EFFECT OF DEXAMETHASONE TREATMENT ON THE EXPRESSION AND FUNCTION OF TRANSPORT PROTEINS IN SANDWICH-CULTURED RAT HEPATOCYTES

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ABSTRACT:

Dexamethasone (DEX) is a well established inducer of CYP3A. These studies examined the influence of DEX treatment on transport protein expression and function in sandwich-cultured (SC) rat hepatocytes. Freshly isolated hepatocytes were cultured between two layers of gelled collagen and maintained in Dulbecco’s modified Eagle’s medium supplemented with DEX (0.1 μM, 0–48 h and 0.1–100 μM, 48–96 h). The expression of sinusoidal (organic anion transporting polypeptide 1a1 (Oatp1a1), Oatp1a4, multidrug resistance-associated protein 3 (Mrp3), and Na+-dependent taurocholate cotransporting polypeptide (Ntcp)) and canalicular [bile salt export pump (Bsep), multidrug resistance protein 1a/b (Mdr1a/b), and Mrp2] transport proteins was determined by Western blot analysis. The accumulation and biliary excretion index (BEI; percentage of accumulated substrate in canalicular networks) of the probe substrates taurocholate (TC; 1 μM, 10 min), rhodamine 123 (Rh123; 10 μM, 30 min), and carboxy-2’-7’-dichlorofluorescein (CDF; 10 μM, 10 min) were employed as measures of canalicular transport protein function in SC rat hepatocytes. DEX treatment increased CYP3A1/2, Oatp1a4, and Mrp2 expression, decreased the expression of Ntcp, and did not seem to alter the expression of Oatp1a1, Mdr3, Mdr1a/b, or Bsep. The BEI of CDF, an Mrp2 substrate, increased from 18 to 37% after DEX treatment (100 μM). The accumulation of TC, an Ntcp substrate, was reduced (<50% of control), whereas the BEI of TC, also a Bsep substrate, was unchanged. Treatment of SC rat hepatocytes with DEX resulted in alterations in the expression of CYP3A1/2 and some hepatic transport proteins. Modest alterations in hepatic transport protein function were consistent with changes in protein expression.

Hepatic uptake of xenobiotics via active or passive translocation processes is prerequisite to hepatic metabolism. Recently, the hepatobiliary disposition of a series of metabolically stable substrates in sandwich-cultured (SC) rat hepatocytes (i.e., hepatocytes maintained between two layers of gelled collagen) was shown to predict in vivo biliary excretion (Liu et al., 1999). Induction of phase I metabolic enzymes in SC rat hepatocytes to levels representative of in vivo expression and function may extend the utility of this in vitro model to facilitate the prediction of hepatobiliary disposition of metabolically labile substrates. However, the effects of dexamethasone (DEX), a known inducer of CYP3A (Schuetz and Guzelian, 1984; Huss and Kasper, 2000) and a common medium supplement, on hepatic transport protein expression and function have not been investigated in SC rat hepatocytes.

Many investigators have evaluated culture conditions to optimize the expression and function of cytochrome P450 and uridine diphosphate glucuronosyltransferase activity (Kern et al., 1997; LeCluyse et al., 2000) and transport proteins (Annaert et al., 2001; Chandra et al., 2001) in primary hepatocytes. The addition of DEX to incubation media improves attachment, survival, and morphology, including the enhancement of bile canalicular networks (Laishes and Williams, 1976; Yamada et al., 1980). DEX has been shown to alter mRNA and protein expression levels of hepatic transport proteins in rat hepatocytes maintained under conventional culture configurations (Kern et al., 1997; Courtois et al., 1999; Warskulat et al., 1999). In addition, the effects of DEX on mRNA of hepatic transport proteins in SC rat hepatocytes also have been evaluated (Luttering et al., 2002). The in vivo hormonal and xenobiologic regulation of the bile salt export pump (Bsep), the multidrug resistance-associated protein 2 (Mrp2), and the multidrug resistance protein 1a/b (Mdr1a/b) recently was reviewed by Fardel et al. (2001), and in vitro investigations of the regulation of these transport proteins also have been reviewed (Courtois et al., 1999; Cast et al., 2002). Mrp2 and Mdr1a/b function in SC rat hepatocytes has been evaluated with the probe substrates carboxy-

ABBREVIATIONS: SC, sandwich-cultured; DEX, dexamethasone; Bsep, bile salt export pump; Mrp, multidrug resistance-associated protein; Rh123, rhodamine 123; Mdr, multidrug resistance protein; GR, glucocorticoid receptor; PXR, pregnane X receptor; Ntcp, Na+-dependent taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; FXR, farnesoid X receptor; DMEM, Dulbecco’s modified Eagle’s medium; ITS, insulin-transferrin-sodium selenite; FBS, fetal bovine serum; TC, taurocholate; CDF-DA, carboxydichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; BEI, biliary excretion index; HBSS, Hanks’ balanced salt solution; BCA, bicinchoninic acid; BC, bile canaliculi.
flourescein and rhodamine 123 (Rh123), respectively (Annaert et al., 2001; Zamek-Gliszczynski et al., 2003).

CYP3A is responsible for the metabolism of approximately 50% of all new drugs. Originally, DEX was thought to modulate CYP3A expression in rats via the glucocorticoid receptor (GR). However, it is now recognized that DEX modulates CYP3A expression in vitro in rat hepatocytes by the preganne X receptor (PXR), resulting in increased expression of CYP3A12 (also termed CYP3A23) mRNA and protein (Burger et al., 1992; Huss and Kasper, 2000). Further studies using a novel reverse transcription-polymerase chain reaction method demonstrated that PXR mediated the induction of the CYP3A1 isoyme but not CYP3A2, CYP3A9, or CYP3A18 (Hoen et al., 2000).

Recently, DEX has been shown to increase mRNA levels of Mrp2 in SC rat hepatocytes (Luttringer et al., 2002), corroborating an earlier report of increased Mrp2 protein expression by DEX both in primary rat hepatocytes and in vivo (Courtour et al., 1999). In SC rat hepatocytes, neither Mdr1a/b nor mRNA levels were influenced by DEX treatment (Annaert et al., 2001; Luttringer et al., 2002). Additionally, Luttringer et al. (2002) reported decreased mRNA for the Na+-dependent taurocholate cotransporting polypeptide (Ntcp) and increased mRNA for the organic anion tranporting polypeptide 2 (Oatp1a4), but no change in mRNA for Oatp1 (Oatp1a1) or Mrp3 following DEX treatment in SC rat hepatocytes. Guo et al. (2002a,b) and Staudinger et al. (2001) have reported that protein and mRNA expression of Oatp1a4 were modulated by PXR ligands. The modulation of Bsep by DEX in rats and in primary hepatocytes was reported by Fardel et al. (2001), who suggested that biliary transporters might be regulated via the GR. DEX also increased cholesterol 7α-hydroxylase (CYP7A1) (Princen et al., 1989) and sterol 27-hydroxylase (CYP27A1) (Stravitz et al., 1996), enzymes responsible for bile acid synthesis, in primary rat hepatocytes. Bile acids have been shown to be ligands of the farnesoid X receptor (FXR). FXR down-regulation of Ntcp (Denson et al., 2001) and up-regulation of Bsep (Ananthanarayanan et al., 2001) and Mrp2 (Kast et al., 2002) have been reported.

The induction of CYP3A1/2 is prerequisite for using the SC rat hepatocyte model as an in vitro surrogate of in vivo hepatobiliary disposition. Functional changes related to observed alterations in mRNA expression levels of transport proteins in DEX-treated SC rat hepatocytes have not been reported. The effects of metabolic enzyme modulation on transport protein expression and function must be taken into consideration, because transport proteins govern intracellular drug concentration and thus the availability of substrate for metabolism. Results of the present studies document that treatment of SC rat hepatocytes with DEX results in an expected increase in CYP3A1/2 expression and modest alterations in the expression and function of select hepatic transport proteins.

Materials and Methods

**Chemicals.** Collagenase (type 1, class 1) was obtained from Worthington Biochemicals (Freehold, NJ). Dulbecco's modified Eagle's medium (DMEM) and insulin were purchased from Invitrogen (Carlsbad, CA). ITS* culture supplement and rat tail collagen (type I) were purchased from BD Biosciences Discovery Labware (Bedford, MA). DMEM (10×), penicillin-streptomycin solution, fetal bovine serum (FBS), taurocholic acid (TC), DEX, Triton X-100, and soybean trypsin inhibitor were purchased from Sigma-Aldrich (St. Louis, MO). Carboxy-dichlorofluorescein diacetate (CFDA), CDF, and Rh123 were purchased from Molecular Probes (Eugene, OR). All other chemicals and reagents were of analytical grade and readily available from commercial sources.

**Animals.** Male Wistar rats (270–325 g) obtained from Charles River Laboratories, Inc. (Raleigh, NC) were used for hepatocyte isolation from whole liver. Animals had free access to water and food prior to surgery. All animal procedures complied with the guidelines of the Institutional Animal Care and Use Committee (University of North Carolina at Chapel Hill, Chapel Hill, NC).

**Isolation and in Vitro Culture of Primary Rat Hepatocytes.** Hepatocytes were isolated from male Wistar rats using a collagenase perfusion as described previously (Liu et al., 1999). Following perfusion, the liver was removed from the rat and immersed in ice-cold medium (DMEM containing 5% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 4 mg/l insulin, and 1 μM DEX). Hepatocytes were filtered through a 70-μm mesh filter followed by centrifugation through a Percoll density gradient. Hepatocyte viability was >85% as determined by trypan blue exclusion. Hepatocytes were plated at a density of ~1.25 × 10^6 cells/cm^2 in 60-mm dishes previously coated with 0.2 ml of rat tail collagen type 1 solution (1.5 mg/ml, pH 7.4). Unattached cells were removed by replacing the cell plating medium with DMEM containing 5% FBS, 4 mg/l insulin, and 0.1 μM DEX 1 h to 3 h after plating hepatocytes. After 24 h, the medium was aspirated, and cells were overlaid with 200 μl of rat tail collagen type 1 solution (1.5 mg/ml, pH 7.4) to achieve a sandwich configuration. The medium (DMEM supplemented with 1% ITS*, antibiotics, and 0.1 μM DEX) was changed every 24 h for 96 h (day 4) as described below.

**DEX Treatment of SC Rat Hepatocytes.** DEX (1, 10, and 100 μM) or vehicle (DMSO) was added to the media after 48 h after plating. The final concentration of DMSO in the culture media was <0.1%. SC rat hepatocytes were treated with DEX or vehicle for 48 h beginning on day 2.

**Accumulation and Biliary Excretion Experiments (Day 4).** Methods to determine the accumulation and biliary excretion index (BEI) of substrates in SC rat hepatocytes have been described previously (Liu et al., 1999). Briefly, cells were rinsed twice with 3 ml of warm standard HBSS or Ca2+-free HBSS and incubated with 3 ml of the same buffer for 10 min at 37°C. The medium was removed, and SC rat hepatocytes were incubated with 3 ml of [3H]JTC (1 μM, 10 min), CDF-DA (10 μM, 10 min), or Rh123 (10 μM, 30 min) in standard HBSS. Following incubation, dishes were rinsed vigorously three times with 3 ml of ice-cold HBSS. Hepatocytes were lysed with 2 ml of 0.5% Triton X-100 by orbital shaking (20 min, room temperature). For the determination of Na+-dependent TC accumulation, HBSS was replaced with choline buffer in which sodium chloride was replaced with choline chloride on an equal molar basis. Accumulation was initiated by adding HBBS or choline buffer containing [3H]TC to each dish as described previously (Liu et al., 1998).

**Sample Analysis.** Cell lysates from TC accumulation experiments were analyzed by liquid scintillation spectroscopy in a Packard Tri-Carb scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Cell lysates from CDF-DA (measured as CDF) and Rh123 accumulation experiments were analyzed by fluorescence spectroscopy using a Bio-Tek FL600 plate reader (Bio-Tek Instruments, Winooski, VT) (λ_em 485 ± 20; λ_ex 530 ± 25). Data were normalized to the protein content of the hepatocytes in each dish as determined with a commercially available BCA protein assay kit (Pierce Chemical, Rockford, IL) based on instructions provided by the manufacturer. Bovine serum albumin served as a standard (supplied with the BCA kit). All accumulation data were corrected for nonspecific binding to gelled collagen-coated hepatocyte-free culture dishes.

**Western Blot Analysis of Canalicular Transport Proteins.** Hepatocytes were cultured as described above. On day 4, the medium was removed, and the cells were rinsed with cold HBBS. Subsequently, 0.8 ml of lysing buffer (1 mM EDTA, 1% SDS, pH 8) was added to each 60-mm dish, the cells were scraped using a cell lifter, and homogenization was achieved by repeated pipetting. Samples were stored at −80°C until analysis.

**Immunoblot analysis.** Cell homogenates were thawed rapidly, and protein concentrations were determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Samples (15 or 30 μg/well) were separated by SDS-polyacrylamide gel electrophoresis, and proteins were electrotransferred to polyvinyldene difluoride membranes. Blots were blocked overnight with Tris-buffered saline containing 5% nonfat dry milk and 0.3% Tween 20. Mrp2 expression was determined using an anti-Mrp2 primary monoclonal antibody M_III-6 (Alexis Biochemicals, Carlsbad CA). Bsep expression was determined with an anti-Bsep primary polyclonal antibody (Gerloff et al., 1998). The polyclonal Mdr1a/b antibody Ab-1 (Calbiochem, San Diego, CA) was used to determine Mdr1a/b expression. Primary polyclonal antibodies for Oatp1a1, Oatp1a4, and a monoclonal antibody for β-actin were obtained from Chemicon
International (Tomecula, CA). Primary polyclonal antibody for CYP3A1/2 was obtained from Xenotech (Lenexa, KS). Ntcp expression was determined with an anti-Ntcp primary polyclonal antibody (Stieger et al., 1994). The primary polyclonal antibody used to detect Mrp3 was kindly provided by Dr. Yuichi Sugiyama (The University of Tokyo, Tokyo, Japan) (Ogawa et al., 2000). Molecular mass standards (SeeBlue Plus2, Invitrogen) were loaded with samples on each gel. Immunoreactive protein bands were detected by chemiluminescence or using a Bio-Rad VersaDoc imaging system. Densitometry analysis was achieved using the Quantity One software package (Bio-Rad).

Data Analysis. The amount of each substrate in cell lysates was determined in triplicate from three individual livers. The BEI (percent) was determined by dividing the difference in substrate accumulation in cells + bile canaliculi (BC) (determined in SC rat hepatocytes preincubated in standard HBSS) and cells (determined in SC rat hepatocytes preincubated in Ca\(^{2+}\)-free HBSS) by the accumulation in cells + BC multiplied by 100. BEI was calculated as the mean of BEI values from the individual livers and is expressed as mean ± S.E.M. Statistical differences between mean accumulation values of substrate in cells + BC versus cells and between BEI values determined for control and DEX-treated day 4 SC rat hepatocytes were determined by Student’s t test with Bonferroni’s correction for multiple comparisons where noted.

Results

Expression of CYP3A1/2 in SC Rat Hepatocytes. The expression of CYP3A1/2 in SC rat hepatocytes maintained for 48 h in the presence of 1.0, 10, and 100 μM DEX was increased compared with control (0.1 μM DEX) (Fig. 1).

Effect of DEX on Canalicular Membrane Transport Protein Expression. The expression of Mrp2 increased in SC rat hepatocytes treated for 48 h with DEX (Fig. 2). The treatment of SC rat hepatocytes with 10 μM DEX resulted in a maximal expression of Mrp2. The expression of Bsep was similar to control in SC rat hepatocytes treated with all DEX concentrations (Fig. 2). In agreement with our earlier published work (Annaert et al., 2001), Mdr1a/b expression was not influenced by DEX treatment (data not shown).

Effect of DEX on Basolateral Membrane Transport Protein Expression. The expression of Mrp3 and Oatp1a1 was similar in day 4 SC rat hepatocytes incubated with 0.1 or 100 μM DEX for 48 h (Fig. 3). The expression of Oatp1a4 was higher, whereas Ntcp expression was lower, in day 4 SC rat hepatocytes maintained in 100 μM DEX for 48 h compared with control.

Accumulation of TC, CDF, and Rh123 in DEX-Treated SC Rat Hepatocytes. The results of accumulation experiments in control and DEX-treated SC rat hepatocytes are shown in Fig. 4. TC accumulation in cells + BC of SC rat hepatocytes was significantly lower (<50% of control) following DEX treatment. However, the BEI of TC was not significantly different between control (66 ± 9%) and DEX-treated SC rat hepatocytes (63 ± 3%). The accumulation of CDF (administered as CDF-DA) in cells + BC as well as in cells was significantly lower in DEX-treated day 4 SC rat hepatocytes compared with control (Fig. 4). The BEI of CDF was significantly greater (p < 0.05) in DEX-treated (37 ± 5%) versus control (18 ± 2%) day 4 SC rat hepatocytes. The accumulation of Rh123 in cells + BC as well as in cells was similar in control and DEX-treated SC rat hepatocytes (Fig. 4). The BEI values of Rh123 in control and DEX-treated SC rat hepatocytes were similar (21 ± 5 and 17 ± 4%, respectively).

Discussion

There is a general consensus that low concentrations of DEX in SC rat hepatocytes are useful in the maintenance of cellular functions (LeCluyse et al., 1996). The effects of increased concentrations of DEX on mRNA of both metabolic enzymes (Kern et al., 1997) and transport proteins (Luttringer et al., 2002) in primary rat hepatocytes have been reported. Furthermore, the regulation of hepatic transport proteins by mechanisms other than via GR has been explored both in vivo and in vitro (Fardel et al., 2001; Gerk and Vore, 2002). To date, alterations in the expression of hepatic transport proteins following DEX treatment in SC rat hepatocytes have not been reported, nor have such alterations been related to functional changes.

CYP3A1 mRNA levels were reported to decrease to 10 to 20% of initial levels in freshly isolated hepatocytes during the first 24 h in culture (Hoen et al., 2000). The expression of CYP3A1/2 in day 4 SC rat hepatocytes (Fig. 1; DEX, 0.1 μM) was consistent with low metabolic activity during investigations of hepatic transport such as described by Liu et al. (1999); the level of immunoreactive CYP3A generally predicts activity (LeCluyse et al., 1996). DEX treatment (10 μM) has been shown to cause an 8-fold increase in the level of CYP3A1/2 mRNA and a 10-fold increase in protein expression in primary rat hepatocytes maintained in a conventional configuration (Schuetz and Guzelian, 1984) that is equivalent to expression at the time of hepatocyte isolation (Hoen et al., 2000) and yields a rate of CYP3A1/2-mediated testosterone 6β-hydroxylation comparable to that observed in vivo (LeCluyse et al., 1996). A similar increase in CYP3A1/2 expression in SC rat hepatocytes was observed in the present study following treatment with 10 μM DEX (Fig. 1). The regulation and modulation of CYP3A1/2 by DEX has been attributed to transcriptional activation via PXR (Burger et al., 1992; Huss and Kasper, 2000).
Historically, DEX treatment of primary hepatocytes has been shown to reduce, or have no effect on, Mdr1a/b expression (Fardel et al., 1992; Chieli et al., 1994). We previously reported that treatment of SC rat hepatocytes with low concentrations of DEX did not influence the expression of Mdr1a/b (Annaert et al., 2001). Luttringer et al. (2002) subsequently showed that mRNA levels of Mdr1a and Mdr1b were unchanged by DEX treatment in SC rat hepatocytes. The expression of Mdr1a/b following DEX treatment (100 μM, 48 h) was not different from control (data not shown). As measured by the BEI of the P-glycoprotein substrate Rh123, the function of Mdr1a/b in day 4 SC rat hepatocytes treated with a high concentration of DEX was not different from control (Fig. 4), consistent with the lack of effect on Mdr1a/b expression.

Mrp2 expression in cultured rat hepatocytes increased following DEX treatment (Fig. 2). These results were consistent with the reported increase of 2- to 3-fold in Mrp2 mRNA expression levels in primary rat and mouse hepatocytes treated with DEX (1 μM) (Kast et al., 2002; Luttringer et al., 2002). Treatment with high concentrations of DEX (10 and 100 μM) resulted in increased Mrp2 protein expression in SC rat hepatocytes. The administration of DEX in vivo was also reported to cause increased mRNA and protein expression of Mrp2 in rat liver (Courtot et al., 1999). The levels of Mrp2 and CYP3A1/2 protein expression were increased to a similar extent in DEX-treated SC rat hepatocytes as compared with the reported PXR modulation of both proteins. DEX-treated primary rat hepatocytes exhibited equivalent induction of Mrp2 mRNA compared with the established PXR ligand pregnenolone-16α-carbonitrile, suggesting that DEX may modulate Mrp2 via the PXR nuclear receptor (Kast et al., 2002). Additionally, ligands for the FXR and constitutive androstane receptor can modulate the expression of Mrp2 in primary rat hepatocytes (as reviewed by Kast et al., 2002). In the present study, Mrp2 expression increased to a greater extent in DEX-treated SC rat hepatocytes compared with Mrp2 function. Mrp2 function was measured by the BEI of the Mrp2 substrate CDF (Zamek-Gliszczynski et al., 2003). Mrp2 localizes to subapical compartments in hepatocytes (Kipp and Arias, 2002). Further studies are required to determine more specifically the extent of changes in Mrp2 expression on the canalicular domain following DEX treatment in SC rat hepatocytes; post-translational modifications also may impact overall Mrp2 expression levels.

As discussed by Stravitz et al. (1996), Wang et al. (1999), and Ananthanarayanan et al. (2001), the transcriptional regulation of bile acid transport proteins is highly complex. Warskulat et al. (1999) demonstrated that the maintenance of Bsep expression was dependent on the presence of DEX (0.1 μM) in the medium. Luttringer et al. (2002) reported that mRNA levels of Bsep were unchanged in SC rat hepatocytes treated with DEX (1 μM) compared with control (0.1 μM); however, those studies were conducted at concentrations of DEX that were not capable of maximal induction of CYP3A1/2.
Results from the present investigation suggest that high concentrations of DEX, resulting in increased CYP3A1/2 expression, do not alter the expression of Bsep in SC rat hepatocytes. The function of Bsep, as measured by the BEI of TC, was not influenced by DEX treatment in SC rat hepatocytes.

Ntcp expression (Fig. 3) decreased in SC rat hepatocytes treated with DEX concentrations of 100 μM for 48 h. Previous data demonstrated that the expression of Ntcp was decreased in day 4 SC rat hepatocytes compared with day 0 (Liu et al., 1998; Rippin et al., 2001). Decreased expression of Ntcp protein in SC rat hepatocytes was similar to expression patterns observed in an in vivo model of cholestasis in the rat (Geier et al., 2003). The appearance of multiple Ntcp bands in the molecular weight range around 51 kilodaltons (Fig. 3) is similar to previous observations using this antibody (Liu et al., 1998; Rippin et al., 2001). Further investigation is required to determine whether multiple bands reflect the phosphorylation state, and thus functional activity, of Ntcp (Mukhopadhyay et al., 1998). In the current experiments, DEX treatment resulted in reduced accumulation of TC on day 4 compared with control (Figs. 4 and 5). Na+-independent accumulation of TC was not lower in DEX-treated SC rat hepatocytes compared with control (Fig. 5), suggesting that decreased TC accumulation correlated with decreased Ntcp protein expression.

Our results were consistent with the recently reported decrease in Ntcp mRNA levels in SC rat hepatocytes treated with DEX (1 μM) (Lutrigger et al., 2002). These results have been explained at the nuclear receptor level in a series of experiments where it was shown that the negative feedback regulation of Ntcp is mediated by shp, an orphan nuclear receptor that is up-regulated by bile-acid induction of the rat bile bile transporter, ntcp. Gastroenterology 121:140–147.


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