACCACTG-3′ (nucleotides 323–343); and ARNT reverse primer, 5′-CAGTAGCCACACAACGATGA-3′ (nucleotides 865–845). Identification of PCR products to previously published sequences of rat *mdr*, AHR, and ARNT genes [35, 36] was checked by sequencing using Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, OH, U.S.A.). Aliquots (10 μL) of RT-PCR products were then subjected to electrophoresis in 2% agarose gel. AHR and ARNT RT-PCR fragments were visualised by staining the gel with ethidium bromide; *mdr* PCR products were transferred to hybond-N+ sheets by capillary and hybridized with mouse *mdr*1 BBpG4, *mdr*2 HIpG3 and *mdr*3 H3pG3 [^{32}P]-labelled probes [37] (a generous gift from Dr. P. Gros, McGill University, Montreal, Canada). After hybridization, sheets were washed, dried, and autoradiographed at −80°C. Several negative control reactions were included in each experiment (e.g., PCRs carried out using water or RNA instead of cDNA). Positive controls for *mdr* RT-PCRs were performed using cDNAs prepared from rat hepatoma RHC1 cells and freshly isolated rat hepatocytes; RHC1 cells are known to express both *mdr*1 and *mdr*3 genes [38] and high levels of *mdr*2 transcripts are found in hepatocytes [35].

Preparation of Membranes and Immunoblotting

Crude membrane were prepared from RLE cells by differential centrifugation as described by Germann *et al.* [39]. Membrane proteins were separated on a 7% polyacrylamide gel and electrophoretically transferred to a nitrocellulose sheet. The nitrocellulose sheet was blocked for 2 hr with Tris-buffered saline containing 3% bovine serum albumin and sequentially incubated with C219 monoclonal antibody (Centocor Inc., Malvern, PA, U.S.A.) raised against P-gp [40] and [^{125}I]-labelled protein A (Amersham). After washing, nitrocellulose sheets were dried and autoradiographed at −80°C. A control blot was performed using the same protocol with nonimmune myeloma cell ascite as primary antibody.

Evaluation of P-gp Activity

P-gp activity was assessed by determination of cellular Rh 123 efflux. Indeed, the fluorescent dye Rh 123 is a substrate for P-gp and its transport out of the cell has been demonstrated to reflect P-gp function [41, 42]. RLE cells were preincubated with Rh 123 (2 μg/mL) for 15 min, washed 3 times with ice-cold PBS and reincubated in Rh 123-free medium for 15 or 30 min in the absence or presence of verapamil (25 μM), a known inhibitor of P-gp function [43]. Intracellular Rh 123 concentration was further determined by fluorimetry using a Titertek Fluoroscan spectrofluorometer (Flow Laboratories, Puteaux, France); excitation and emission wavelengths were 485 and 538 nm, respectively. Intracellular Rh 123 retention values were expressed as percentages of initial Rh 123 accumulation and were analysed by the Student's *t*-test. The criterion of significance of the differences between the means (±SD) was $P < 0.05$. Cellular Rh 123 efflux was also investigated in some experiments by flow cytometry. Briefly, cells were stained by Rh 123 as described above, washed, and reincubated in Rh 123-free medium for 30 min; the fluorescent signal of intracellular Rh 123 was then measured using a Cytoron Absolute flow cytometer (Ortho Diagnostic Systems) as previously reported [42].

Assay of Protein Synthesis

Protein synthesis was determined by measuring incorporation of [^{14}C] leucine in trichloroacetic acid precipitable macromolecules as previously described [44]. Results are expressed relative to total cellular protein contents measured by the Bio-Rad protein assay [45].

RESULTS

Nonparenchymal RLE SDVI and FIII cells were cultured in the absence or presence of 5 μM MC for 48 hr and then analysed for *mdr* expression by Northern blotting (Fig. 1). Hybridization with pCHP1 probe, which does not discriminate between the different *mdr* genes [31], evidenced a strong increase in 4.5 kb *mdr* mRNA levels in SDVI cells exposed to the PAH when compared to their untreated counterparts. A similar induction also occurred in FIII cells (Fig. 1); the two RLE cell lines, thus, appeared to display

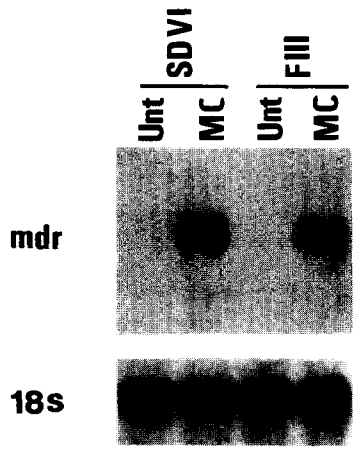

FIG. 1. Induction of *mdr* mRNA levels in RLE cells by MC treatment. Each well contains 10 μg total RNAs isolated from nonparenchymal SDVI and FIII liver cells either untreated (Unt) or exposed to 5 μM MC for 48 hr. The RNAs were then transferred to Hybond-N sheets after electrophoresis and hybridized with an *mdr* cDNA probe and then with an 18S probe to demonstrate equal gel loading.

similar responses when treated by MC, and only SDVI cells were retained for further studies.

The precise nature of the *mdr* genes regulated by MC was then determined by RT-PCR using *mdr* gene-specific primers (Fig. 2). Results demonstrated that SDVI cells exposed to MC showed a huge induction of *mdr*1 mRNA expression. By contrast, *mdr*2 and *mdr*3 gene transcripts were not detected in either untreated or MC-treated RLE cells. Positive controls for *mdr*1 and *mdr*3 expression were provided using drug-resistant RHC1 cells and *mdr*2 mRNAs were evidenced in freshly isolated rat hepatocytes (Fig. 2).

Crude membrane fractions were further prepared from SDVI cells exposed or not to 5 μM MC, and were used to investigate P-gp expression by Western blot analysis (Fig. 3). A C219 antibody-reactive band of 140 kDa corresponding to P-gp was markedly overexpressed in MC-treated RLE cells when compared to their untreated counterparts; this band was not present in control blots performed using non-immune myeloma cell ascite as primary antibody instead of C219 (data not shown). The effect of MC treatment on P-gp activity in SDVI cells was then evaluated using analysis of cellular Rh 123 efflux. As shown in Fig. 4A, Rh 123 concentrations measured spectrofluorometrically were found to strongly decrease in MC-treated RLE cells during postincubation in Rh 123-free medium. By contrast, RLE cells not exposed to MC only slightly lost Rh 123 staining. In addition, flow cytometry experiments indicated that the whole population of RLE cells exposed to MC displayed low cellular retention of Rh 123 when compared to their untreated counterparts (Fig. 4B). Moreover, addition of 25 μM verapamil in the postincubation medium resulted in a strong inhibition of Rh 123 efflux in MC-treated SDVI cells, but it had no effect on cellular levels of the fluorescent dye in untreated cells (Fig. 4A).

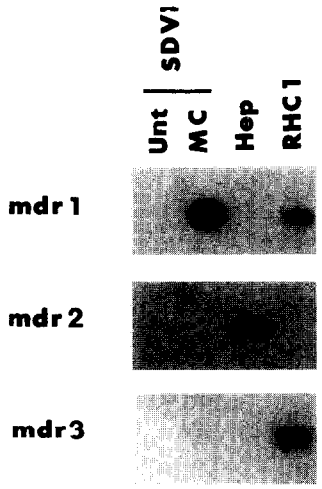

FIG. 2. Effect of MC treatment on *mdr* gene-specific mRNA levels in RLE cells. Expression of *mdr*1, *mdr*2 and *mdr*3 genes was analyzed in SDVI cells either untreated (Unt) or exposed to 5 μM MC for 48 hr by RT-PCR as described in Materials and Methods. Drug-resistant RHC1 cells and freshly isolated rat hepatocytes (Hep) were used as positive controls for *mdr*1 and *mdr*3, and *mdr*2 gene expression, respectively.

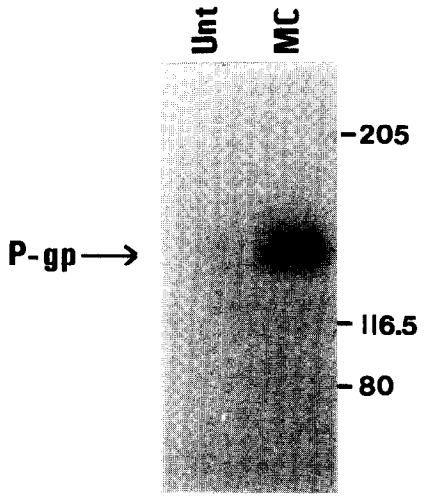

FIG. 3. Western blot analysis of membrane proteins obtained from RLE cells exposed to MC. Crude membrane fractions were prepared from SDVI cells untreated (Unt) or exposed to 5 μM MC for 48 hr. Membrane proteins were then separated on 7% polyacrylamide gel and transferred onto a nitrocellulose sheet. After incubation with C219 antibody raised against P-gp, the blot was developed with [^{125}I]-labelled protein A. Arrow indicates the position of P-gp. The position of molecular mass standards in kDa is indicated on the right.

The effects of various doses of MC on *mdr* mRNA levels in RLE cells were further determined by Northern blot analysis (Fig. 5A). Results indicated that 48-hr treatments by low concentrations of the PAH (0.01 and 0.1 μM) did not alter *mdr* gene expression. Exposure to 1 μM MC resulted in a slight induction of *mdr* mRNA levels and the use of higher MC concentrations (5 to 50 μM) much more strongly increased *mdr* transcripts (Fig. 5A). The time-course of *mdr* gene induction by 5 μM MC was then characterized (Fig. 5B). *Mdr* mRNAs in SDVI cells were clearly increased after a 24-hr treatment and reached higher levels when cells were exposed to the PAH for 48 to 96 hr. To determine whether or not this MC-mediated *mdr* induction was reversible, SDVI cells were treated by 5 μM MC for 48 hr, washed, and incubated in MC-free medium for 24 to 120 hr, and then analysed for *mdr* gene expression by Northern blot (Fig. 6). *Mdr* mRNA amounts were found to remain high 24 hr after MC withdrawal; they clearly decreased thereafter and returned to low levels after 120 hr.

Previous studies have demonstrated that cycloheximide treatment of cultured rat hepatocytes resulted in a strong induction of *mdr* transcripts, thus suggesting a link between inhibition of cellular protein synthesis and increase in *mdr* mRNAs levels in liver cells [46, 47]. To investigate whether or not MC-mediated *mdr* induction in RLE cells could also involve, at least partly, alteration of protein synthesis, we compared [^{14}C] leucine incorporation in SDVI cells exposed or not to 5 μM MC for 48 hr. As shown in Fig. 7B,

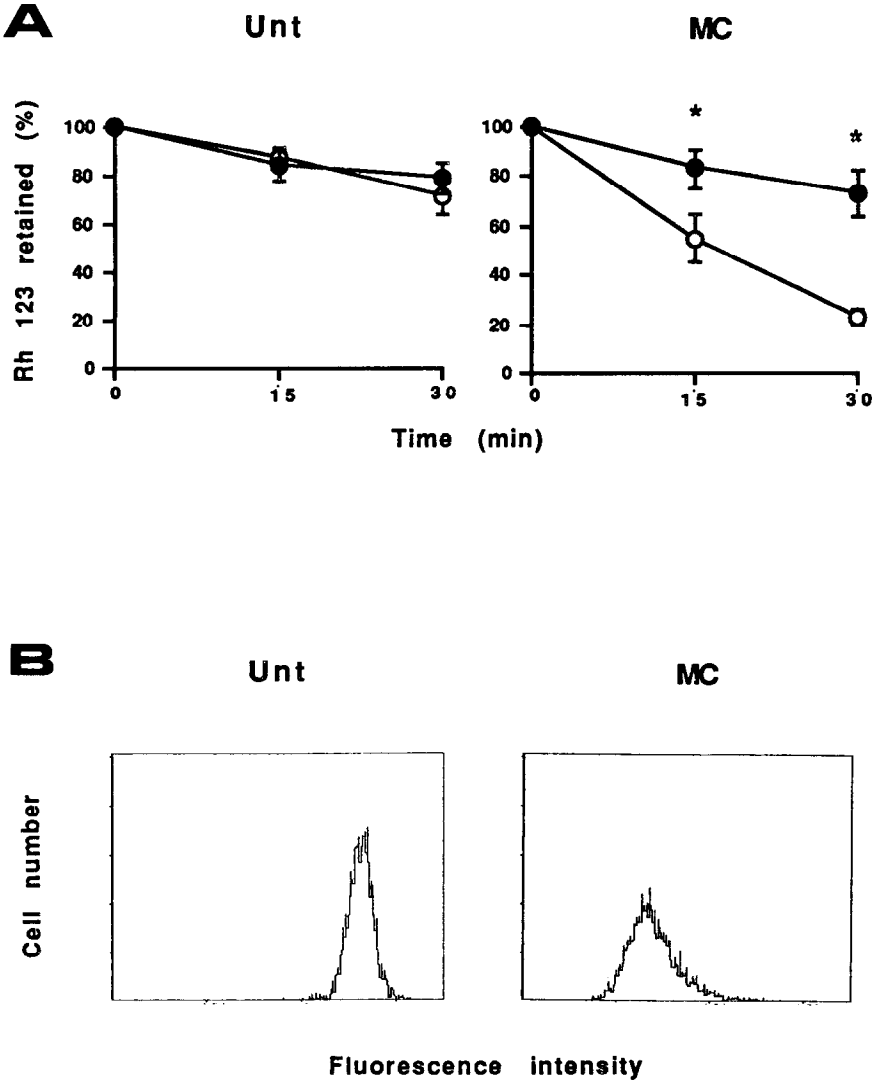

FIG. 4. Effect of MC treatment on P-gp activity in SDVI cells. A. SDVI cells either untreated (Unt) or exposed to 5 μM MC for 48 hr were incubated with Rh 123 (2 μg/mL) for 15 min, washed, and reincubated in Rh 123-free medium for 15 or 30 min in the absence (—○—) or presence (—●—) of verapamil (25 μM). Intracellular Rh 123 concentrations were further measured by fluorimetry using a Titertek Fluoroscan spectrofluorometer and were expressed as percentages of initial Rh 123 accumulation. The values are the mean ±SD of 3 independent experiments in triplicate. *, $P < 0.05$. B. SDVI cells either untreated (Unt) or exposed to MC for 48 hr were incubated with Rh 123 (2 μg/mL) for 15 min, washed and reincubated in Rh 123-free medium for 30 min. The fluorescent signal of residual intracellular Rh 123 was then analyzed by flow cytometry using a Cytoron Absolute flow cytometer.

[^{14}C] leucine incorporation was similar in untreated and MC-treated SDVI cells; in both situations, protein synthesis strongly decreased only after an 8-hr cycloheximide (60 μg/mL) treatment. Moreover, although exposure to either cycloheximide or MC resulted in an increase in *mdr* mRNA levels, cotreatment with the two compounds led to a superinduction of *mdr* gene transcripts in cultured RLE cells (Fig. 7A).

To determine whether or not the P-gp increase observed in RLE cells treated by MC also occurred in response to other AHs, SDVI cells were exposed to 25 μM benzo(a)pyrene, 25 μM benzo(e)pyrene, or 10 nM TCDD for 48 hr; at these concentrations, all these compounds have previously been demonstrated to be active on the expression of some detoxifying enzymes in liver cells [48, 49]. As shown in Fig. 8, benzo(a)pyrene, like MC, was found to be a strong inducer of *mdr* transcripts in RLE cells. By contrast, neither TCDD nor benzo(e)pyrene altered *mdr* mRNA levels (Fig. 8).

We then examined the expression of CYP 1A1 and CYP 1A2 in RLE cells in response to MC and TCDD treatment (Fig. 9). These CYPs are known to be induced by AHs in liver parenchymal cells [36] and have been hypothesised to be coordinately regulated with P-gp [14]. CYP 1A1 and CYP 1A2 transcripts were not detected in untreated, MC- or TCDD-treated RLE cells, even after long-time exposure of the autoradiograms (Fig. 9). By contrast, mRNA levels of both CYPs 1A1 and 1A2 were demonstrated to be strongly increased in primary rat hepatocytes exposed to 5 μM MC or 10 nM TCDD for 48 hr. Expression of AHR and ARNT, two proteins directly involved in AH-mediated CYP 1A regulation [36], was then investigated in both RLE cells and rat hepatocytes using RT-PCR assays. As indicated in Fig. 10, both AHR and ARNT mRNAs were demonstrated to be present in SDVI cells and primary hepatocytes.

DISCUSSION

Previous reports on P-gp regulation in liver cells in response to acute treatment by carcinogens have resulted in conflict-

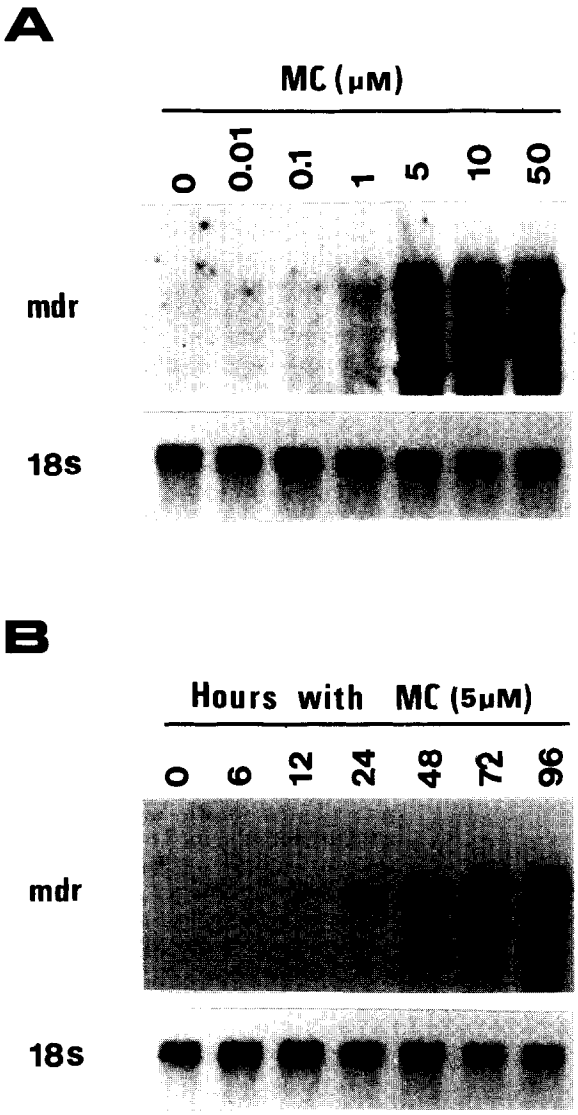

FIG. 5. Dose-dependence (A) and time-course (B) of *mdr* mRNA induction in response to MC treatment. Each well contained 10 μg total RNAs isolated from SDVI cells exposed to various doses of MC (0.01–50 μM) for 48 hr (A) or to 5 μM MC for various lengths of time (6–96 hr) (B). RNAs were then transferred to Hybond-N sheets after electrophoresis and hybridized with an *mdr* cDNA probe and then with an 18S probe to demonstrate equal gel loading.

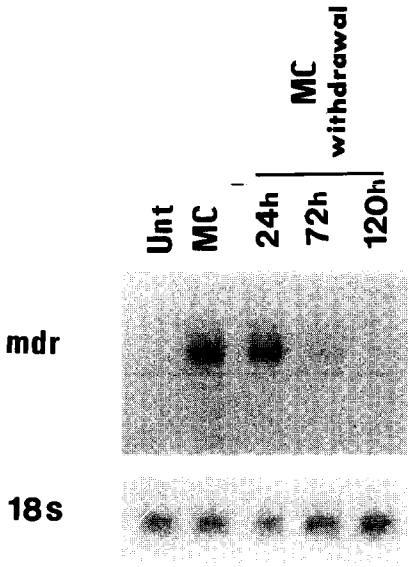

FIG. 6. Downregulation of *mdr* mRNA levels after MC withdrawal. SDVI cells were either untreated (Unt) or exposed to 5 μM MC for 48 hr, followed by recovery after withdrawal of the PAH for 24 hr, 72 hr, and 120 hr. RNAs were then isolated as described in Materials and Methods, transferred to Hybond-N sheets after electrophoresis, and hybridized with an *mdr* cDNA probe and then with an 18S probe in order to demonstrate equal gel loading.

ing data [14–18]. In particular, analyses of P-gp expression and activity in xenobiotic-treated primary hepatocyte cultures led to apparently contradictory results [15, 18], which could be linked to the marked overexpression of functional P-gp occurring spontaneously in untreated cultured hepatocytes [19, 20]. In the present study, we used nonparenchymal RLE cells displaying no detectable basal P-gp activity [21] to evidence and characterize carcinogen-mediated regulation of P-gp. The RLE cells likely deriving from bile ductules are not considered as equivalent to hepatocytes [26, 50]; however, like parenchymal cells, they may be targets for genotoxic compounds [22] and have been shown to allow the investigation of some liver detoxification pathways, such as conjugating enzymes [23]. Our results demonstrated that acute exposure to some PAHs led to a strong induction of P-gp in RLE cells, supporting the view that, in agreement with previous studies [14, 15], carcinogens can directly regulate P-gp expression in rat liver cells, including nonparenchymal cells. Indeed, exposure to either MC or benzo(a)pyrene resulted in a strong induction of *mdr* mRNA levels in RLE SDVI liver cells. This increase in *mdr* transcript amounts in MC-treated SDVI cells was shown to be dose-dependent and was associated with elevated expression of a 140 kDa P-gp as demonstrated by Western blotting. MC also enhanced cellular efflux of the P-gp substrate Rh 123, which was found to be strongly inhibited by verapamil, a known inhibitor of P-gp function [43], thereby indicating that MC-induced P-gp was fully active in RLE cells. Moreover, the low cellular retention of Rh 123 observed by flow cytometry analysis in all SDVI cells exposed to MC demonstrated that the whole cell population expressed high P-gp activity in response to the PAH. In addition, MC-induced *mdr* gene expression also occurred in RLE FIII cells, supporting the view that this effect is not a unique phenomenon restricted to SDVI cells.

Two *mdr* genes, *mdr*1 and *mdr*3, are involved in anticancer drug transport in rodents [6] and the *mdr*2 gene has recently been demonstrated to encode a phospholipid transporter [51]. RT-PCR experiments using rat *mdr* gene-specific primers revealed a strong induction of *mdr*1 mRNA levels in MC-treated SDVI cells, but *mdr*2 and *mdr*3 transcripts were not detected. These results, therefore, clearly indicate that P-gp expression in response to PAHs is spe-

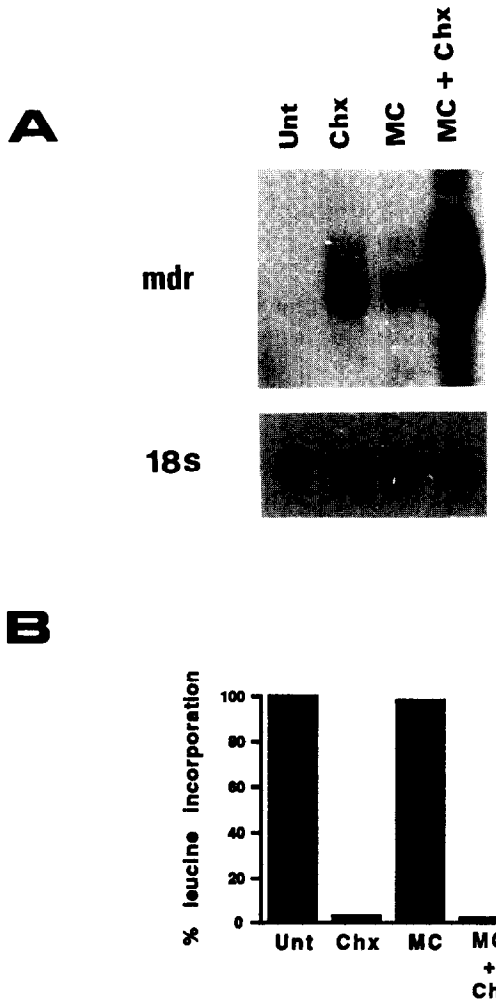

FIG. 7. Effect of MC and cycloheximide treatments on *mdr* gene expression (A) and protein synthesis (B) in SDVI cells. A. SDVI cells maintained in the absence or presence of 5 μM MC for 48 hr were exposed, or not, to cycloheximide (Chx) (60 μg/mL) for 8 hr. Total RNAs were then extracted, transferred to Hybond-N sheets after electrophoresis, and hybridized with *mdr* and 18 S probes. Unt, untreated cells. B. Protein synthesis in SDVI cells maintained as described above, in the presence or absence of MC and Chx, was analysed by determination of the incorporation of [^{14}C] leucine in trichloroacetic-precipitable macromolecules as reported in Materials and Methods. The values normalized to total cellular protein content are expressed as the percentage of protein synthesis in untreated cells (Unt) and are the mean of 3 independent experiments; the standard deviation was <10% in all cases.

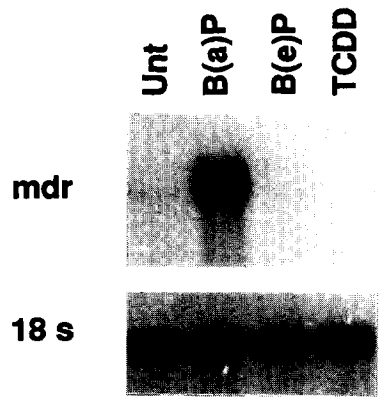

FIG. 8. Effect of TCDD, benzo(a)pyrene, and benzo(e)pyrene on *mdr* mRNA levels in SDVI cells. Each well contained 10 μg total RNAs isolated from SDVI cells untreated (Unt) or exposed to 25 μM benzo(a)pyrene (BaP), 25 μM benzo(e)pyrene (BeP), or 10 nM TCDD for 48 hr. RNAs were then transformed to Hybond-N sheets after electrophoresis and hybridized with an *mdr* cDNA probe and then with an 18S probe to demonstrate equal gel loading.

cifically regulated in RLE cells through the *mdr*1 gene. Overexpression of the *mdr*1 gene also occurred in rat liver cells during carcinogenesis, regeneration after partial hepatectomy and cholestasis [35, 38, 52]. Taken together, these data suggest that the *mdr*1 gene plays a critical role in P-gp regulation in the liver in various circumstances, including exposure to carcinogenic compounds and cell growth.

Elevated P-gp expression was initially observed in multidrug-resistant tumoral cell lines obtained by stepwise exposure to increased sublethal doses of xenobiotics, such as cytotoxic and genotoxic agents [1–4]. This drug-selection process leading to multidrug resistance cannot account for MC-mediated induction of P-gp in RLE cells. Indeed, increased *mdr* gene expression was detected as early as 24 hr after MC addition and was maximal when cells were maintained in the presence of the PAH for 48 to 96 hr, as indicated by time-course analysis of P-gp induction; by contrast, several months of treatment with increasing concentrations of cytotoxic chemicals were usually required to select multidrug resistant cells overexpressing P-gp [1]. More-

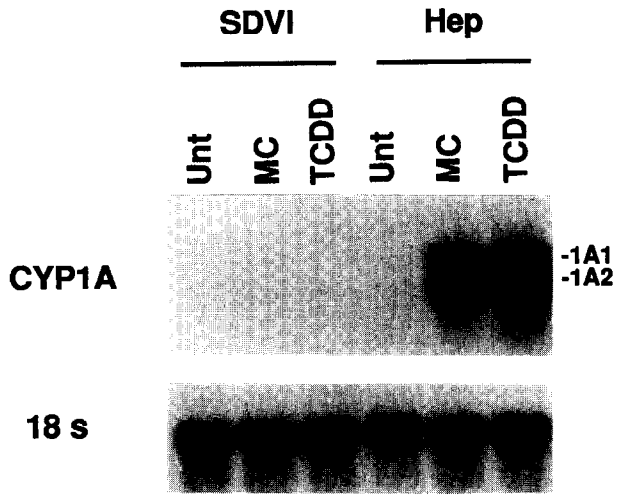

FIG. 9. Effect of MC and TCDD treatment on CYP 1A expression in SDVI cells and primary cultured rat hepatocytes. Each well contained 10 μg total RNAs isolated from SDVI cells or cultured rat hepatocytes (Hep) either untreated (Unt) or exposed to 5 μM MC or 10 nM TCDD for 48 hr. RNAs were then transferred to Hybond-N sheets after electrophoresis, and hybridized with CYP 1A and 18S probes. The CYP 1A probe used detected both CYP 1A1 and CYP 1A2 mRNAs.

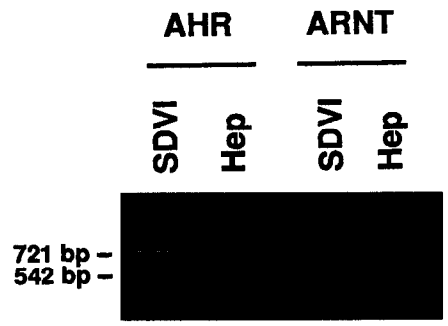

FIG. 10. Analysis of AHR and ARNT expression in RLE cells and hepatocytes by RT-PCR. Expression of AHR and ARNT in SDVI cells and rat hepatocytes (Hep) was analyzed by RT-PCR as described in Materials and Methods. The PCR products were resolved on an agarose gel and visualized by ethidium bromide. The sizes of AHR and ARNT PCR-generated fragments are indicated on the left of the figure.

over, *mdr* mRNA levels in MC-treated cells decreased to low levels within 120 hr after the PAH was removed from the culture medium, thus suggesting that the MC effect on P-gp is, at least in part, rapidly reversible, and drug-selected resistant cells displayed much more stable expression of P-gp when cultured in the absence of drug [4]. In addition, the whole population of SDVI cells, and not a fraction of putatively selected cells, showed elevated P-gp activity when exposed to MC for 48 hr, as demonstrated by flow cytometric determinations of Rh 123 efflux. All these data suggest that P-gp induction in RLE cells by MC treatment probably corresponds to an acute and specific response to the PAH.

Cellular incorporation of [^{14}C] leucine was found to be unaffected by MC treatment, thus suggesting that this PAH altered P-gp expression without inducing major cellular disturbances such as alteration of protein synthesis. MC-mediated P-gp induction, thus, differs from *mdr* mRNA increase due to cycloheximide treatment [47]; decreased synthesis of a putative labile *mdr* gene repressor, thought to explain the cycloheximide effect on P-gp [46], can probably not account for increased P-gp expression in response to MC. Moreover, cotreatment of SDVI cells with cycloheximide and MC led to a superinduction of *mdr* mRNAs, thus suggesting that the effects of the two xenobiotics on *mdr* mRNA contents were, at least partly, additive.

P-gp induction in the rat liver in response to chemical compounds was initially shown to be associated with increased expression of CYPs 1A, suggesting a coordinate regulation of these liver detoxifying systems [14]. However, our failure to evidence an induction of CYPs 1A in RLE cells exposed to MC demonstrates that P-gp and CYPs are, at least in part, not strictly coregulated in response to chemicals. This lack of CYPs 1A induction in RLE cells in response to AHR agonists such as MC and TCDD occurred even though both AHR and ARNT were present as assessed by RT-PCR experiments. It is, however, noteworthy that the AHR could be present in an inactive form in RLE cells, which may account for the failure to detect AHR in several RLE cell lines using ligand-binding studies [23]. Alternatively, RLE cells could contain proteins that repress CYP 1A1 expression and prevent its induction in response to AHs; rat CYP 1A1 gene has, thus, been demonstrated to display a negative regulatory element to which three different liver nuclear proteins bind [53]. Similarly, mutants of murine hepatoma Hepa-1 cells have been shown to express a repressor that inhibits TCDD-dependent stimulation of CYP 1A1 gene transcription [54].

The precise mechanism involved in P-gp regulation in response to chemical compounds remains to be determined. The AHR does not seem to be involved; indeed, we have failed to evidence any *mdr* gene induction in RLE cells exposed to TCDD, known to be one of the most potent agonists of the AHR [48]. In fact, according to Thorgeirsson *et al.* [55], xenobiotic-mediated induction of *mdr* gene expression could involve a yet-unindentified receptor sharing overlapping substrate specificity with the AHR, including some PAHs; moreover, it could be hypothesised that the activation of these two distinct receptors required similar concentrations of PAHs because the doses of MC (1–50 μM) acting on P-gp levels in RLE cells increased CYPs 1A expression in cultured hepatocytes [15]. A candidate for the putative receptor mediating *mdr*1 induction may be the 4S PAH-binding protein recently demonstrated to be identical to glycine N-methyltransferase [56]. This protein binds to PAHs such as MC and benzo(a)pyrene and is thought to contribute to CYP 1A1 regulation independent of the AHR; moreover, this protein does not bind halogenated AHs, such as TCDD, which may explain why TCDD failed to induce P-gp. However, benzo(e)pyrene, a PAH that is referred to as a specific ligand for the 4S PAH-binding protein because it interacts with this protein, but not with the AHR [49], was demonstrated to be unable to increase *mdr* mRNA levels in RLE cells. Hence, further studies are needed to bring definitive evidence that P-gp induction by xenobiotics in liver cells is mediated by a receptor and to characterize this putative factor.

The pathophysiological consequences of P-gp induction in response to PAHs in RLE cells remain to be determined. It is, however, noteworthy that RLE cells, thought to putatively represent liver epithelial stem cells [57], have been demonstrated to be targets of chemical carcinogens and the source of some liver tumors, including hepatocarcinomas and cholangiocarcinomas [22, 58]. Overexpression of P-gp in RLE cells in response to carcinogenic xenobiotics may, therefore, be interpreted as an adaptive reaction that could result in decreased cellular accumulation of toxic drugs, including carcinogens known to be transported by P-gp and, therefore, confer protection on these compounds. Thus, P-gp induction in response to benzo(a)pyrene may secondly lower cellular retention of this carcinogenic PAH that was recently demonstrated to be a substrate for P-gp [59, 60].

In summary, the results reported here demonstrate that nonparenchymal liver RLE cells showed induced functional

P-gp levels through increased expression of the *mdr*1 gene when exposed to some PAHs. These RLE cell lines may, therefore, represent useful cell systems to better understand cellular and molecular mechanisms leading to P-gp overexpression and multidrug resistance in response to acute treatment by xenobiotics.

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