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Modulation of P-Glycoprotein Expression by Cytochrome P450 3A Inducers in Male and Female Rat Livers

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ABSTRACT. A strong overlap between P-glycoprotein (Pgp) and cytochrome P450 3A (CYP3A) substrates and modulators has been reported. To test the hypothesis that CYP3A and Pgp are coordinately regulated, we examined the effects of known inducers of CYP3A (triacetyloleandomycin, rifampicin, dexamethasone, pregnenolone 16α-carbonitrile) on Pgp expression in rat liver. We also investigated the gender-specific expression of Pgp and compared its response to dexamethasone between male and female rats. In male rats, western blot analyses showed that rifampicin and dexamethasone caused 50% and 5-fold increases in Pgp levels, respectively. RNase protection assays using gene-specific probes for the three Pgp isoforms revealed a 3-fold increase in mdr2 mRNA levels after dexamethasone administration and a 2-fold increase following rifampicin treatment. Triacetyloleandomycin and pregnenolone 16α-carbonitrile had no effect on Pgp expression and mRNA levels. We also observed that the basal level of Pgp was 40% lower in male rats than in females and that mdr2 mRNA levels in male rats were one-half those in females. As opposed to the results in male rats, dexamethasone reduced Pgp expression by approximately 60% and caused a 30% decrease in mdr2 mRNA levels in female rats. Mdr1a was not affected and mdr1b was not detected in female or male rats. We conclude that, at the dosage regimen used, CYP3A and Pgp responses to CYP3A inducers are regulated independently in rat liver. In addition, this study shows that Pgp expression and regulation are gender specific. BIOCHEM PHARMACOL 55;4:387–395, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. P-glycoprotein; CYP3A; rat liver; gender; induction; dexamethasone

Cytochrome P450 3A (CYP3A) is the major phase I drug-metabolizing enzyme, accounting in humans for the metabolism of more than half of the drugs subject to CYP† biotransformation [1]. CYP3A represents about 30 and 70% of total CYP in liver and intestine, respectively [2]. Pgp is a multidrug transporter that has been studied mostly for its prominent role in the development of cross-resistance to cancer chemotherapy [3–6]. Pgp is located in the plasma membrane, on the apical side in many eliminating organs (liver, kidney, intestine) [7], and participates in the blood–brain barrier function [8]. These locations suggest that Pgp may also play a major role in drug absorption and disposition.

Humans possess two MDR genes, *MDR1* and *MDR2*, while three genes are present in rat, mouse, and hamster, *mdr1a*, *mdr1b* and *mdr2* [6, 9]. *MDR1* and *mdr1a* and *1b* encoded Pgps are drug transporters. The *mdr2* genes encode a phospholipid translocator [10, 11]. A recent article from our laboratory pointed out that CYP3A and Pgp share a large number of substrates and modulators, among which are cyclosporine, nifedipine, etoposide, vinblastine, and ketoconazole [12]. These two proteins are also expressed at similar locations including liver and intestine. We hypothesized that CYP3A and Pgp could be coordinately regulated.

Many studies have investigated the regulation of Pgp in cultured cells. Pgp (mdr1b) was found to be overexpressed in rat primary hepatocytes in the absence of any treatments [13, 14]; this increase was repressed by Dex. In contrast, Schuetz *et al.* [14] reported that Dex increases Pgp and mdr1b levels in the rat hepatoma cell line H35. Furthermore, Zhao *et al.* [15] observed that Dex is able to induce mdr1a and mdr1b in a mouse hepatoma cell line and in HepG2 cells, whereas no changes were noted in NIH3T3 and HeLa cells. *In vivo* studies in rats have investigated the effects of carcinogens on Pgp expression [16]. A coordinated induction of CYP1A and Pgp through the Ah receptor was first hypothesized by Burt and Thorgeirsson [17]. Later studies in the same laboratory [18] found that the Ah receptor was not involved in the induction of mdr and postulated an overlap in substrate specificity between the Ah receptor and a different receptor (mdr-R) involved in xenobiotic regulation of mdr expression. The parallel regulation of CYP3A and Pgp has also been examined in the

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† *Abbreviations:* CYP, cytochrome P450; Pgp, P-glycoprotein; Dex, dexamethasone; Rif, rifampicin; TAO, triacetyloleandomycin; PCN, pregnenolone 16α-carbonitrile; mdr, multidrug resistance; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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human colon carcinoma cell line LS180 [19], where many xenobiotics including phenobarbital, reserpine, and rifampicin were found to coordinately induce both Pgp and CYP3A. However, Dex and TAO, although potent inducers in primary hepatocytes and *in vivo*, did not increase CYP3A expression in this cell line.

We chose an *in vivo* approach to test the hypothesis and investigate the response of Pgp to CYP3A inducers. We also examined the gender-specific expression of Pgp and its response to Dex.

MATERIALS AND METHODS

Chemicals

Dex, Rif, PCN, TAO, corn oil, and DMSO were purchased from the Sigma Chemical Co. Isopropanol was obtained from Fisher Scientific.

In Vivo *Treatments*

Adult male and female Sprague–Dawley rats (280–300 g) were obtained from the Charles River Animal Farm and maintained on a 12-hr light/dark cycle. Rats were fed standard laboratory chow *ad lib*. The xenobiotics were administered using the following regimens: Dex (100 mg/kg/day), TAO (500 mg/kg/day), PCN (50 mg/kg/day), all dissolved in corn oil, and Rif (100 mg/kg/day), dissolved in DMSO.

The drugs were administered i.p. (0.5 mL) for 3–4 days, and the rats were killed 24 hr after the last treatment. Control rats received i.p. injection of the vehicle. The animals were fasted following the last injection. Plasma membranes, microsomes, and RNA were prepared from each rat liver.

Preparation of Plasma Membrane Fraction and Microsomes

For the preparation of plasma membranes, the livers were homogenized in 100 mM Tris–HCl, pH 7.5, containing a protease inhibitor mixture (Boehringer–Mannheim). The homogenate was centrifuged at 1,500 × *g* for 15 min, and the supernatant was then centrifuged at 100,000 × *g* for 35 min. The pellet was washed, resuspended in Tris buffer, and stored at −80° until analysis. The microsomes were prepared by homogenization of liver and differential centrifugation following established protocols [20]. All procedures were carried out at 4°. Protein concentrations were determined using the Bio-Rad protein assay kit with BSA as a standard.

Western Blot Analyses

Proteins were dissolved in Laemmli buffer and denatured at 90° for 3 min (microsomes only). Then, 10 μg of plasma membrane and 2 μg of microsomal proteins were loaded onto 6% and 12.5% SDS–polyacrylamide gels, respectively, using a 4% stacking gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane in a 25 mM Tris/192 mM glycine/20% methanol buffer at 100 V for 1 hr. Prestained markers were myosin (204 kDa), β-galactosidase (121 kDa), and BSA (82 kDa) (Bio-Rad). The membrane was then blocked in 3% non-fat milk (Bio-Rad) in PBS-Tween 0.1% for 1 hr and washed for 10 min with PBS. The primary antibodies, C219 (Signet) or 1G8 (provided by Dr. S. Wrighton, Lilly Research Laboratories) were incubated at 1/500 and 1/10,000 dilutions, respectively, in blocking buffer overnight at 4°. The membrane was washed subsequently (3 × 15 min) with PBS-Tween 20 0.1% and the secondary antibody, goat anti-mouse horseradish peroxidase-conjugated (Life Technologies, Inc.), was applied in blocking buffer for 1 hr. The membrane was washed as described above, and the bands were revealed using the ECL detection system (Amersham) following the manufacturer's instructions.

Ribonuclease Protection Assay

RNA was isolated using TRIzol® Reagent (Life Technologies), a monophasic solution of phenol and guanidinium isothiocyanate, according to the manufacturer's instructions.

The plasmids for the riboprobes corresponding to *mdr1a*, *mdr1b*, *mdr2*, and *GAPDH* were provided by Dr. J. A. Silverman (NCI) and have been described [21]. The antisense probes were transcribed with T7 polymerase using the Ambion Maxiscript kit (Ambion, Inc.) and purified on a 5% polyacrylamide gel. Total RNA (20 μg) was hybridized to antisense *mdr* (100,000 cpm) and *GAPDH* (10,000 cpm) probes in 20 μL of hybridization buffer [80% formamide, 100 mM sodium citrate (pH 6.4), 300 mM sodium acetate (pH 6.4), 1 mM EDTA] for 16–24 hr at 42°. The mixture was digested with RNase A (0.33 Kunitz U/mL) and RNase T1 (100 U/mL) at 37° for 45 min. The digestion was stopped, and the protected fragments were precipitated by addition of a precipitation solution (4 M guanidinium isothiocyanate, 100 mM Tris–HCl, pH 7.4; 200 μg/mL yeast tRNA, 0.5% Sarkosyl, 1% β-mercaptoethanol) and isopropanol [22]. The tubes were stored at −20° for 30 min and spun at 16,000 × *g* for 30 min. The supernatants were removed and the pellets resuspended in gel loading buffer (Ambion), denatured at 90° for 5 min, and loaded onto a 5% polyacrylamide gel. The gel was exposed to X-ray film at −80°. Mdr1a, mdr1b, mdr2, and GAPDH gave protected fragments of 127, 313, 357, and 249 nucleotides, respectively.

Analyses of western blots, as well as quantitation of mRNA and normalization of mdr to GAPDH levels, were performed using a Pharmacia UltroScan XL (Pharmacia Biotech).

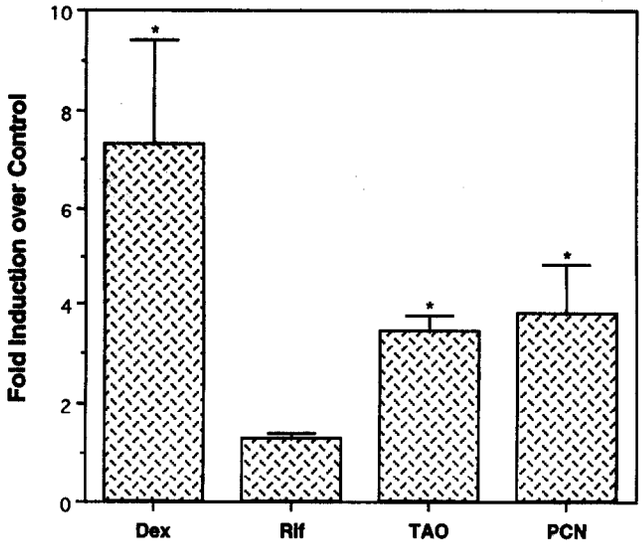


FIG. 1. Induction of CYP3A in male rat liver following administration of Dex (100 mg/kg/day), Rif (100 mg/kg/day), TAO (500 mg/kg/day), and PCN (50 mg/kg/day). Quantitation was performed by densitometric analysis. Fold induction was calculated based on CYP3A expression in control male rats. The values are reported as means ± SD ($N = 4$). Key: (*) $P < 0.05$ vs control.

Data Analysis

Results are given as means ± SD. Statistical comparisons were made using Student's *t*-test. Statistical significance was accepted as $P < 0.05$.

RESULTS

Male Rats

Sprague–Dawley rats were treated i.p. with CYP3A inducers for 3–4 days. Microsomes were prepared and the expression of CYP3A was investigated by western blot analyses using a rabbit polyclonal antibody that cross-reacts with CYP3A1 and CYP3A2.

In control male rats, CYP3A was readily detectable. Dex, TAO, and PCN increased CYP3A levels by 7-, 3-, and 4-fold, respectively. In contrast, Rif caused only a slight, nonsignificant increase in CYP3A expression (Fig. 1). These results are in agreement with previous reports [23]. The monoclonal antibody C219 used for western blot analyses revealed a band of 150–155 kDa corresponding to Pgp. Treatment of the rats with Dex caused a 5-fold increase in Pgp expression (Figs. 2A and 3). In contrast, administration of Rif resulted in a modest, but significant ($P < 0.05$), 50% increase in the Pgp level (Fig. 2B). PCN and TAO had no effect on Pgp expression (Figs. 2C, 2D, and 3).

We then used gene-specific probes for *mdr1a*, *mdr1b* and *mdr2* in an RNase protection assay to determine whether the changes observed in protein levels were matched by variations in mRNA. To confirm that the probe synthesized was able to recognize mdr1b mRNA, the least abundant Pgp in rat liver [24], we used the GCE rat hepatoma subline which expresses this gene [25]. As shown on Fig. 4A, the *mdr1b* probe gave a protected fragment of the expected size. Mdr1b was not detected in control or treated animals (Fig. 4B). Dex and Rif treatments of rats resulted in a 3- and 2-fold increase in mdr2 mRNA, respectively (Fig. 5, A and B). Furthermore, PCN and TAO treatments did not cause any change in the level of mdr2 expression. These data are consistent with our western blot analyses described above. The level of expression of mdr1a was not affected by any of the drugs used in the study (Fig. 6, A and B).

The stronger induction observed for CYP3A and Pgp in male rats was caused by Dex. Since it is established that CYP3A shows gender-specific expression and regulation [26, 27], we investigated the effects of Dex in female rats.

Gender Differences

In control female rats, CYP3A was not detectable whereas it was strongly induced by Dex treatment (data not shown), which agrees with what has been reported previously [26]. Basal levels of Pgp were approximately 40% higher in female than in male rats ($P = 0.035$, $N = 4$). Contrary to what was observed in males, Dex treatment repressed Pgp expression in female rats to one-third that measured in female controls ($P = 0.01$, $N = 4$) (Fig. 7). The mdr2 mRNA levels in control females were more than 2-fold higher than those in males. But, as with western blot Pgp measurements, pretreatment with Dex produced a decrease (30%) in the female rats (Fig. 8, A and B). In contrast to these results, mdr1a expression in female rats was lower than in male rats and no effect of Dex was observed (Fig. 6, A and B).

DISCUSSION

In the present study we used an *in vivo* approach to test the hypothesis that CYP3A and Pgp could yield coordinated responses to drugs previously reported to induce the enzyme. Sprague–Dawley rats were treated with inducers of hepatic CYP3A, and the levels of these two proteins were quantitated. Dex produced a strong induction of both Pgp and CYP3A in male rats, whereas Rif increased Pgp expression to a lesser extent than Dex and barely affected CYP3A expression. Dex also appeared to suppress higher and lower molecular weight bands in males and females when compared with controls (Fig. 8). The higher molecular weight bands (around 170,000) could correspond to different glycosylated forms of Pgp, as has been reported previously, [28, 29], while the lower molecular weight bands could result from proteolytic degradation as has been shown more recently [30, 31]. The absence of these bands after Dex treatment might indicate that Dex interferes with both processes. No effect on Pgp levels was observed after PCN and TAO treatments, although these drugs, as expected, induced CYP3A expression. Rif was reported pre-

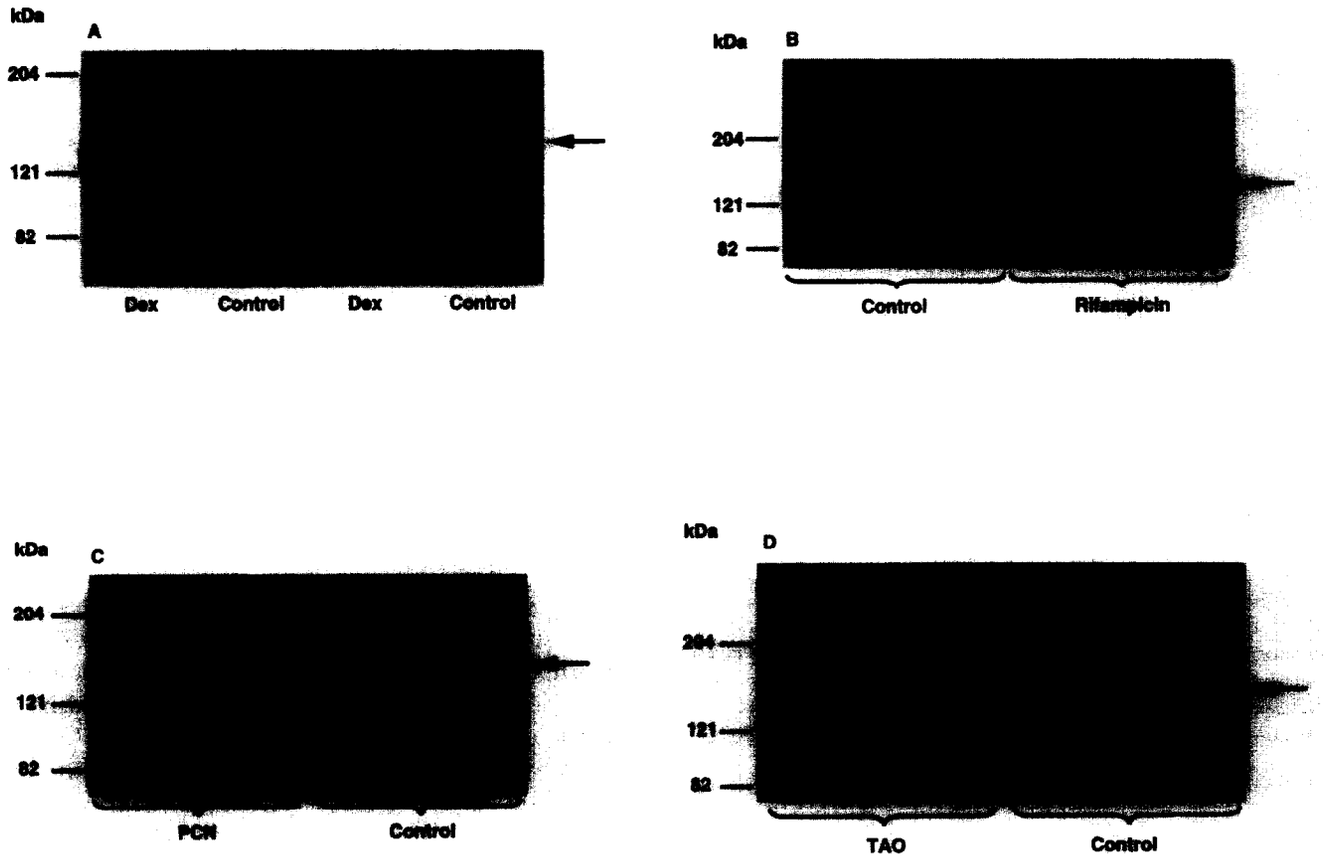


FIG. 2. Induction of Pgp in male rat liver. (A) Four rats were treated with Dex for 3 days and four control rats were administered corn oil for the same period. Plasma membrane proteins (10 μg) were separated on a 6% polyacrylamide gel and transferred onto nitrocellulose. The membrane was probed with the monoclonal antibody C219, and the blot was developed with the ECL detection reagent. (B) Four rats were treated with Rif for 4 days and four control rats were injected with DMSO. Plasma membrane proteins (10 μg) were separated as described in (A). (C) Four rats were treated with PCN for 4 days. For each treatment, four control rats were given the vehicle (corn oil). Analysis was then carried out as described in (A). (D) Four rats were treated with TAO for 4 days. For each treatment, four control rats were administered with corn oil. Analysis was carried out as described in (A).

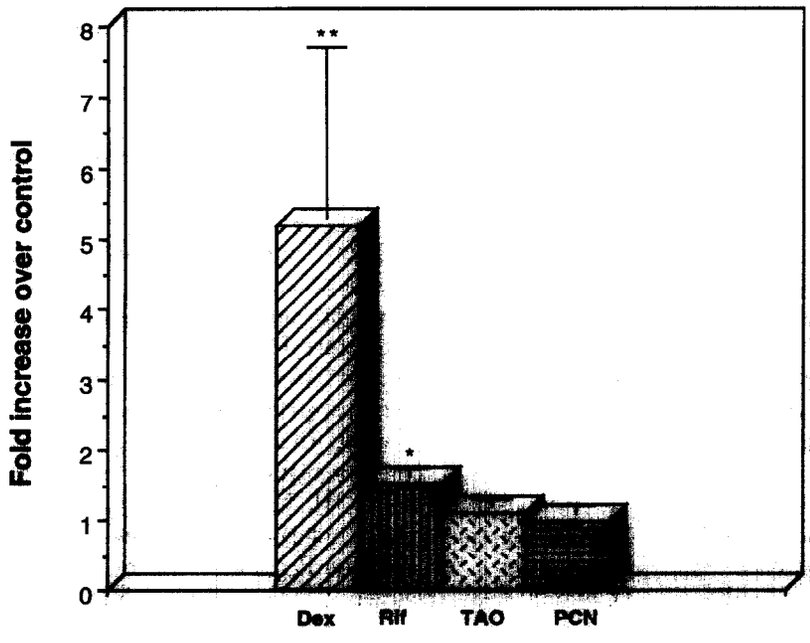


FIG. 3. Induction of Pgp following treatment of male rats with Dex, Rif, TAO, and PCN. Western blots were quantitated by densitometry. Fold induction was calculated based on Pgp expression in control rats. The values are reported as means ± SD ($N = 4$). Key: (*) $P = 0.024$, and (**) $P = 0.005$.

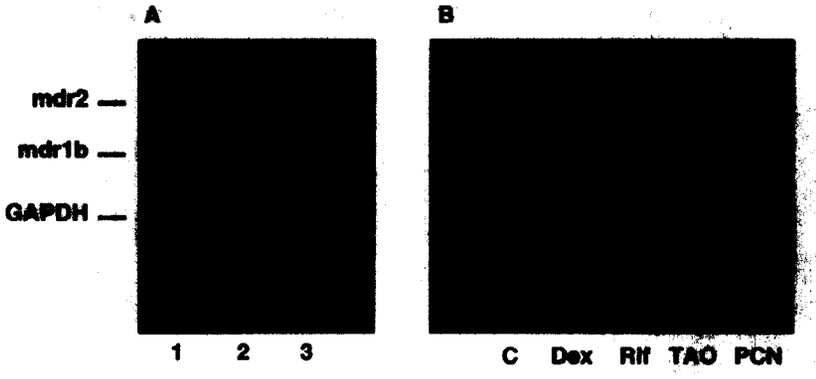
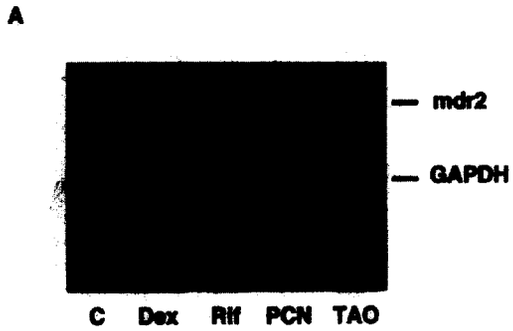


FIG. 4. Expression of mdr1b in male rat liver. (A) RNase protection assay of control samples with rat mdr probes. Lane 1: 20 μg of total RNA extracted from GCE cells was hybridized with *mdr1b* and GAPDH probes. Lane 2: 20 μg of total liver RNA was hybridized to *mdr2* probe. Lane 3: 20 μg of total liver RNA was hybridized to GAPDH probe. (B) Total liver RNA (20 μg) from control (C) and treated animals was hybridized with the *mdr1b* and GAPDH probes, as described in "Materials and Methods."



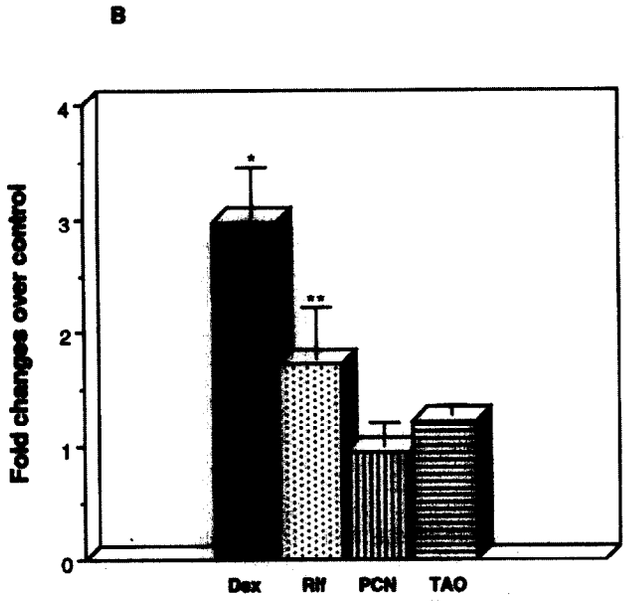


FIG. 5. Effects of Dex, Rif, PCN and TAO on mdr2 expression compared with control (C) in liver of male rats. (A) Representative RNase protection assay of 20 μg of total mRNA. (B) Induction of mdr2 was calculated by quantitation of mRNA and normalization of mdr2 to GAPDH expression using a densitometer. Fold induction was calculated based on mdr2 expression in control animals. The values are reported as means ± SD of three independent experiments. Key: (*) $p < 0.05$ and (**) $P = 0.052$ vs control.

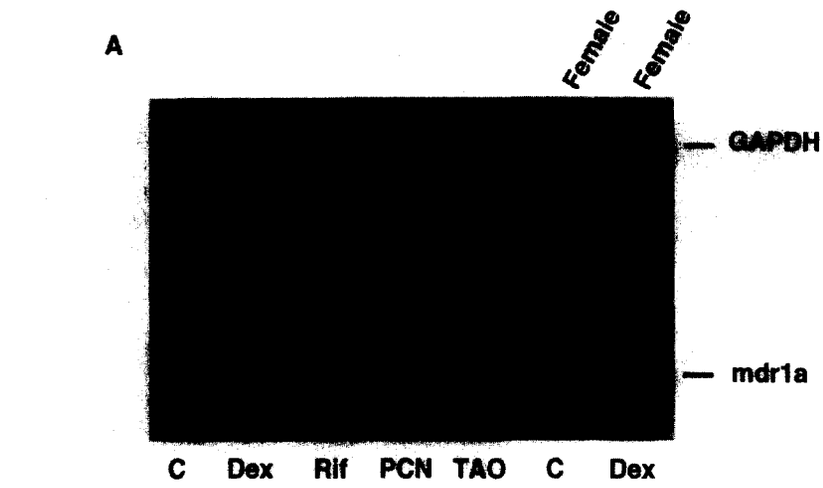
viously to be a potent inducer of CYP3A in humans and rabbits but a poor inducer in rats, unless used at high doses [23, 32]. In this study, we administered 100 mg Rif/kg/day for 4 days to Sprague–Dawley rats. This dose is able to induce CYP3A in Long–Evans rats [26]. The smaller induction of CYP3A obtained in our study might point to strain differences in the response to Rif, as has been observed for phenobarbital [33]. Interestingly, strain differences have also been reported in Pgp induction by carcinogens [34]. However, no study examining simultaneously the regulation of CYP3A and Pgp across strains has been carried out.

Using gene-specific probes for *mdr1a*, *mdr1b* and *mdr2*, we detected increased levels of mdr2 mRNA, relative to GAPDH mRNA, after Dex and Rif administration to male rats. These data suggest that these drugs modulate Pgp expression by transcriptional or post-transcriptional mechanisms. Parallel changes were also observed in female rats treated with Dex, where a reduction in Pgp was accompanied by a diminution in mdr2 mRNA relative to GAPDH. Mdr1a mRNA was not affected by the treatments used, in male and female rats, which agrees with the independent regulation of the three isoforms previously reported [24, 35].

The results obtained here with Dex contrast with previous reports using rat primary hepatocytes [13, 14, 36]. In those studies, mdr1b displayed a dramatic increase 8 hr after plating, whereas mdr2 exhibited a gradual decline to become the least expressed Pgp. The addition of Dex to the culture medium markedly reduced mdr1b mRNA and protein overexpression, the mechanism of which was found to be post-transcriptional, with a shortened mRNA half-life [14].

Previous studies in rats have focused on the effects of carcinogens or pathological states, showing increases of mdr1a and mdr1b but not mdr2 in cholestasis, and overexpression of the three Pgp isoforms after carbon tetrachloride treatment [35, 37, 38]. Our study shows a specific induction of mdr2. Such a response has also been observed in monkeys following administration of biliary excreted drugs [39] and in mice treated with hypolipidemic fibrates [40].

We observed a gender-specific expression of Pgp consistent with the findings of Furuya *et al.* [41]. Such a dimorphic expression has also been noted in human livers



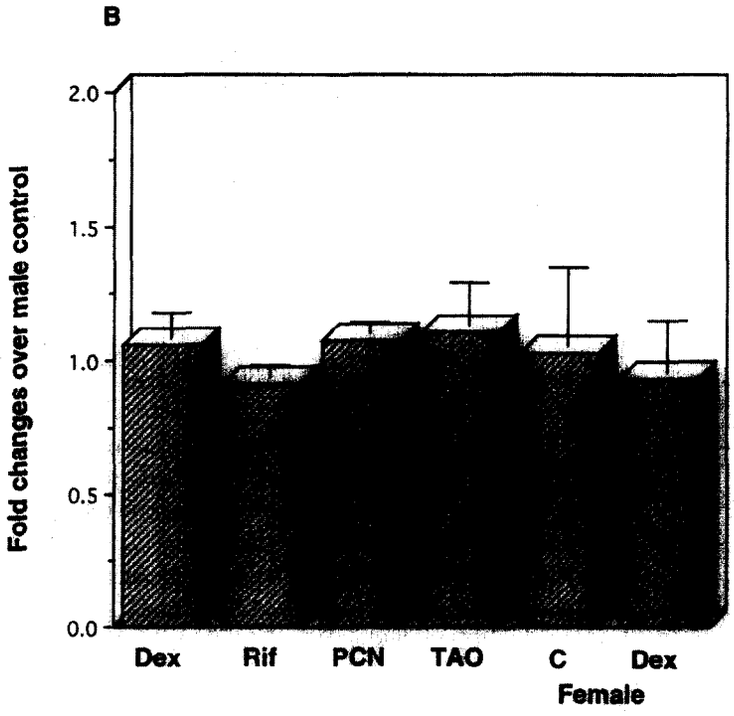


FIG. 6. Effects of Dex, Rif, PCN, and TAO on mdr1a expression in male and female rat liver. (A) The level of mdr1a mRNA was determined by the RNase protection assay using 20 μg of total RNA, as described in "Materials and Methods." (B) Changes in mdr1a mRNA levels were calculated by quantitation of mRNA and normalization of mdr1a to GAPDH expression using a densitometer. Fold changes were calculated based on mdr1a expression in control male rats. The values are reported as means ± SD of three independent experiments.

where Pgp levels are higher in males [42], in hamster adrenal glands [43], and in mouse kidney where mdr1b showed a much higher expression in females [8]. However, here we report for the first time opposite effects of a drug, Dex, on Pgp expression which is gender dependent. Although the mechanisms involved are not elucidated, the gender-specific response to Dex could result from effects on a hormonal regulatory pathway. By inducing CYP3A, Dex modifies steroid metabolism in male and female rats. Steroids have been shown to interact with Pgp [44]; thus, it is conceivable that this induction affects Pgp expression, enhancing a regulatory pathway in males and antagonizing it in females. However, for such a mechanism, we would anticipate an induction of Pgp in males with all the CYP3A inducers used. An alternate possibility could be that Dex targets directly a hormonal mechanism which, once it is affected, leads to opposite responses in males and females. Growth hormone has been shown to determine CYP2C11, 2C12, and 3A2 expression, depending on the pattern of secretion in male and female rats [27]; it was also able to

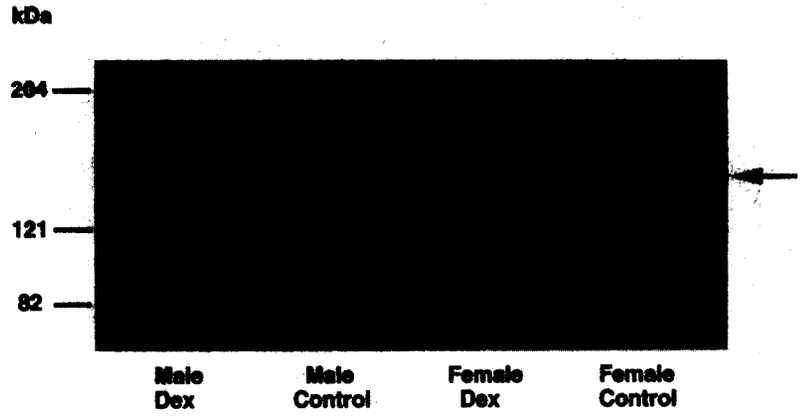


FIG. 7. Representative western blot of the effects of Dex on Pgp expression in male and female rat livers. Each lane represents a different rat. Plasma membrane proteins (10 μg) were separated on a 6% polyacrylamide gel and transferred onto nitrocellulose. The membrane was probed with the monoclonal antibody C219 and developed with the ECL detection reagent.

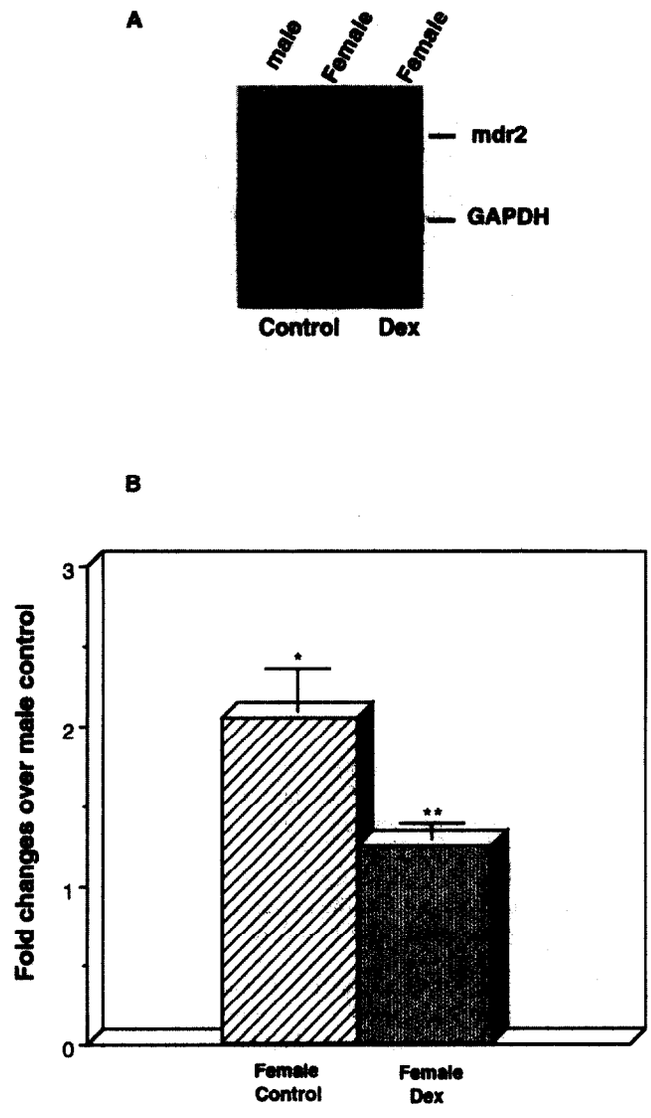


FIG. 8. Effects of Dex treatment on mdr2 expression in male and female rat livers. (A) Representative RNase protection assay of 20 μg of total RNA. (B) Induction of mdr2 was calculated by quantitation of mRNA and normalization of mdr2 to GAPDH expression using a densitometer. Fold changes were calculated based on mdr2 expression in control male rats. The values are reported as means ± SD of three independent experiments. Key: (*) $P < 0.05$ vs male control; and (**) $P < 0.05$ vs female control.

modify steroid metabolism in male rats [45]. Growth hormone might similarly have a significant influence on the gender-specific expression of Pgp.

In summary, these results demonstrated that CYP3A and Pgp responses to xenobiotics are likely to be driven by independent mechanisms. However, both proteins present gender-specific expression and regulation. Further studies are needed to clarify the mechanisms involved in this dimorphism and what, if any, common factors may be involved.

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