Dexamethasone- and osmolarity-dependent expression of the multidrug-resistance protein 2 in cultured rat hepatocytes[1]

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Expression of the conjugate export pump multidrug-resistance protein 2 (MRP2) in liver is regulated by endotoxin and anti-tumour agents. This paper reports on the effects of dexamethasone and osmolarity on MRP2 expression. MRP2 expression was studied at the protein, mRNA, immunocytochemical and functional levels in cultured rat hepatocytes. Protein and mRNA expression of MRP2 in rat hepatocytes 24 and 48 h after isolation were largely dependent on the presence of dexamethasone (100 nmol/l) in the culture medium. MRP2 was localized at the pseudocanalicular membrane and increased expression of MRP2 was accompanied by a widening of the pseudocanaliculi. In presence of dexamethasone, hypo-osmolarity (205 mosmol/l) led to a strong induction of MRP2 mRNA and protein, whereas expression was decreased by hyperosmolarity (405 mosmol/l). Also, a decay of MRP2 protein and mRNA following dexamethasone withdrawal was osmosensitive. Expression of dipeptidylpeptidase IV, another canalicular protein, was unaffected by dexamethasone and osmolarity. It is concluded that glucocorticoids are strong inducers of MRP2 in liver. Besides short-term carrier insertion/retrieval, osmoregulation of MRP2 also involves a long-term effect on MRP2 expression.

Key words: ABC transporter, bile secretion, cell volume, cholestasis, glucocorticoid.

INTRODUCTION

Canalicular secretion of glutathione and glucuronide conjugates is accomplished by the conjugate export pump MRP2 (multidrug-resistance protein 2, an isoform expressed in liver, kidney and gut) [1–4]. Defective expression of MRP2 underlies the jaundice in the human Dubin–Johnson syndrome [5] and the transporter deficient (TR^-) and Eisai hyperbilirubinaemic mutant (EHBR) rats [6–8]. MRP2 is regulated on a short-term basis by an osmo-dependent insertion/retrieval of the carrier molecules into/from the canalicular membrane [9]. On a long-term scale, MRP2 expression is modulated by ethinyl oestradiol, chemotherapeutic agents, barbiturates and bile-duct ligation [10–13], but nothing is known about the role of osmolarity on MRP2 expression. Endotoxin downregulates MRP2 at the protein and mRNA levels [13], in addition to a short-term retrieval of MRP2 from the canalicular membrane [14]. Cholestasis induced by endotoxin is counteracted by glucocorticoids [14,15]. We therefore studied the effects of dexamethasone and osmolarity on MRP2 expression.

MATERIALS AND METHODS

Materials

Cell-culture medium (Dulbecco's modified Eagle's medium, DMEM, with or without NaCl), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Biochrom (Berlin, Germany). Dexamethasone was from Merck (Darmstadt, Germany), and insulin from bovine pancreas, FITC-labelled phalloidin and the molecular-mass markers (205, 116, 97.5, 66, 45 and 29 kDa) were from Sigma (Deisehofen, Germany).

The EAG15 polyclonal antibody was raised in a rabbit against the 12-amino-acid peptide sequence at the C-terminus of the rat MRP2 sequence [16]. This was a generous gift from Professor D. Keppler (Deutsches Krebsforschungszentrum, Heidelberg, Germany), who also provided a sample of a preparation of rat canalicular membrane. The mouse monoclonal antibody MAB13.4 was raised against the rat dipeptidylpeptidase IV (DPPIV) and was a generous gift from Professor W. Reutter (University of Berlin, Berlin, Germany) [17]. Rat anti-mouse ZO-1 [a polypeptide associated with the tight junction (zonula occludens)] [18] was from Biozol (Eching, Germany). Cy3™-conjugated AffiniPure goat anti-rabbit IgG (heavy and light chains) and fluorescein-conjugated AffiniPure goat anti-rat IgG (heavy and light chains) were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). Sheep anti-rabbit IgG peroxidase was from Boehringer Mannheim (Mannheim, Germany), goat anti-mouse IgG horseradish peroxidase was from Bio-Rad (München, Germany) and 5-chloromethylfluorescein diacetate (CMFDA) was from Molecular Probes (Eugene, OR, U.S.A.). Enhanced chemiluminescence detection kit and the oligonucleotide-labelling kit were purchased from Amersham-Pharmacia (Freiburg, Germany), Duralon-UV nylon membranes were from Stratagene (Heidelberg, Germany) and [α-^{32}P]dCTP (3000 Ci/mmol) was from ICN (Meckenheim, Germany). The cDNA probe for the rat MRP2 was a 347-bp fragment, as described in [16], which represented 22.5% of the coding region of MRP2 and was also a gift from Professor D. Keppler. The 1.0-kb fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, which was used for standardization, was from Clontech (Palo Alto, CA, U.S.A.).

Isolation and culture of rat hepatocytes

Isolated rat hepatocytes were prepared from livers of male Wistar rats (150–180 g, fed *ad libitum*) by collagenase perfusion,

Abbreviations used: DPPIV, dipeptidylpeptidase IV; CMFDA, 5-chloromethylfluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MRP2, multidrug-resistance protein 2; TBS-T, Tris-buffered saline + 0.1% Tween 20; ZO-1, polypeptide associated with the tight junction (zonula occludens).

[1] This paper is dedicated to Professor Dr. K. H. Meyer zum Büschenfelde on the occasion of his 70th birthday.

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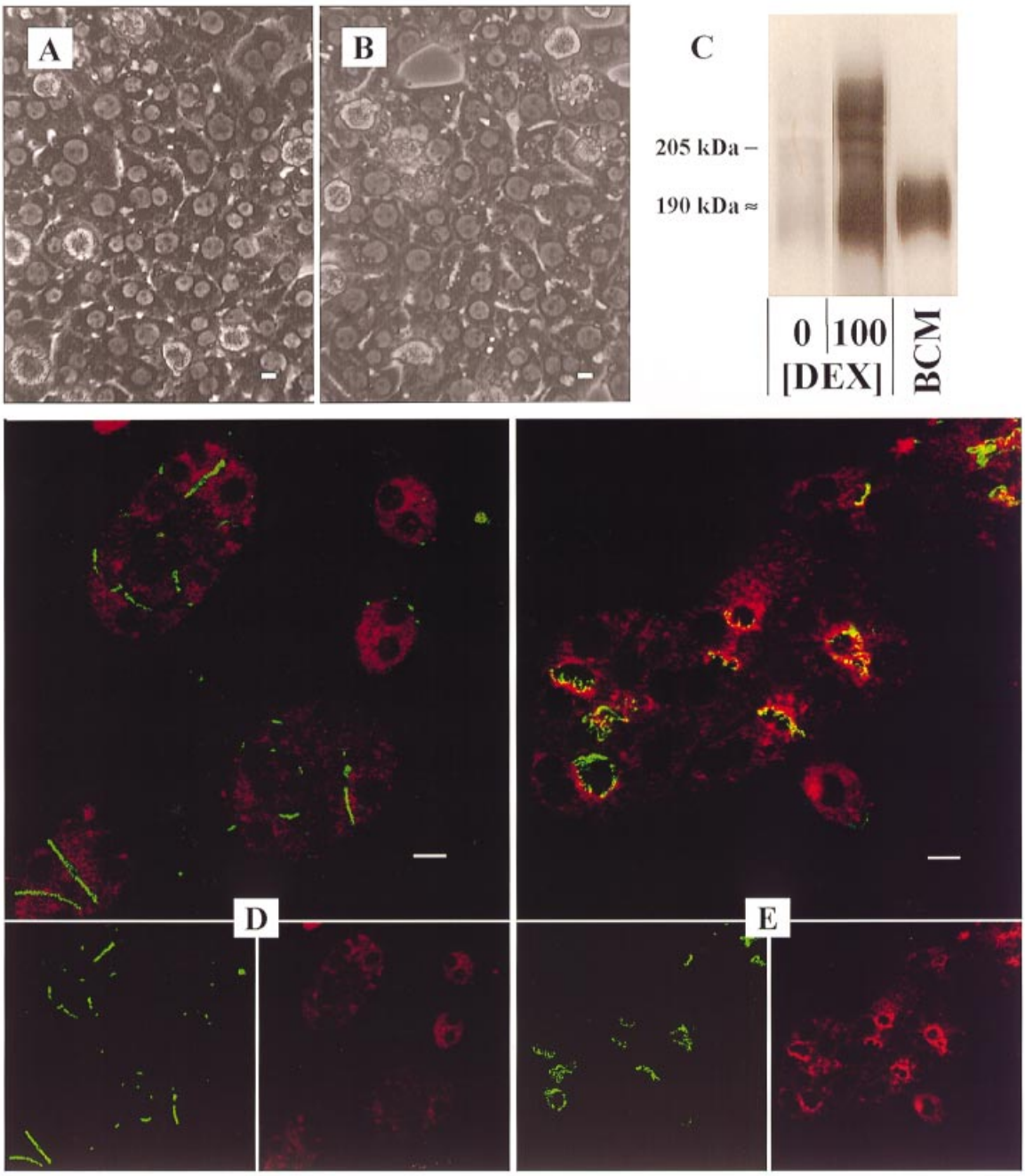

Figure 1 MRP2 expression in rat hepatocytes cultured with or without dexamethasone

Freshly isolated rat hepatocytes were cultured in DMEM for 48 h in the absence (**A**) or presence (**B**) of 100 nmol/l dexamethasone. Their light-microscopic appearances were similar but pseudocanaliculi in dexamethasone-treated cells appeared broadened. (**C**) MRP2 protein separated by SDS/PAGE was hardly detectable in hepatocytes grown for 48 h without dexamethasone ([DEX], 0 nmol/l) compared with cells grown under the influence of dexamethasone (100 nmol/l). The BCM lane shows a preparation of bile canalicular membrane (BCM) from rat liver, with an estimated molecular mass of $\approx$ 190 kDa [16] (see the Materials and methods section). Only minimal immunostaining of MRP2 (red) was found in hepatocytes grown for 24 h in DMEM lacking dexamethasone (**D**), whereas strong (peri)canalicular immunoreactivity for MRP2 was found in cells cultured with 100 nmol/l dexamethasone (**E**). Tight-junction-associated protein ZO-1 (green) marks the pseudocanaliculi. Note that pseudocanaliculi in dexamethasone-treated cells are enlarged. Cells were visualized by high amplification of the red channel, thereby increasing the background slightly. Large pictures, co-localization; small pictures, single channels; scale bars, 10 μm.

as described previously [19]. Cell viability was more than 90 % as assessed by Trypan Blue exclusion. Cell purity 24 h after isolation was approx. 99 %, as assessed after staining for F-actin by FITC-labelled phalloidin (for method see [20]), which allowed an easy discrimination of the non-parenchymal cells from hepatocytes by their morphology. Hepatocytes were seeded at a density of 0.1×10^6 cells/cm^2 on collagen type-VII-coated six-well plates (Becton-Dickinson, Heidelberg, Germany) or on glass coverslips. They were cultured in DMEM containing glucose (6 mmol/l), penicillin (100 units/ml) and streptomycin (100 μg/ml). In some

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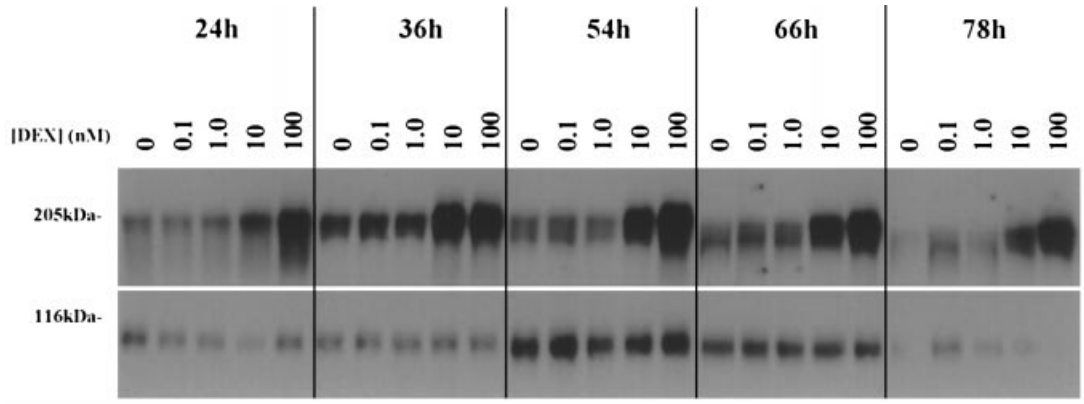

Figure 2 Dexamethasone increases the expression of MRP2 but not of DPPIV

Rat hepatocytes were cultured in DMEM for up to 78 h with increasing concentrations of dexamethasone ([DEX], 0–100 nmol/l). After 24 h of culture with or without dexamethasone there was a pronounced and concentration-dependent difference in the expression of MRP2 (upper row). Expression of DPPIV showed a culture-time but no dexamethasone dependency (lower row).

experiments the medium was supplemented with 10 % FBS/dexamethasone (100 nmol/l)/insulin (100 nmol/l)/aprotinin (1 μg/ml)/sodium selenite (30 nmol/l), according to the protocols described in the Results section. In some experiments, cells were exposed to hypo-osmotic (205 mosmol/l) or hyperosmotic (405 mosmol/l) media. Osmolarity was changed by appropriate alteration of the NaCl concentration of the DMEM.

Western-blot analysis

For Western-blot analysis of the rat MRP2, hepatocytes grown in six-well plates were lysed in 150 μl of a buffer containing 0.5 % SDS and 10 mmol/l Tris/HCl (pH 7.4) supplemented with a cocktail of protease inhibitors (Complete™, Boehringer Mannheim). Protein contents of the individual samples were determined in triplicates with the Bio-Rad protein assay (Bio-Rad) according to the manufacturer's guidelines. Cell lysates were mixed with loading buffer [final concentrations: 55 mmol/l Tris/HCl (pH 7.8), 2.3 % SDS, 0.4 mol/l sucrose, 5 mmol/l EDTA, 20 mmol/l dithiothreitol, 0.03 % Bromophenol Blue] and incubated at 37 °C for 30 min. Protein (30 μg per sample) and 1 μl of a preparation of bile-canalicular membrane as a reference (preparation described in [21,22]) were loaded on to a 7.5 % gel and separated by SDS/PAGE. Proteins were then blotted on to nitrocellulose membranes (0.45 μm; Schleicher & Schuell, Dassel, Germany) by semi-dry transfer with transfer buffer composed of 38.6 mmol/l glycine, 47.8 mmol/l Tris and 0.03 % SDS. Even transfer of proteins was checked routinely by staining of the membranes with Ponceau S solution (Sigma). The membranes were blocked overnight at 4 °C with 5 % BSA in Tris-buffered saline + 0.1 % Tween 20 (TBS-T). EAG15 antibody (anti-rat MRP2, 1:25000) or MAB13.4 (anti-DPPIV, 1:10) was applied for 2 h to the membranes in 1 % BSA/TBS-T. After washing, sheep anti-rabbit peroxidase or goat anti-mouse horseradish peroxidase was applied for another 2 h. Binding of the secondary antibody was detected by the enhanced chemiluminescence detection kit according to the manufacturer's instructions. Incubation with MAB13.4 was performed on the membranes after detection of MRP2 and after a second blocking period with 5 % BSA/TBS-T overnight.

Northern-blot analysis

Total RNA from culture plates of liver parenchymal cells was isolated by using a total-RNA extraction kit (Qiagen, Hilden, Germany). RNA samples were electrophoresed in 0.8 % agarose/3 % formaldehyde and then blotted on to Duralon-UV nylon membranes with 20 × SSC (where 1 × SSC is 0.15 mol/l NaCl/0.015 mol/l sodium citrate). After brief rinsing with water and crosslinking with UV light (Hoefer UV-crosslinker 500; Hoefer, San Francisco, CA, U.S.A.), blots were subjected to hybridization as described in [23]. Suitably exposed autoradiograms were then analysed with densitometry scanning (E. A. S. Y. RH-System, Herolab, Wiesloch, Germany) to determine the densities of the mRNA levels. Relative mRNA levels were determined by standardization of the density of GAPDH mRNA.

Immunocytochemistry

For immunocytochemistry, cells cultured on glass coverslips were fixed with pure methanol (−20 °C, 10 min). They were permeabilized with Triton-X 100 (0.1 % in PBS, 10 min) and then washed with PBS. Unspecific binding was blocked with BSA (1 % in PBS, 1 h). Either a mixture of the primary antibodies [i.e. anti-rat MRP2 (EAG15), 1:5000 plus anti-ZO-1, 1:500], PBS alone or a single primary antibody for control staining was applied for 2 h of incubation in a wet chamber. After rinsing and washing, a combination of Cy3™-conjugated goat anti-rabbit IgG (1:500) plus FITC-conjugated goat anti-rat IgG (1:100) was applied for 2 h. Finally, the cells were washed and mounted on slides.

Phase-contrast and confocal laser-scanning microscopy

Before cells were harvested for Western- or Northern-blot analyses, they were photographed with a Seescan 3CCD low-light camera (Intas, Göttingen, Germany) on an inverted IM35 microscope (Zeiss, Oberkochem, Germany) with a phase-contrast facility. Immunostained cell samples were analysed using a Leica TCS-NT confocal laser-scanning system with an argon/krypton laser on a Leica DM IRB inverted microscope (Leica, Bensheim, Germany). Images were acquired from two channels at wavelengths of 488 and 568 nm. Only samples that were prepared in

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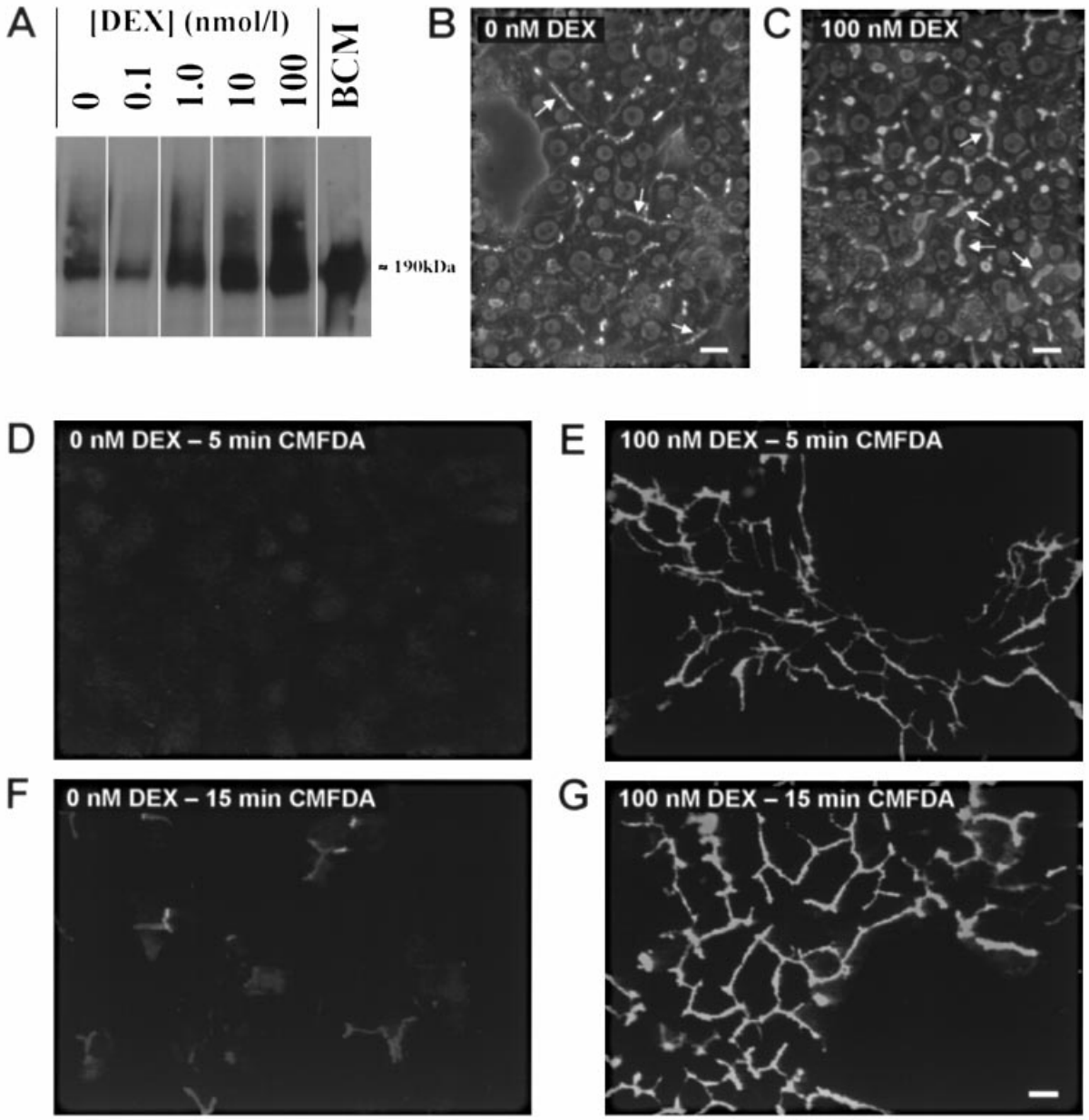

Figure 3 Withdrawal of dexamethasone decreases the expression of MRP2

Rat hepatocytes were cultured for 36 h in DMEM supplemented with 10% FBS, 100 nmol/l dexamethasone, 100 nmol/l insulin, 1 μg/ml aprotinin and 30 nmol/l sodium selenite. Thereafter, medium was changed to DMEM containing no supplements or just dexamethasone (DEX) in different concentrations, as indicated. MRP2 expression was reduced dose-dependently 24 h later (**A**). Formation of pseudocanaliculi (arrows) was identical in cells cultured without (**B**) or with 100 nmol/l dexamethasone (**C**) but they were enlarged in dexamethasone-treated cells. In cells cultured without dexamethasone for 48 h no canalicular filling was detectable 5 min after addition of 2 μmol/l CMFDA (**D**; for details see the Materials and methods section), whereas dexamethasone-treated cells rapidly secreted the fluorescent substrate (**E**). After CMFDA application (15 min) there was some pseudocanalicular filling in the dexamethasone-free conditions (**F**), with no further increase for up to 30 min (results not shown). In contrast, dexamethasone-treated hepatocytes showed a further strong accumulation of the dye (**G**) at that time point. BCM, preparation of bile-canalicular membrane of rat liver; scale bars, 20 μm.

parallel in all steps were compared, and identical adjustments were used.

Evaluation of functional MRP2 in rat hepatocytes

The secretory activity of MRP2 in cultured hepatocytes was assessed by the use of CMFDA. The lipophilic compound CMFDA is taken up by diffusion into the cells, where it becomes hydrophilic and fluorescent through cleavage of the acetates by unspecific cytosolic esterases. Thereafter, the compound is conjugated with glutathione by glutathione S-transferase [24]. The glutathione-conjugated 5-chloromethylfluorescein is a substrate for MRP2 [25].

Cultured hepatocytes grown on glass coverslips were incubated with Krebs–Henseleit buffer at 37 °C and 5% CO_2 1 h before measurement. Thereafter, the coverslips were placed in a bath

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holder, covered with 200 μl of Krebs–Henseleit buffer and mounted on the inverted microscope of the confocal laser-scanning system. After adjustment of the optical plane, CMFDA (dissolved in Krebs–Henseleit buffer) was added to the bath, resulting in a final concentration of 2 μmol/l CMFDA. Cells were scanned every 5 min for up to 30 min (exciting light, 488 nm; emission measured at > 515 nm) with constant adjustments for all conditions.

Statistics

Data were reproduced in at least three independent hepatocyte preparations. Relative absorbances of MRP2 mRNA were compared using Student's t test, with $P < 0.05$ considered to be statistically significant.

RESULTS

Dexamethasone-dependent expression of MRP2

Freshly isolated rat hepatocytes (0.1×10^6 cells/cm^2) cultured for 48 h in DMEM with (Figure 1B) or without (Figure 1A) dexamethasone had similar light-microscopic appearances, but the pseudocanaliculi appeared wider in dexamethasone-treated hepatocytes. Cells cultured for 48 h without dexamethasone expressed scarcely any MRP2 protein, as evidenced by Western-blot analysis, whereas hepatic parenchymal cells, which were grown in the presence of 100 nmol/l dexamethasone, expressed high amounts of MRP2 (Figure 1C). Compared with the MRP2 detected from a preparation of bile-canalicular membrane, a shift of the MRP2 band above 190 kDa was found regularly in cultured rat hepatocytes (compare Figure 1 and Figure 3, see below). The cause for this was not further investigated.

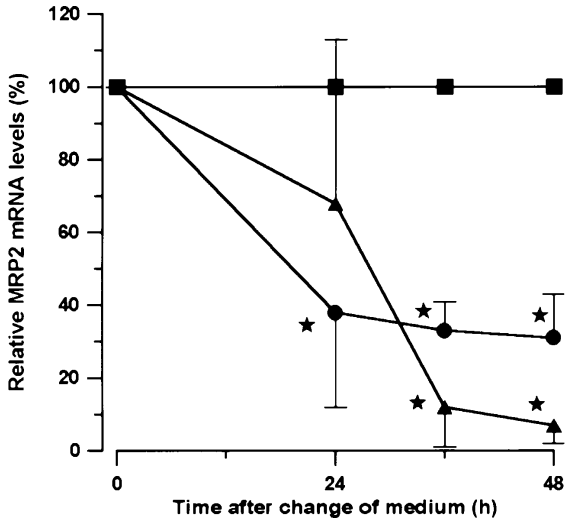

Figure 4 MRP2 mRNA decreases after withdrawal of dexamethasone

Rat hepatocytes were cultured for 36 h in DMEM (with FBS, 100 nmol/l dexamethasone, insulin, aprotinin and sodium selenite), and then the medium was changed to DMEM with 0, 1 or 100 nmol/l dexamethasone. RNA was isolated 24–48 h later and analysed by Northern blotting. Whereas MRP2 mRNA levels in cells cultured in the presence of 100 nmol/l dexamethasone remained relatively unchanged, a continuous decline of MRP2 mRNA was observed when dexamethasone was displaced. Reduction of the relative amount of MRP2 mRNA (as determined by standardization to GAPDH mRNA) was dose- and time-dependent. The relative MRP2 mRNA levels found in incubations with 100 nmol/l dexamethasone at each time point were set to 100% (■) and the relative MRP2 mRNA levels found in media with 1 nmol/l (●) or without (▲) dexamethasone were expressed as percentages thereof. Data are given as means ± S.E.M. and are from three independent hepatocyte preparations. *, Significantly different from the incubations with 100 nmol/l dexamethasone ($P < 0.05$).

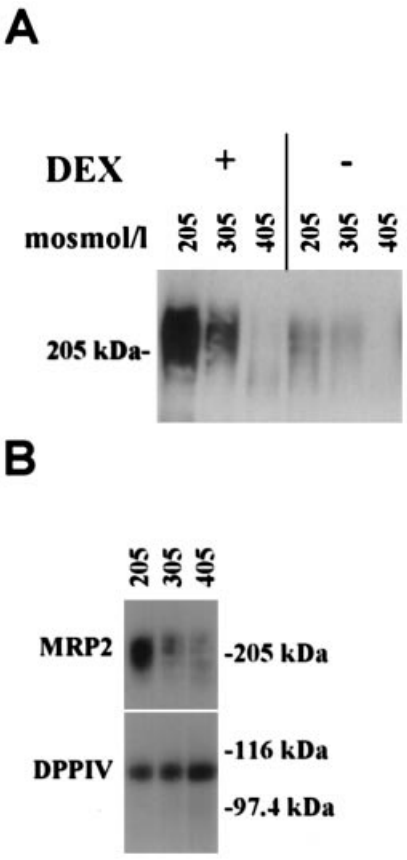

Figure 5 Hypo-osmolarity increases and hyperosmolarity decreases MRP2 protein

Cells were incubated for 36 h in normosmotic DMEM (305 mosmol/l, plus FBS/100 nmol/l dexamethasone/insulin/aprotinin/sodium selenite). Then they were exposed to aniso-osmolar DMEM with (+) or without (−) 100 nmol/l dexamethasone (DEX). Expression of MRP2 (as demonstrated by Western blotting) was increased strongly by hypo-osmotic (205 mosmol/l) and decreased by hyperosmotic (405 mosmol/l) medium (**A**; 36 h aniso-osmolarity). Expression of DPPIV was not affected by aniso-osmolar incubation (**B**; 30 h aniso-osmolarity).

Cells grown in the absence of dexamethasone showed a stripe-like immunostaining of the tight-junction-associated protein ZO-1, which delineates the (pseudo)canaliculus *in vivo* and in culture [26], but hardly any MRP2 immunoreactivity could be detected (Figure 1D). Cells cultured in the presence of dexamethasone (100 nmol/l) showed a strong staining of MRP2 at the pseudo-canalicular membranes (delineated by ZO-1, Figure 1E). Under the experimental conditions used (bile-salt free), secretion of endogenous MRP2 substrates, such as glutathione conjugates or GSSG, represents the main driving force for bile secretion. Therefore, broadening of the pseudocanaliculi is consistent with the appearance of active MRP2 in the canalicular membrane with increased secretion.

MRP2 expression was dependent on the concentration of dexamethasone (Figure 2). Differences in the amount of MRP2 protein due to dexamethasone were already found after 24 h and persisted for at least 3 days of culture. Expression of DPPIV (another canalicular protein, with a molecular mass of 110 kDa [17]) was culture-time-dependent but was not affected by dexamethasone, as shown by incubation of the same blots with the

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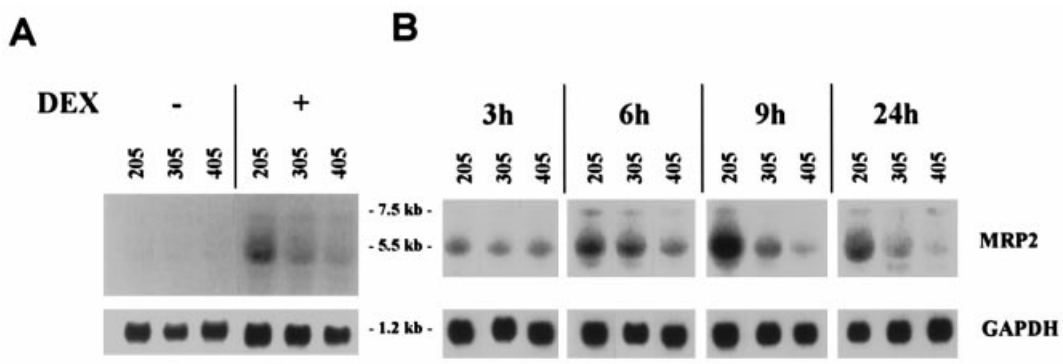

Figure 6 Regulation of MRP2 mRNA by osmolarity

After 12 h of normosmotic and another 12 h of aniso-osmotic incubation, MRP2 mRNA was only detectable when dexamethasone (DEX, 100 nmol/l) was present throughout the incubation. Media of 205 mosmol/l increased MRP2 mRNA, 405 mosmol/l decreased it (**A**). This effect was time-dependent and most pronounced after 9 h of aniso-osmolarity (**B**; in the presence of 100 nmol/l dexamethasone). Numbers along the top, osmolarity; + and −, presence and absence of dexamethasone, respectively.

monoclonal antibody MAB13.4 directed against DPPIV (Figure 2).

In order to exclude the possibility that differences in dexamethasone-dependent MRP2 expression were due to effects of dexamethasone on cell growth, hepatic parenchymal cells were cultured for 36 h in DMEM supplemented with 10% FBS, 100 nmol/l dexamethasone, 100 nmol/l insulin, 1 μg/ml aprotinin and 30 nmol/l sodium selenite until they reached confluence. At that time the cell layers formed pseudocanaliculi, as shown in Figure 1(B).

Upon lowering or withdrawal of dexamethasone, MRP2 protein expression declined within 24 h (Figure 3A). Concentrations of dexamethasone $\geqslant$ 1 nmol/l were sufficient to maintain the expression of MRP2. In addition to the downregulation of MRP2 protein induced by dexamethasone withdrawal (Figure 3A), there was a decrease of pseudocanalicular filling, as shown by phase-contrast microscopy (Figures 3B and 3C, arrows). Furthermore, secretion of the fluorescent MRP2 substrate 5-chloromethylfluorescein (see the Materials and methods section for details) into the pseudocanaliculi was strongly reduced when dexamethasone was withdrawn, suggesting a correlation of the MRP2 detected by Western blots and the activity of MRP2.

Likewise, the MRP2 mRNA levels were decreased dose-dependently and MRP2 mRNA was hardly detected in cells cultured for 48 h in the absence of dexamethasone (Figure 4). When MRP2 mRNA levels were standardized to GAPDH mRNA there was a 20-fold difference in cells grown with and without dexamethasone.

Osmo-dependent expression of MRP2

Hepatocytes were grown for 36 h in normosmotic DMEM (305 mosmol/l) supplemented with 10% FBS, 100 nmol/l dexamethasone, 100 nmol/l insulin, 1 μg/ml aprotinin and 30 nmol/l sodium selenite. Thereafter they were exposed to aniso-osmotic media (i.e. DMEM with appropriately reduced or increased NaCl concentrations) for up to 48 h Expression of MRP2 was increased under hypo-osmotic conditions (i.e. when cells were exposed to medium of 205 mosmol/l) and reduced when they were kept in hyperosmotic (405 mosmol/l) medium. This effect was already seen after 12 h of aniso-osmotic incubation (results not shown) and increased largely after 24 and 36 h (Figure 5A). The expression of DPPIV was not effected by aniso-osmolarity (Figure 5B), suggesting a specific action of aniso-osmolarity on the expression of MRP2. The effect of hypo-osmolarity on protein levels of MRP2 was diminished strongly when dexamethasone was withdrawn (Figure 5A).

The effect of anisotonicity on MRP2 expression also occurred at the mRNA level. Figure 6(A) shows MRP2 mRNA of hepatocytes that were grown with or without dexamethasone throughout the entire cultivation. The finding that hypo-osmolarity increases the amount of MRP2 mRNA suggests that the osmo-dependent MRP2 expression is regulated pre-translationally, at least in part. However, hypo-osmolarity did not induce MRP2 mRNA in the absence of dexamethasone (Figure 6A). This proposes that the osmolarity modifies MRP2 expression in a dexamethasone-dependent way.

Induction of MRP2 mRNA levels by hypo-osmolarity in the presence of 100 nmol/l dexamethasone (12 h norm-osmotic pre-incubation) was observed after 6 h of aniso-osmolarity and was most prominent after 9 h (Figure 6B). At that time point, hypo-osmotic exposure (205 mosmol/l) increased the relative amount of MRP2 mRNA (standardized with GAPDH) by 101 ± 23%, whereas hyperosmotic exposure (405 mosmol/l) decreased it by 46 ± 5%, compared with normo-osmotic incubation (mean $\pm$S.E.M., $n = 3$ preparations).

DISCUSSION

This study demonstrates that dexamethasone regulates the expression of MRP2 at the mRNA and protein levels. This was observed during early culture as well as in confluent cells (i.e. after 36 h of culture), suggesting that dexamethasone not only stimulates MRP2 expression in proliferating cells but also maintains a high level of MRP2 expression in confluent cells after formation of new pseudocanaliculi. MRP2 is localized largely in the pseudocanalicular membrane, as demonstrated by immuncytochemical staining. Functional activity of MRP2 is suggested by the higher filling state of the pseudocanaliculi in dexamethasone-treated hepatocytes and by a more rapid and much stronger filling of the pseudocanaliculi with conjugated 5-chloromethylfluorescein, which is a fluorescent MRP2 substrate [25].

The expected molecular mass of MRP2 is $\approx$ 190 kDa [16]. In cultured hepatocytes, a large amount of MRP2 detected by Western-blot analysis had a higher molecular mass than the

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MRP2 from a rat canalicular membrane preparation (compare Figures 1C, 2, 3A and 5). Whether all of these bands reflect functionally active MRP is unclear. However, the relationship between protein levels of MRP2 and secretory activity suggests that the MRP2 with the higher molecular mass may also represent an active form of MRP2.

Expression of DPPIV showed a culture-time dependency but no regulation by dexamethasone. This demonstrates a specific effect of dexamethasone on the expression of MRP2.

Regulation of MRP2 by dexamethasone is in line with the finding that 11 glucocorticoid-responding elements in the promotor region of the *mrp2* gene have been reported [12]. The action of dexamethasone on MRP2 expression could participate in the inhibition of the endotoxin-induced decrease of MRP2 function and expression *in vivo* [14,15]. Thus not only modulation of the cytokine release by non-parenchymal cells but also a counteraction of the level of MRP2 expression might explain the effect of dexamethasone in endotoxinaemia.

The concentrations of dexamethasone used in this study were close to the physiological concentrations of glucocorticoids in humans, because a concentration of 0.5–2.5 nmol/l dexamethasone is equivalent to a concentration of 15–75 nmol/l cortisol with regard to glucocorticoid potency. Higher cortisol concentrations (10-fold) are reached when pharmacological doses of hydrocortisone are administered [27]. As shown in Figure 4, 1 nmol/l dexamethasone is enough to maintain expression of MRP2 mRNA in confluent cells.

Induction of MRP2 by glucocorticoids might be physiologically relevant. One example is the conjugated hyperbilirubinaemia in neonates with pan-hypopituitarism [comprising corticotropin (adrenocorticotrophic hormone) and cortisol deficiency], which rapidly resolves following cortisol administration [28–30]. The primary adrenal insufficiency due to a defect of 11-hydroxylase was reported to produce cholestasis [30].

As shown recently, changes of the ambient osmolarity regulate MRP2 activity on a short-term scale by carrier retrieval and reinsertion from and into the canalicular membrane [9,14]. In addition to this short-term regulation, the present study suggests that osmolarity also exerts long-term effects at the level of MRP2 expression. However, hypo-osmolarity in the absence of dexamethasone was not able to induce MRP2 mRNA (Figure 6), suggesting a permissive role of dexamethasone for the osmo-dependent regulation of MRP2 expression. Although several osmosignalling pathways have been characterized in liver [19,31,32], further studies are required to identify steps towards MRP2 expression.

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