

Up-regulation of multidrug resistance-associated protein 2 (MRP2) expression in rat hepatocytes by dexamethasone

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Abstract Regulation of multidrug resistance-associated protein (MRP2) expression in response to dexamethasone (DEX) was analyzed using mainly primary rat hepatocytes. Enhanced levels of MRP2 mRNAs associated with increased amounts of a 190 kDa MRP2 were found in cultured DEX-treated hepatocytes; similarly, administration of DEX to rats (100 mg/kg, i.p.) led to a marked increase of hepatic amounts of MRP2 mRNAs. Maximal induction of MRP2 expression in DEX-treated primary hepatocytes was reached with 10^{-5} M DEX, a concentration higher than that (10^{-7} M) required for maximal up-regulation of tyrosine aminotransferase (TAT), a typical glucocorticoid receptor-regulated enzyme. In addition, the anti-glucocorticoid compound RU486 failed to inhibit MRP2 induction caused by DEX whereas it fully blocked that of TAT. These findings therefore demonstrate that DEX is a potent inducer of MRP2 expression in rat hepatocytes through a mechanism that seems not to involve the classical glucocorticoid receptor pathway.

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Key words: Dexamethasone; Hepatocyte; Multidrug resistance-associated protein 2; RU486; Tyrosine aminotransferase

1. Introduction

Multidrug resistance-associated protein 2 (MRP2), also called canalicular multi-specific organic anion transporter (cMOAT), is a 190 kDa plasma membrane phosphoglycoprotein [1,2]. It is predominantly expressed at the biliary pole of hepatocytes, but has also been evidenced in kidney and intestine [3]. MRP2 is thought to mediate the transport of various organic anions, including glucuronate, sulfate and glutathione conjugates, across the canalicular hepatocyte membrane [1,2]. Mutant strains of rats lacking MRP2 expression such as the transport deficient (TR⁻) wistar rat and the Eisai hyperbilirubinemic rat (EHBR) therefore display a deficiency in the biliary secretion of conjugates of xenobiotics or endogenous compounds such as bilirubin [1,3]. Similarly, in humans, the Dubin-Johnson-syndrome, an autosomal dominant hereditary disease characterized by a chronic hyperbilirubinemia, is also caused by an absence of MRP2 due to a mutation in the gene [4].

MRP2 belongs to the ATP-binding cassette (ABC) transporter superfamily which comprises other drugs efflux pumps such as P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1) [5]. Like these latter membrane pro-

teins, MRP2 is thought to transport some anti-cancer drugs such as vinblastine out of cells and may therefore confer some drug resistance to tumor cells [6].

Regulation of hepatic expression of MRP2 is poorly understood. Recent studies have however demonstrated that MRP2 levels are down-regulated in the liver in response to endotoxin whereas exposure of cultured rat hepatocytes to 2-acetylaminofluorene or cycloheximide results in an increased expression of MRP2 [7–9]. Such treatments have also been found to alter the levels of P-gp found in rat hepatocytes [8,10,11], thus suggesting that some factors, i.e. xenobiotics or endogenous compounds, may regulate both MRP2 and P-gp. Interestingly, expression of the latter pump has been shown to be strongly down-modulated in primary cultured rat hepatocytes treated by DEX [12]. This potent glucocorticoid is also known to regulate other liver proteins such as tyrosine aminotransferase (TAT) through its binding to the glucocorticoid receptor (GR) and subsequent interaction with glucocorticoid response elements found in the 5'-flanking region of responsive genes [13,14]. In the present study, we have investigated whether DEX may also regulate MRP2 levels using mainly primary rat hepatocyte cultures that are well recognized as a suitable cellular model for analyzing regulation of hepatic transporters [9,10,12]. Our results indicated that the glucocorticoid compound strongly induced MRP2 expression in rat hepatocytes through a mechanism that seems not to involve the GR pathway.

2. Materials and methods

2.1. Chemicals

Dexamethasone, actinomycin D, clotrimazole and pregnenolone 16 α -carbonitrile (PCN) were purchased from Sigma (St Louis, MO, USA). RU-486 (also termed mifepristone) was kindly provided by Roussel-Uclaf (Romainville, France).

2.2. Animal treatment

Male Sprague-Dawley rats weighing 180–200 g were treated with DEX (100 mg/kg, i.p.) dissolved in corn oil. Control rats received only corn oil. Rats were killed 8 h after administration of the glucocorticoid. Livers were removed and immediately stored at -80°C until use.

2.3. Cell isolation and culture

Hepatocytes from adult male Sprague-Dawley rats weighing 180–200 g were isolated by perfusion of the liver as previously described except that a liberase solution was used for dissociation of liver cells instead of a collagenase solution [12]. Cells were seeded at a density of 10^5 cells/cm² in plastic dishes in Williams' E medium supplemented with 0.2 mg/ml bovine serum albumin, 10 mg/ml bovine insulin and 10% (v/v) fetal calf serum. The medium was discarded 4 h after cell seeding and cells were thereafter maintained in serum-free medium. For treatment of the cultures, DEX, actinomycin D, clotrimazole, PCN and RU486, dissolved in dimethylsulfoxide, were added in the serum-free medium; controls received the same volume of solvent which did not exceed 0.1% (v/v). At the concentrations used, neither

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DEX nor the xenobiotics used were found to have cytotoxic effects towards primary hepatocytes as assessed by light microscopic examination of the cultures.

2.4. Isolation of total RNAs and Northern blot analysis

Total RNAs were extracted from cultured hepatocytes or liver fragments by the guanidinium thiocyanate/cesium chloride method of Chirgwin et al. [15] as modified by Raymondjean et al. [16]. For Northern blotting, 10 μ g of total RNAs was subjected to electrophoresis in a denaturing formaldehyde/agarose gel and transferred onto Hybond-N+ sheets (Amersham). The sheets were pre-hybridized, hybridized with 32 P-labeled probes, washed, dried and autoradiographed at -80°C . MRP2 mRNAs were analyzed with a 0.86 kb rat MRP2 cDNA probe generated by reverse transcription-polymerase chain reaction as previously reported [17] whereas TAT and P-gp mRNAs were detected using a rat TAT cDNA probe, kindly provided by Dr. L. Corcos (INSERM U517, Dijon, France), [18] and the pCHP1 probe [19], respectively. 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 were prepared from cultured hepatocytes by differential centrifugation as described by Germann et al. [20]. Membrane proteins were then separated on a 7.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose sheets. Sheets were blocked for 2 h with Tris-buffered saline containing 3% bovine serum albumin and 1% milk and were next incubated with the polyclonal anti-MRP2 antibody RM2. This antibody was developed through rabbit immunization against the peptide EAGIENVNHTL corresponding to the C-terminal portion of rat MRP2. The anti-MRP2 EAG15 antibody, kindly provided by Dr. D. Keppler (Deutsches Krebsforschungszentrum, Heidelberg, Germany) was also used as primary antibody instead of RM2. A peroxidase-conjugated anti-rabbit 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 rabbit serum.

3. Results

Primary rat hepatocytes were first treated by various concentrations of DEX ranging from 10^{-12} to 10^{-4} M for 48 h. Northern blot analysis indicated that the glucocorticoid compound strongly enhanced MRP2 mRNA levels in a dose-dependent manner (Fig. 1). Indeed, this effect started with 10^{-8} M DEX and was maximal when the glucocorticoid was used at 10^{-5} M or 10^{-4} M. As previously reported [1,2], several MRP2 mRNAs of different sizes, especially of 5.5 kb and 7.5 kb, were evidenced on the blot (Fig. 1); they probably represent alternative mRNA splicing variants with different 3'-untranslated region lengths [21] and their relative proportion appeared not to be obviously modified in response to DEX whatever doses were used. Dexamethasone was also found to down-regulate P-gp mRNA levels and to increase those of TAT mRNAs in primary hepatocytes (Fig. 1). Analysis of the dose-response corresponding to this induction of TAT transcripts indicated that, in contrast to MRP2 mRNAs, those of TAT were maximally induced when DEX was used at 10^{-7} M and higher concentrations of the glucocorticoid did not result in an higher increase of TAT mRNA amounts.

Time-course of the induction of MRP2 mRNA levels occurring in response to 10^{-4} M DEX in primary hepatocytes was further determined by Northern blot analysis. As indicated in Fig. 2, MRP2 mRNA amounts were clearly up-regulated in primary hepatocytes as early as a 4 h exposure to the glucocorticoid compound whereas they were not obviously altered by shorter treatments. Longer exposures (24–48 h) to

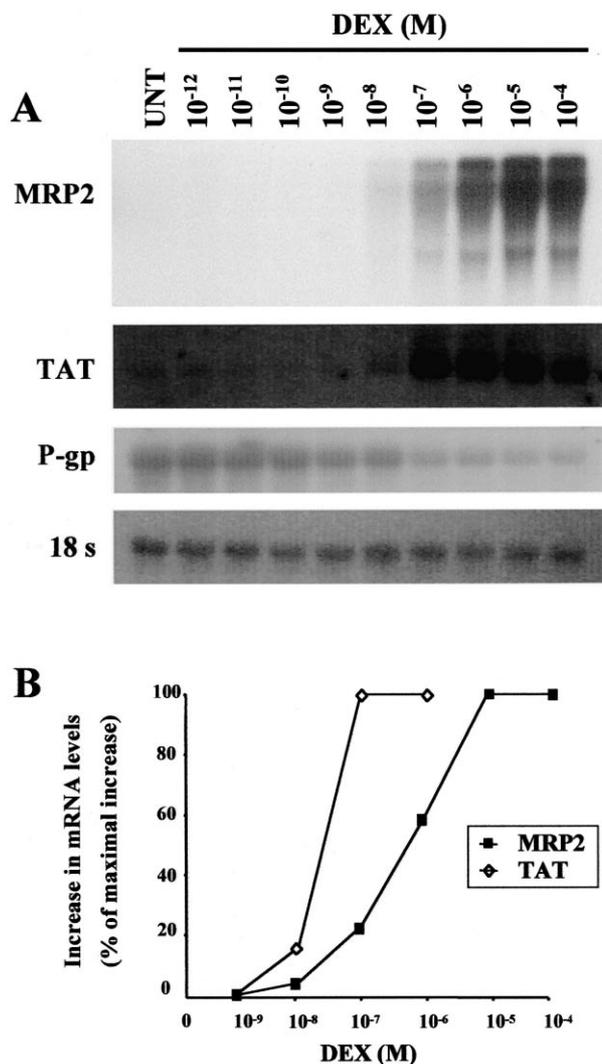


Fig. 1. Dose-dependent induction of MRP2 mRNAs in primary hepatocytes exposed to DEX. A: Each lane contained 10 μ g total RNAs isolated from primary rat hepatocytes either untreated (UNT) or exposed to various concentrations of DEX ranging from 10^{-12} to 10^{-4} M. RNAs were transferred to Hybond N+ sheets after electrophoresis and hybridized with MRP2, TAT, P-gp and 18S probes. B: Increases in MRP2 and TAT mRNA levels occurring in response to various doses of DEX were quantified by densitometric analysis. Results were expressed as percentage of maximal increase of MRP2 or TAT mRNAs, obtained with 10^{-5} and 10^{-7} M DEX, respectively. The data shown are representative of two independent experiments.

DEX resulted in maximal induction of MRP2 transcripts (Fig. 2).

Total RNAs were next prepared from livers of rats treated by i.p. injection of DEX. As indicated in Fig. 3, DEX-treated rats displayed enhanced hepatic amounts of MRP2 mRNAs when compared to control rats.

Crude membrane fractions were prepared from cultured hepatocytes exposed to 10^{-4} M DEX for 48 h and were used to investigate MRP2 expression by Western blot analysis (Fig. 4). An RM2 antibody-reactive band of 190 kDa corresponding to MRP2 was markedly overexpressed in DEX-treated hepatocytes when compared to their untreated counterparts. A similar 190 kDa band was also observed in DEX-treated rat hepatocytes when the polyclonal antibody EAG15

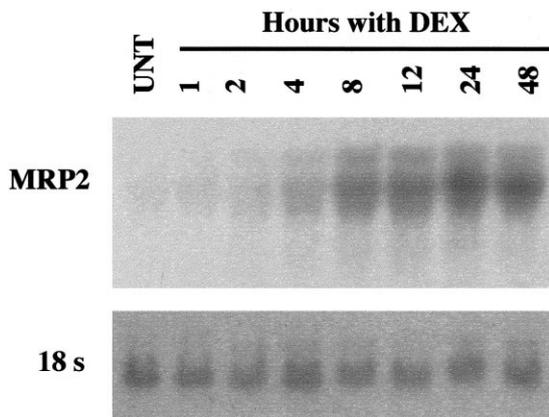


Fig. 2. Time-course of MRP2 mRNA induction in response to DEX. Each lane contained 10 µg total RNAs isolated from primary rat hepatocytes either untreated (UNT) or exposed to 10⁻⁴ M DEX for various lengths of time (1–48 h). RNAs were transferred to Hybond N+ sheets after electrophoresis and hybridized with MRP2 and 18S probes.

was used instead of RM2 whereas this band was not present in control blots performed using non-immune rabbit serum as primary antibody (data not shown).

The effect of actinomycin D, a known inhibitor of transcription, on DEX-mediated induction of MRP2 mRNA levels was further evaluated by Northern blot analysis. In this experiment, actinomycin D was used at 3 µg/ml for 8 h and such a treatment has been previously shown to decrease RNA synthesis levels to less than 1% of values found in untreated liver cells [22]. As indicated in Fig. 5A, actinomycin D fully suppressed MRP2 mRNA increase occurring in response to DEX in primary hepatocytes whereas it did not affect basal levels of MRP2 transcripts.

We then examined the effect of RU486, an anti-glucocorticoid compound acting through interaction with the GR receptor [23], on DEX-related increase of MRP2 mRNA levels. RU486, used at 10⁻⁵ M, did not block induction of MRP2 transcripts occurring in response to DEX in cultured hepatocytes (Fig. 5B). Moreover, RU486-treated hepatocytes were found to display increased amounts of MRP2 mRNAs when compared to their untreated counterparts; such an increase was however weaker than that occurring in response to DEX. By contrast, treatment by the anti-glucocorticoid com-

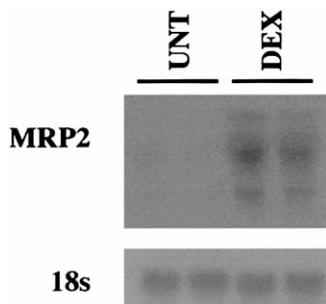


Fig. 3. Induction of MRP2 mRNA levels in liver of DEX-treated rats. Each lane contained 10 µg total RNAs isolated from the liver of control rats (UNT) or DEX-treated rats. RNAs were transferred to Hybond N+ sheets after electrophoresis and hybridized with MRP2 and 18S probes. The data shown are representative of the analysis of four rats in each group (control and DEX-treated).

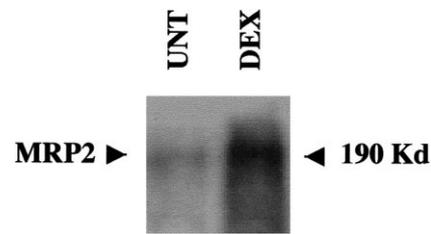


Fig. 4. Western blot analysis of crude membrane proteins obtained from DEX-treated hepatocytes. Crude membrane fractions were prepared from primary rat hepatocytes either untreated (UNT) or exposed to 10⁻⁴ M DEX for 48 h. Membrane proteins (50 µg) were transferred onto a nitrocellulose sheet after electrophoresis. After incubation with RM2 antibody raised against MRP2, the blot was developed as described in Section 2.

ound did not up-regulate TAT mRNA levels in primary hepatocytes and was found to strongly inhibit induction of TAT mRNA levels caused by DEX (Fig. 5B).

Finally, we analyzed MRP2 mRNA levels in primary hepatocytes treated by the antimycotic drug clotrimazole or the

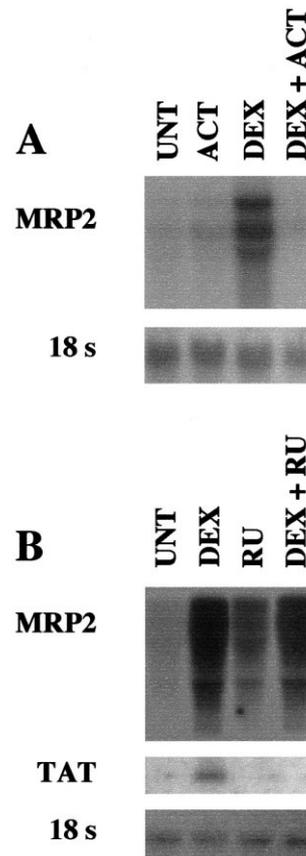


Fig. 5. Effect of actinomycin D (A) and RU486 (B) on DEX-mediated MRP2 mRNA induction. A: Each lane contained 10 µg total RNAs isolated from primary rat hepatocytes either untreated (UNT) or exposed to 10⁻⁴ M DEX or 3 µg/ml actinomycin D (ACT) or co-treated with DEX and ACT for 8 h. RNAs were transferred to Hybond N+ sheets after electrophoresis and hybridized with MRP2 and 18S probes. B: Each lane contained 10 µg total RNAs isolated from primary rat hepatocytes either untreated (UNT) or exposed to 10⁻⁷ M DEX or 10⁻⁵ M RU486 or co-treated with DEX and RU486 for 48 h. RNAs were transferred to Hybond N+ sheets after electrophoresis and hybridized with MRP2, TAT and 18S probes.

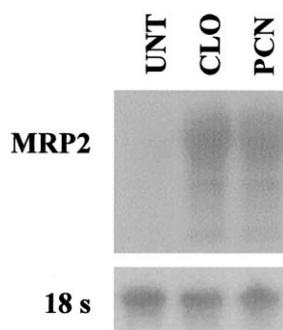


Fig. 6. Effect of clotrimazole and PCN on MRP2 mRNA levels in primary rat hepatocytes. Each lane contained 10 μ g total RNAs isolated from primary rat hepatocytes either untreated (UNT) or exposed to 2×10^{-5} M clotrimazole (CLO) or to 2×10^{-5} M PCN. RNAs were transferred to Hybond N+ sheets after electrophoresis and hybridized with MRP2 and 18S probes.

anti-glucocorticoid PCN. Indeed, these two drugs have previously been shown to induce expression of some DEX-regulated detoxifying enzymes such as cytochromes P450 (CYP) 3A1/2 [24]. As shown in Fig. 6, clotrimazole and PCN were also found to increase MRP2 mRNA levels in primary rat hepatocytes.

4. Discussion

The results reported in the present study demonstrated that DEX, a potent glucocorticoid compound, markedly up-regulates MRP2 expression in rat hepatocytes. Indeed, treatment by DEX was found to strongly enhance MRP2 mRNA levels in both DEX-treated primary hepatocytes and liver of DEX-treated rats. Such an increase in MRP2 transcripts occurred relatively rapidly since it started in primary hepatocytes after a 4 h exposure to the glucocorticoid compound. Moreover, it was associated with elevated levels of a 190 kDa MRP2 in cultured hepatocytes exposed to DEX as assessed by Western blot analysis; similarly, DEX-treated rats were also found to display enhanced hepatic levels of MRP2 (data not shown). By contrast, DEX was demonstrated to down-regulate expression of the cationic drug transporter P-gp in primary hepatocytes as previously reported [12]. Taken together, these data therefore indicate that DEX differently alters expression of drug transporters in hepatocytes. Similarly, levels of drug metabolizing enzymes found in rat hepatocytes may be either up-regulated or down-regulated in response to glucocorticoids according to the enzyme. Expression of some cytochromes P-450 (CYP) such as CYP 3A1/2 is thus increased by DEX [13,24] whereas levels of glutathione *S*-transferase pi are decreased in DEX-treated rat hepatocytes [12].

The action of DEX on MRP2 mRNA levels in primary hepatocytes requires active transcription since it was fully blocked by actinomycin D. By contrast, it was not inhibited by the anti-glucocorticoid RU486 whereas this compound fully blocked DEX-mediated induction of TAT mRNAs. These results suggest that GR, which plays a major role in regulation of TAT by DEX [25], is likely not involved in the induction of MRP2 expression by the glucocorticoid compound; such a regulation may therefore be included in non-classical glucocorticoid-mediated processes. This conclusion is moreover fully supported by the comparison of the dose-

responses for MRP2 and TAT, which clearly demonstrated that maximal induction of MRP2 required higher doses of DEX than those needed for TAT, a typical GR-regulated enzyme.

The profile of the dose-response for MRP2 up-regulation in response to DEX is in fact close to that observed for the induction of CYP 3A1/2 in rat liver cells. Indeed, maximal induction of CYP 3A1/2 in primary rat hepatocytes also required the use of elevated concentrations of glucocorticoids [13]. Another feature shared by both hormone-mediated MRP2 and CYP 3A1/2 regulations is linked to the effect of RU486; indeed, treatment by this anti-glucocorticoid compound, that was demonstrated in the present study to increase MRP2 transcript levels in primary hepatocytes, has also been found to up-regulate CYP3 A1/2, although, as for MRP2, to a smaller extent than exposure to DEX [24]. Interestingly, the increase of CYP 3A1/2 contents in DEX- or RU486-treated hepatocytes has been recently shown to be mediated by a new member of the nuclear hormone receptor superfamily termed pregnane X receptor (PXR) [26]. Indeed, DEX and RU486 have been demonstrated to activate PXR bound to a hormone response element located in the 5'-flanking region of the rat CYP 3A1/2 gene and subsequently to induce the transcription of this gene [26,27]; glucocorticoids and anti-glucocorticoids also up-regulate human CYP 3A4 levels by this way [28]. The similarities between DEX-related regulations of rat CYP 3A1/2 and MRP2 strongly favor the idea that PXR might play a role in glucocorticoid-mediated induction of MRP2. This hypothesis is moreover supported by the facts that (1) a putative consensus sequence for PXR, i.e. two copies of the nuclear receptor half site sequence TGAAC organized as an everted repeat and separated by 8 bp, is present in the promoter of the rat MRP2 gene at position -404 [29] and (2) clotrimazole and PCN, two known activators of PXR [28], were demonstrated to up-regulate MRP2 expression. In addition, we have detected substantial levels of PXR mRNAs in primary rat hepatocytes (data not shown), suggesting that PXR is well expressed in such cells. Further studies are therefore required to validate the putative involvement of PXR in DEX-mediated regulation of MRP2.

Dexamethasone and other glucocorticoids are drugs commonly used in patients, often in multicomination therapy, and the up-regulation of MRP2 expression that they may induce may increase MRP2-dependent biliary secretion of co-administered drugs, therefore potentially altering the efficacy of these compounds. Elimination of physiologically important substrates for MRP2 such as leukotriene C4 and bilirubin glucuronides may also be enhanced. In this context, it may be noteworthy that DEX treatment has been reported to up-regulate elimination of the contrast agent ioglycemic acid [30] and also to decrease bilirubin levels in some patients [31]. In addition, administration of DEX to rats has been recently shown to increase MRP2 expression in kidney [32], therefore suggesting that MRP2-mediated renal elimination of drugs may also be altered in response to glucocorticoids. It is however noteworthy that the doses of DEX that maximally induce MRP2 expression in primary hepatocytes are super-physiological, therefore suggesting that endogenous glucocorticoids may contribute to *in vivo* MRP2 regulation in a limited manner.

In summary, the present study indicates that DEX is a potent inducer of MRP2 expression in rat hepatocytes,

through a mechanism that requires active transcription and likely does not involve the classical GR pathway.

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