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Farnesoid X receptor suppresses constitutive androstane receptor activity at the multidrug resistance protein-4 promoter

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ABSTRACT

Multidrug resistance protein-4 (MRP4) is a member of the multidrug resistance associated gene family that is expressed on the basolateral membrane of hepatocytes and undergoes adaptive up-regulation in response to cholestatic injury or bile acid feeding. In this study we demonstrate that farnesoid X receptor (FXR) regulates MRP4 in vivo and in vitro. In vivo deletion of FXR induces MRP4 gene expression. In vitro treatment of HepG2 cells with FXR ligands, chenodeoxycholic acid (CDCA), cholic acid (CA) and the synthetic ligand GW-4064 suppresses basal mRNA level of the MRP4 gene as well as the co-treatment with CDCA and 6-(4-Chlorophenyl) imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime (CITCO), an activator of constitutive androstane receptor (CAR). We found in the human MRP4 promoter a CAR responsive element (CARE) embedded within an FXR responsive element (FXRE). We cloned this region and found that FXR suppresses CAR activity in luciferase assay. Finally, we demonstrated that FXR competes with CAR for binding to this overlapping binding site. Our results support the view that FXR activation in obstructive cholestasis might worsen liver injury by hijacking a protective mechanism regulated by CAR and provides a new molecular explanation to the pathophysiology of cholestasis.

1. Introduction

The multidrug resistance protein 4 (MRP4) is a member of the C-subfamily of ATP-binding cassette (ABC) transporters [1,2]. It is expressed in a variety of epithelia, including the basolateral and apical plasma membranes of the liver and kidney cells. A unique characteristic of MRP4/ABCC4 is its remarkable ability to transport a range of endogenous molecules that have a key role in cellular communication and signaling, including cyclic nucleotides, ADP, eicosanoids, urate and conjugated steroid hormones [3–6]. Other potentially relevant physiological substrates are folate, bile acids and glutathione, which is co-transported with bile acids [7,8].

In the liver, MRP4 protects from accumulation of toxic bile acids during cholestasis by facilitating their efflux into the blood for ultimate renal excretion. Consistent with this interpretation, Mrp4-knockout mice are sensitized to cholestatic-induced liver injury [9,10]. The basolateral localization of MRP4 in hepatocytes and the apical localization in renal proximal tubule cells are compatible with an alternative elimination pathway under conditions of impaired canalicular bile salt excretion. Accordingly, increased urinary levels of bile salts were detected after bile duct ligation, accompanied by elevated renal MRP4 expression in mice [9,10].

The expression of hepatic MRP4 gene is positively regulated by a network of nuclear receptors that includes the peroxisome proliferator-activated receptor-α (PPARα) [11], the Aryl hydrocarbon receptor (AhR) [12], the NF-E2 related factor (Nrf2) [12] and the constitutive androstane receptor (CAR) [13]. While it has been demonstrated that PPARα, AhR and NRf2 specifically bind to the MRP4 promoter and activate its transcription [11,12,19], the mechanism by which CAR activates MRP4 is poorly defined [13,14]. Conversely, there is a body of evidence implicating the bile acid sensor farnesoid X receptor (FXR) in the negative regulation of MRP4 [15]. Support to this view comes from mice harboring a disrupted FXR gene: these mice show an increased expression of MRP4 mRNA in the liver [16,17] and are protected against liver injury in a model of cholestasis induced by bile duct ligation (BDL) [18]. Despite data obtained in FXR deficient mice supporting the notion that an opposite regulation of MRP4 by FXR and CAR does exist, the molecular determinants of this interaction are unknown.

In the present study we have identified a novel mechanism of MRP4 regulation based on the competition of FXR and CAR for an overlapping binding site located on the MRP4 promoter. Our results demonstrate that despite CAR and FXR share common target genes, their functional activity on MRP4 is antagonistic and that by hijacking a CAR regulated mechanism, FXR activation impairs hepatocytes'

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basolateral detoxification contributing to bile acid-mediated injury in cholestasis.

2. Materials and methods

2.1. Cell culture

HepG2 cells were maintained at 37 °C in E-MEM containing 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. To value whether FXR activation by bile acids regulates MRP4 gene expression serum starved HepG2 cells were stimulated for 18 h with 50 μM bile acids chenodeoxycholic acid (CDCA) and cholic acid (CA) or with 1 μM synthetic ligand GW-4064. To investigate whether FXR activation suppresses CAR induction of MRP4, serum starved HepG2 cells were treated 18 h with the CAR ligand CITCO (10 μM) alone or in combination with the FXR ligand CDCA (50 μM).

2.2. Isolation and culture of primary hepatocytes from FXR wild type and FXR deficient mice

Primary hepatocytes were isolated from C57BL6 wild type and FXR knockout animals (obtained from Sinal et al. [16]) anesthetized with pentobarbital sodium solution (50 mg/kg ip). Briefly, the inferior vena cava was cannulated and the liver was first perfused in situ with an oxygenated Hanks' buffer salt solution (HBSS) containing 100 U/ml penicillin/streptomycin and 0.04% collagenase-D (Roche) pH 7.4 (8 ml/min, 37 °C for 10 min), followed by perfusion with oxygenated HBSS containing 1 mM Ca^{2+} and Mg^{2+}, penicillin/streptomycin (100 U/ml), and 0.04% collagenase type II (Sigma), pH 7.4 for 10 min. The liver was removed and then gently minced in HBSS containing 1 mM Ca^{2+} and Mg^{2+}, penicillin/streptomycin (100 U/ml), and 1×10^{-7} M insulin (Sigma), pH 7.4. The liver cell suspension was then filtered with Falcon cell strainers (40, 70, and 100 μm; Becton Dickinson) and centrifuged at 50*g* for 2 min. From the isolation of one mouse liver, a typical yield was about $50–60\times10^6$ hepatocytes. Cell viability, as determined by trypan blue exclusion, was generally >90%, and cell purity was >95% hepatocytes. Cells were plated on 6-well plates (6×10^5 cells/well) or 24-well plates (8×10^4) (Biocoat collagen I cellware plates; Becton Dickinson) in Williams's Medium E (Invitrogen) containing 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 1×10^{-7} M insulin and cultured at 37 °C with 5% CO_2. After an initial 4-h attachment period, cultures were washed with phosphate-buffered saline (PBS) and then primed with: (i) the murine CAR agonist 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene (TCPBOP) (10 μM); (ii) the FXR ligand CDCA (50 μM); and (iii) the combination of TCPBOP (10 μM) and CDCA (50 μM).

2.3. Real-time PCR (RT-PCR) analysis

Total RNA was isolated from HepG2 and primary hepatocytes using the TRIzol reagent according to the manufacturer's specifications (Invitrogen). One microgram RNA was purified of the genomic DNA by DNase I treatment (Invitrogen) and random reverse-transcribed with Superscript II (Invitrogen) in 20 μl reaction volume. Fifty nanogram template was added to the PCR mixture (final volume 25 μl) containing the following reagents: 0.2 μM of each primer and 12.5 μl of 2X SYBR Green qPCR master mix (Invitrogen). All reactions were performed in triplicate and the thermal cycling conditions were: 2 min at 95 °C, followed by 40 cycles of 95 °C for 20 s, 55 °C for 20 s and 72 °C for 30 s in iCycler iQ instrument (Biorad). The relative mRNA expression was calculated and expressed as $2^{-(\Delta\Delta Ct)}$. Primers used for qRT-PCR were human GAPDH: gaaggtgaaggtcggagt and catgggtggaatcatattggaa; human MRP4: ggcgaattgttagctgtggt and cagggctgctgagacacata; mouse GAPDH: ctgagtatgtcgtggagtctac and gttggtggtgcaggatgcattg; mouse MRP4: gcaaagcccatgtaccatct and accacggctaacaactcacc.

2.4. Plasmids construction, mutagenesis and luciferase assays

The human 5′flanking region of MRP4 gene, consisting of −892 to −651 base pairs respect to the transcriptional start site ATG, was amplified by PCR from HepG2 genomic DNA (primers: ttcctttcccaatctaagggg and aagcttggaggctcttcaacctg), sub-cloned into pCR2.1 with TOPO TA CLONING Kit (Invitrogen) and cloned Hind-III into pGL3 promoter vector (Promega). The mutagenesis of ER8 and ER6 responsive elements was performed with the Quik change site directed mutagenesis kit (Stratagene). The primers used for the ER8 mutagenesis were the following: ggataatctgtggctaaacaatgctgtcgtc caaggtcaa and ttgaccttggacgacagcattgtttagccacagattatcc; the primers used for the ER6 mutagenesis were: caaggataatctgtggctaaactttgc taacgtccaaggtcaaat and atttgaccttggacgttagcaaagtttagccacagattatccttg. The transfection experiments were performed using Fugene HD (Roche). Luminescence was measured with the Glomax 20/20 luminometer (Promega).

2.5. Chromatin immunoprecipitation (ChIP)

Serum starved HepG2 cells (10×10^6) were left untreated or stimulated with CDCA (50 μM) or CITCO (10 μM) for 1, 3 and 6 h or the combination of the two (same concentrations) for 1 h. After treatments cells were cross-linked with 1% formaldehyde 10′ at room temperature and then the reaction terminated by the addition of glycine to a final concentration of 125 mM. Cells were washed in ice-cold PBS and lysed with 500 μl ChIP lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris–HCl, pH 8) containing 10 μM PMSF and protease inhibitor cocktail (Sigma), sonicated and centrifuged at 13000 rpm 10′ at 4 °C. Fifty microliters of each supernatant (Input DNA) were reverse-cross-linked by the addition of 150 μl Elution buffer (1% SDS and 0.1 M NaHCO3) and 8 μl NaCl 5M and by heating the mixture to 65 °C for 4 h. DNA was recovered from input by proteinase K treatment at 65 °C for 1 h followed by phenol/chloroform (1:1) extraction, ethanol precipitation and dissolved into 50 μl of molecular biology grade water. Thus, input DNA was spectrophotometrically quantified and 40 μg chromatin was diluted with ChIP dilution buffer (0.01% SDS, 1% Triton-X-100,1.2 mM EDTA pH 8.0, 16.7 mM Tris–HCl pH 8.0, 167 mM NaCl) containing protease inhibitors and 20 μl of ChIP lysis buffer equilibrated Protein A Sepharose (Amersham Bioscience)/Salmon Sperm DNA/1% BSA. After mixing at 4 °C for 1 h, the mixtures were centrifuged at 1000 rpm for 1 min to obtain pre-cleared supernatants. Pre-cleared supernatants were immunoprecipitated overnight at 4 °C with 4 μg specific antibodies: anti-FXR (sc-13063—Santa Cruz, CA), cells untreated and stimulated with CDCA, anti-CAR (sc-13065—Santa Cruz, CA), cells untreated, stimulated with CITCO or with the combination CITCO + CDCA, or anti IgG SA1-36098 (Pierce), all experimental conditions. Immunoprecipitates were washed sequentially with low-salt wash buffer (0.1% SDS, 1% Triton-X-100, 2 mM EDTA pH 8.0, 20 mM Tris–HCl pH 8.0, 150 mM NaCl) and then with high-salt wash buffer (0.1% SDS, 1% Triton-X-100, 2 mM EDTA pH 8.0, 20 mM Tris–HCl pH 8.0, 500 mM NaCl). DNA was eluted by addition of 250 μl Elution buffer and the cross-linking reactions were reversed by heating the mixture to 65 °C overnight. The DNA was recovered from immunoprecipitated material by proteinase K treatment at 65 °C for 1 h followed by phenol/chloroform (1:1) extraction, ethanol precipitation and dissolved into 50 μl of molecular biology grade water. Five microliters of chromatin was used for quantitative real-time PCR. Raw data analysis was performed as follows: ΔCt was calculated versus the input DNA concentration; ΔΔCt was versus unstimulated (or untrasfected) cells immunoprecipitated with the anti-IgG antibody (experimental condition set as