Biochemical Pharmacology 84 (2012) 1651–1659

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

ABCC2 is involved in the hepatocyte perinuclear barrier for small organic compounds

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ARTICLE INFO

Article history:
Received 15 August 2012
Accepted 18 September 2012
Available online 3 October 2012

Keywords:
ABC proteins
Bilirubin
Mitoxantrone
MRP2
Liver toxicity

ABSTRACT

Small organic molecules are believed to freely diffuse across nuclear pores but this may not be so if this route is blocked during protein and nucleic acid transfer. Here we have investigated the existence of transport mechanisms across the nuclear envelope (NE) of hepatocytes. Using nuclei isolated from rat liver cells, evidence for the existence of ATP-dependent transporters of organic compounds was found. In rat hepatocyte NE, with negligible contamination by other membranes, the presence of mature and glycosylated ABCC2, but not other ABC export pumps, was detected. ABCC2 was localized in the same membranes as the conjugating enzyme UGT1A1. Human *ABCC2* ORF was tagged with V5 and transfected to human hepatoma cells. ABCC2-V5 protein was detected at perinuclear ER vesicles and at the NE. Both compartments expressing ABCC2-V5 were able to exclude calcein. ABCC2 abundance at the NE of rat hepatocytes was modified by treatments able to increase or reduce the expression of canalicular ABCC2. The sensitivity to mitoxantrone was higher for hepatocytes obtained from TR- rats whose NE lacked ABCC2. Incubation with mitoxantrone after depletion of ATP resulted in a marked accumulation of mitoxantrone in the nucleus of wild-type, but not TR-, hepatocytes. In sum, ABCC2 is present at the NE and perinuclear ER where, in combination with the activity of conjugating enzymes, this pump may be involved in the perinuclear barrier for small organic molecules, playing a role in protecting DNA from genotoxic compounds and in the control of intranuclear concentrations of ligands for nuclear receptors.

1. Introduction

Many liver functions are regulated by modification in gene expression profiles to adjust the phenotype of hepatocytes and liver cells to continuously changing conditions. This is accounted for by a complex network of nuclear receptors able to respond to changes in the chemical composition of extracellular environment and the intracellular metabolic profile. Accordingly, a large variety of small organic molecules must reach the nucleus of hepatocytes to interact with their nuclear receptors. This is for instance the case of bile acids able to activate the farnesoid X receptor (FXR), which specifically activates the transcription of genes involved in bile acid homeostasis, but which has been recently suggested to be also involved in very different functions, such as the activation of mechanisms of chemoresistance [1]. Translocation to the nucleus after binding of bile acids in the cytosol to carrier proteins, such as the glucocorticoid receptors [2–4] and probably histone deacetylases [5] has been suggested as a mechanism that permits the entrance of bile acids to the nucleus. However, in previous studies, we have demonstrated that the access of small organic molecules ($\leq$1 kDa) to the nucleus is not strictly dependent on cytoplasmic proteins and could be partly accounted for by simple diffusion, probably through the nuclear pore complexes (NPCs) [6]. These are structures formed by 8 lateral channels and 1 central pore [7,8]. For molecules smaller than 10 kDa transit across the NPC probably occurs across the lateral channels, whereas the central pore probably permits the passage of molecules of up to 40 kDa [6,9]. Nevertheless, previous findings also revealed that there is a certain molecular selectivity in the ability of isolated nuclei to retain organic compounds due to mechanisms that are not merely dependent upon the molecular weight or the charge of the compound [6]. Thus, although small organic molecules are believed to freely diffuse across nuclear pores this may not be so if this route is blocked during protein and nucleic acid transfer.

Abbreviations: ABC, ATP-binding cassette; BDL, bile duct ligation; CAT, chloramphenicol acetyltransferase; CDDP, cisplatin; CGamF, cholylglycylamidofluorescein; FITC, fluorescein isothiocyanate; MRP, multidrug resistance-associated protein; MTX, mitoxantrone; NE, nuclear envelope; NPC, nuclear pore complex; UDCGamF, ursodeoxycholylglycylamidofluorescein.

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0006-2952/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcp.2012.09.029

Additionally, regarding the ability of the whole nucleus to take up, retain and export small molecules, the nuclear envelope (NE) constitutes an important compartment that could affect the net exchange of these compounds with the cytoplasm.

A simple explanation for the molecular selectivity in the nucleus–cytoplasm exchange would be the existence of carrier proteins involved in the trafficking across the membranous boundary between both compartments. This is not unexpected because among the more than 70 proteins that have been identified in the NE the functions of only few more than 10 of them have been elucidated [10]. An important type of primary active transporters involved in the barrier function of several epithelia are ATP-binding cassette (ABC) proteins which are able to carry out ATP-dependent pumping of a large variety of substrates [11]. Several of the 48 human genes belonging to the ABC superfamily have been involved in the multidrug resistance (MDR) phenotype characteristic of many tumor cells. One of these genes (*ABCC2*) encodes ABCC2 protein, initially described as the canalicular multiple organic anion transporter (cMOAT) [12] and now usually called the multidrug resistance-associated protein-2 (MRP2). This pump is expressed at the apical membranes of hepatocytes and epithelial cells of renal proximal tubules, gallbladder, small intestine, colon, and lung [13]. Some members of the ABC superfamily of proteins, such as these of family D, are expressed in intracellular membranes and play a role in limiting the net trafficking between different subcellular compartments. In this respect, using immunohistochemical techniques some evidence for the presence of ABCC2 at the nucleus, presumably at the NE, of cells from ovarian [14] and breast [15] carcinoma, and ABCC1, not at the NE but inside the nuclei, of mucoepidermoid carcinoma cells [16] have been suggested but not unambiguously demonstrated.

Since, on one hand, the liver is an important organ regarding drug detoxification, and hence the hepatocyte nuclei are particularly exposed to genotoxic compounds and, on the other hand, ABCC2 is highly expressed in these cells the present study was aimed to investigate the presence of this ABC protein at the NE and the closely connected perinuclear ER compartment of hepatocytes. Moreover, differential sensitivity to genotoxicity of the ABCC2 substrate mitoxantrone of hepatocytes from normal and ABCC2-deficient rats was investigated.

2. Materials and methods

2.1. Chemicals

Glycoursodeoxycholic acid (GUDCA) was from Calbiochem (Darmstadt, Germany). Sodium salt of glycocholic acid (GCA), fluorescein isothiocyanate (FITC), doxorubicin, calcein, rhodamine 123, dexamethasone, cisplatin (CDDP) and mitoxantrone (MTX) were from Sigma–Aldrich (Madrid, Spain). Calcein acetoxymethyl ester (calcein-AM) was from Invitrogen (Carlsbad, CA). Cholylglycyl amidofluorescein (CGamF) and ursodeoxycholylglycyl amidofluorescein (UDCGamF) were synthesized by coupling the amido group of FITC to the carboxyl group of the glycine moiety of GCA or GUDCA, respectively [17].

2.2. Animals and cells

Male Wistar (Animal House, University of Salamanca, Spain) and Wistar TR- (HsdAmc:TR-Abcc2; Harlan, OR) received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication vol. 2, 2nd ed., 2002). The experimental protocols were approved by the University of Salamanca Ethical Committee for the Use of Laboratory Animals. Rat hepatocytes were isolated from Wistar or TR- rats, seeded in collagen-coated plates and incubated in Williams' Medium E as described elsewhere [18]. Alexander (PLC/PRF/5) human hepatoma cells from the American Type Culture Collection (ATCC: CRL-8024, LGC Standards S.L.U., Barcelona, Spain) were cultured with an appropriate medium in a humidified CO_2:air (5:95%) atmosphere at 37 °C.

2.3. Studies on isolated nuclei

Nuclei were obtained from rat liver homogenates as previously described [19,6], purified by serial centrifugation [20], suspended in STM solution (250 mM sucrose, 50 mM Tris HCl, and 5 mM $MgSO_4$, pH 7.4), and counted in a Neubauer chamber. These preparations consisted of clean suspensions of nuclei free of cell debris (Fig. 1), which were kept at 4 °C until use within the following 24 h. Flow cytometry using a FACSort flow cytometer (BD Biosciences, Madrid, Spain) was used to determine nuclear uptake or efflux (after loading) of fluorescent compounds as previously described [6]. In some experiments, the incubation buffer also contained 3 mM ATP plus an ATP-regenerating system (3 mM phosphocreatine plus 100 μg/ml creatine phosphokinase) [21].

2.4. Experiments on isolated hepatocytes

Freshly isolated hepatocytes from Wistar or TR- rats were preincubated with PBS (control) or with ATP-depleting medium (PBS containing 15 mM sodium azide and 50 mM 2-deoxyglucose) [22] at 37 °C in 5% CO_2 for 20 min and then incubated with 5 μM MTX in PBS or in ATP-depleting medium for 30 min. After incubation, cells were analyzed on the flow cytometer. Dead cells, whose identification was based on propidium iodide staining, were excluded from the data analysis. To visualize the subcellular localization of MTX the same treatment was also performed in hepatocytes seeded in collagen-coated plates. After the incubation with MTX, cells were fixed and observed by confocal microscopy. Cell viability in wild type or TR- hepatocytes was determined using the formazan test (CellTiter 96-AQUEOUS Non-Radioactive Cell Proliferation Assay, Promega, Madrid, Spain) after incubation with MTX for 72 h.

2.5. Western blot analyses

Western blot analyses were performed on rat liver homogenates, crude liver membranes, isolated hepatocyte nuclei and membrane nuclear ghosts derived from NE obtained from isolated nuclei by treatment with DNAse I and RNAse A [23]. In some cases samples were incubated with 500 U of PNGase F (New England BioLabs, Ipswich, UK) for 60 min to deglycosylate proteins. In some experiments rats were treated with dexamethasone (50 mg/kg/day, i.p., 4 days) or CDDP (2 mg/kg/day, i.p., 4 days) or subjected to bile duct ligation (BDL) 2 days prior to being used as donors for hepatocyte nuclei isolation.

Immunoblotting analyses were carried out in 7.5% sodium dodecyl sulfate-polyacrylamide gels, loading 25 μg of protein per lane, and then were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The primary antibodies were as follows: rabbit polyclonal antibodies against rat calnexin (ab22595 Abcam, Cambridge, UK), lamin B1 (ab16048, Abcam), ABCC2 (K13, generously donated by Bruno Stieger from Zurich University), ABCC3 (M0318, Sigma–Aldrich), ABCC6 (sc-25505, Santa Cruz Biotechnology, CA) and UGT1A1 (AB10339, Millipore Ibérica, Madrid); and mouse monoclonal antibodies against rat ABCG2 (BXP21, Abcam), GAPDH (SC32233, Santa Cruz Biotechnology), ABCB1 (Mdr1/Mdr2, ab3364, Abcam), ABCC2 (M2III5 and M2III6,