Relationship between the microsomal epoxide hydrolase and the hepatocellular transport of bile acids and xenobiotics

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Recently two different bile-acid carriers for the hepatocellular sodium-dependent uptake of taurocholate have been described. The first transport system was isolated and characterized by functional expression cloning in *Xenopus laevis* oocytes. The corresponding cDNA clone, named Ntcp for Na^+/taurocholate co-transporting polypeptide, codes for a protein of 362 amino acids and shows no similarity to previously known sequences. The transport function of this carrier system is well documented by expression in *Xenopus laevis* oocytes and by transient and stably transfected cell lines. In addition, several lines of evidence implied that the well-known xenobiotic-metabolizing enzyme microsomal epoxide hydrolase (mEH, EC 3.3.2.3) is also able to mediate sinusoidal uptake of taurocholate. Furthermore, it was claimed that the same enzyme also mediates the uptake of the conjugated bile acid into the smooth endoplasmic reticulum (ER). No direct proof of the transport function of mEH by its heterologous expression has yet been published. In the present work we used a stable transfected cell line that expressed high levels of heterologous mEH for uptake studies of various bile acids and the loop diuretic bumetanide. The uptake of the conjugated bile acid taurocholate, of the non-conjugated bile acid cholate and of the organic anion bumetanide was measured in the transfected as well as in the non-transfected parental cell line. These organic anions represent the main substrates of the known transport systems for organic anions in the rat liver. The results show that the microsomal epoxide hydrolase is unable to transport taurocholate, cholate or bumetanide. Furthermore, Western-blot analysis revealed the expression of mEH in hepatoma tumour cell lines, which show no transport activity for these organic anions. These results show that it is unlikely that mEH can mediate the transport of these substrates.

INTRODUCTION

The hepatocellular uptake of organic anions such as bile acids and xenobiotics is mediated by at least three different carrier systems, which were identified by expression of mRNA fractions in *Xenopus laevis* oocytes [1–3]. Two of these transporters, namely the organic anion transporting polypeptide (oatp) and the Na^+/taurocholate cotransporting polypeptide (Ntcp) were recently cloned [4,5] and characterized by using different expression systems [6]. The third organic anion transporter is specific for the loop diuretic bumetanide and possibly for other substrates [2]. Two recent papers, however, have suggested that an enzyme that was formerly identified in the ER as the xenobiotic metabolizing epoxide hydrolase (mEH) is also located in the sinusoidal plasma membrane of hepatocytes and mediates taurocholate uptake [7,8]. From the data presented, the authors concluded that mEH mediates sodium-dependent uptake of taurocholate across the sinusoidal plasma membrane as well as sodium-independent uptake of the same substrate into the smooth ER. This conclusion was drawn from the fact that a monoclonal antibody (mAb) raised against a plasma membrane fraction that had been partly purified by affinity chromatography with glycocholate [9] identified a protein that showed an amino-terminal amino acid sequence identical to that of mEH [7].

These conclusions were further supported by the following. First, the mAb 25D-1 recognized a protein of molecular mass 49 kDa [9]. The molecular mass of the bile acid transporters as estimated by photoaffinity labelling is 48–54 kDa and the molecular mass of Ntcp is 50–51 kDa [10,11]. Secondly, the mAb 25A-3, which apparently recognized the same protein as did the antibody 25D1 and a protein of molecular mass 54 kDa, was able to protect these plasma membrane proteins from inhibition by 4,4′-di-isothiocyanostilbene-2-2′-disulphonate (DIDS), an irreversible inhibitor of organic anion transporters. However, the mAb 25D1 failed to inhibit the transport of taurocholate in plasma membrane vesicles and was also unable to protect the proteins from inhibition by DIDS [9]. Thirdly, the proteolytic fragment pattern of mEH and the protein immunoprecipitated by the mAb 25D-1 was identical [7]. Fourthly, reconstitution of the immunoprecipitated protein into proteoliposomes followed by uptake measurements showed a marked increase in taurocholate transport [12]. Beyond these results there is no direct proof for the transport function of mEH. Because the cDNA clone for mEH was published in 1986 [13], direct-uptake studies with a heterologous expression system were possible. To investigate whether mEH is able to transport bile acids, we used a stable fibroblast cell line expressing mEH heterologously [14] for uptake experiments with different bile acids and the loop diuretic bumetanide.

MATERIALS AND METHODS

Materials

[^{3}H]Taurocholate (specific activity 2 Ci/mmol, 74 GBq/mmol) and [^{14}C]cholate (specific acitivity 54 Ci/mmol, 1998 GBq/mmol) were purchased from Amersham, Braunschweig, Germany. [^{3}H]Bumetanide (specific activity 15 Ci/mmol, 555 GBq/mmol) was prepared as previously described [15]. The reagents for the

Abbreviations used: ER, endoplasmic reticulum; mAb, monoclonal antibody; mEH, microsomal epoxide hydrolase; Ntcp, Na^+/taurocholate co-transporting polypeptide; oatp, organic anion transporting polypeptide; DIDS, 4,4′-di-isothiocyanostilbene-2-2′-disulphonate.

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cell culture (e.g. DMEM, trypsin) were purchased by Gibco–BRL, Eggenstein, Germany. Foetal calf serum was from Technomara, Fernwald, Germany. The reagents for SDS/PAGE were from Sigma, Deisenhofen, Germany; Pefablock SC was from Merck, Darmstadt, Germany.

Methods

Isolation of hepatocytes

Hepatocytes were prepared from male Wistar rats (230–260 g) as described previously [15]. This method is based on the collagenase perfusion technique originally described by Berry and Friend [16].

Cell culture

BHK21 Syrian hamster kidney fibroblasts were transfected with cDNA encoding rat mEH as described previously [14]. One cell clone, BHK21-mEH/Mz1, expressing high levels of mEH as verified by immunoblotting and enzymic activity towards benzo[*a*]pyrene-4-5-oxide [14], was used for the experiments described in the present work. The cell lines were grown in DMEM with 10% FCS, 50 U/ml penicillin/streptomycin, 4mM glutamine, 10 g/ml insulin, 10 g/ml inosin and 1.5 M dexamethasone in an air/CO_2 mixture (95:5 v/v) at 37 °C.

Uptake studies

BHK21- and BHK21-mEH/Mz1 cells were collected by trypsinization. The cells were resuspended in fresh culture medium for 20 min at 37 °C under carbogen (95% O_2/5% CO_2). After washing three times with Tyrode buffer (including 5.5 mM glucose), the cells were equilibrated in the same buffer at 37 °C for 10 min under carbogen; 90–95% of these cells excluded 0.2% Trypan Blue. The uptake of the radiolabelled substrates into the cells was measured in Tyrode buffer at 37 °C under carbogen. After addition of the radiolabelled substrates (10 nM [^{3}H]taurocholate/10 μM taurocholate, 370 nM [^{14}C]cholate/10 μM cholate or 1.34 nM [^{3}H]bumetanide/10 μM bumetanide), 100 μl aliquots of the cell suspension were taken at the indicated time points, and the cells were pelleted in small tubes through silicone oil by rapid centrifugation, according to the method described by Schwarz et al. [17]. The pelleted cells were dissolved in 3 M KOH overnight, and cell-derived radioactivity was measured by liquid scintillation counting in a Pharmacia Wallac 1409 scintillation counter. As a control for the uptake studies, freshly isolated hepatocytes were measured in parallel. In the Figures a representative uptake measurement from at least four different studies is shown.

Western-blot analysis

Western-blot analysis was done according to Honscha et al. [18]. In brief, the cells were collected by trypsinization and washed three times in PBS. The resulting cell pellet was resuspended in PBS with 25 μM Pefablock SC. After homogenization, 20 μg of protein was separated by SDS/PAGE and subsequently blotted onto nitrocellulose. The first antibody, a polyclonal rabbit anti-rat serum directed against mEH (generously given by Dr. M. Arand, Institute of Toxicology, Mainz, Germany), was used at a dilution of 1:1000. A 1:750 dilution of an alkaline phosphatase conjugated goat anti-rabbit serum was used as the second antibody. The blot was stained by Nitroblue Tetrazolium and 4-bromo-3-chloroindolyl phosphate. As a positive control the total cellular protein of freshly isolated hepatocytes was used.

Immunofluorescence

Immunofluorescence analysis with non-permeabilized cells was done as described earlier [18]. In brief, the cells were grown on 12 mm coverslips. After washing with PBS, pH 7.4 (three times for 10 min each), the slides were incubated with a monoclonal antibody against mEH at a dilution of 1:500 for 18 h at 4 °C. The slides were washed again and incubated in the dark with a fluorescein–isothiocyanate conjugated goat anti-mouse IgG at a dilution of 1:200 for 2 h. The slides were mounted after additional washing with a glycerol-based medium containing *p*-phenylenediamine to reduce the photobleaching. The cells were observed with a Zeiss universal microscope, equipped with the appropiate filters.

For studies with permeabilized cells, the slides, after washing with PBS, were incubated in cold (−20 °C) methanol/acetone (1:1, 10 min) and then dried at room temperature. After 5 min the slides were incubated with the first antibody.

RESULTS

First we verified that the BHK21-mEH/Mz1 cell line used for the uptake experiments described below expressed high levels of mEH. In this Western-blot analysis we used a polyclonal antibody that was monospecific for mEH. As shown in Figure 1, in contrast to the non-transfected cell line, the BHK21-mEH/Mz1 cell line showed an immunosignal of the expected molecular

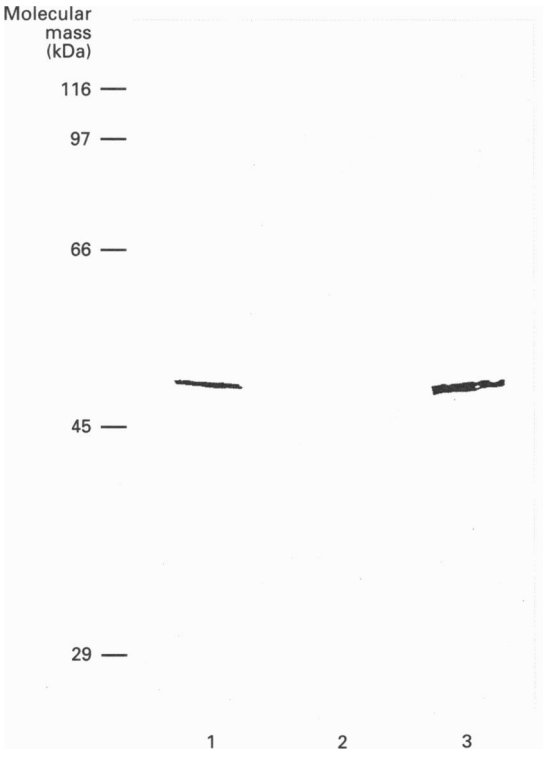

Figure 1 Western-blot analysis of mEH in hepatocytes, in mEH cDNA transfected and in non-transfected BHK21-cells

Cellular protein (20 μg) of hepatocytes, non-transfected BHK21 cells and stably transfected BHK21-mEH/Mz1 cells were separated by SDS/PAGE and the proteins transferred to nitrocellulose. The nitrocellulose filter was incubated with a polyclonal antibody against mEH at a dilution of 1:1500. For details, see the Materials and Methods section. Lane 1, freshly isolated hepatocytes; lane 2, non-transfected BHK21-cells; lane 3, BHK21-mEH/Mz1-cells. Marker proteins are shown at the left.

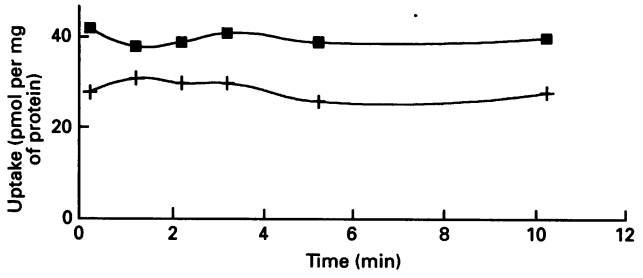

Figure 2 Sodium-dependent uptake of [^{3}H]taurocholate in BHK21− and BHK21-mEH/Mz1 cells

For details see the Materials and Methods section. ■, Taurocholate–BHK-21; +, taurocholate–BHK21-mEH/Mz1.

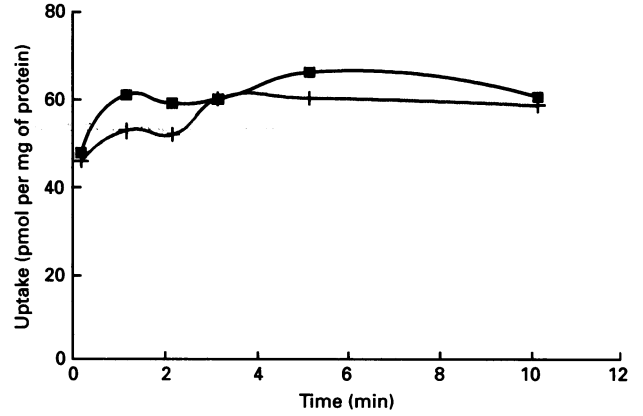

Figure 3 Sodium-dependent uptake of [^{3}H]cholate in BHK21 and BHK21-mEH/Mz1 cells

For details see the Materials and Methods section. ■, Cholate–BHK-21; +, cholate–BHK21-mEH/Mz1.

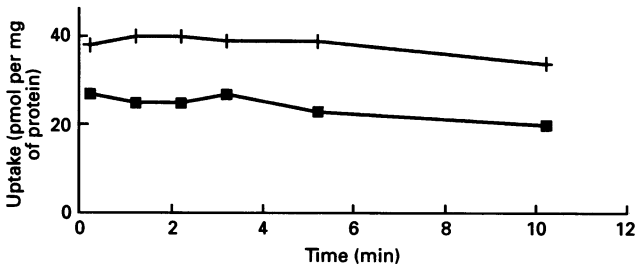

Figure 4 Sodium-dependent uptake of [^{3}H]bumetanide in BHK21− and BHK21-mEH/Mz1 cells

For details see the Materials and Methods section. ■, Bumetanide–BHK-21; +, bumetanide–BHK21-mEH/Mz1.

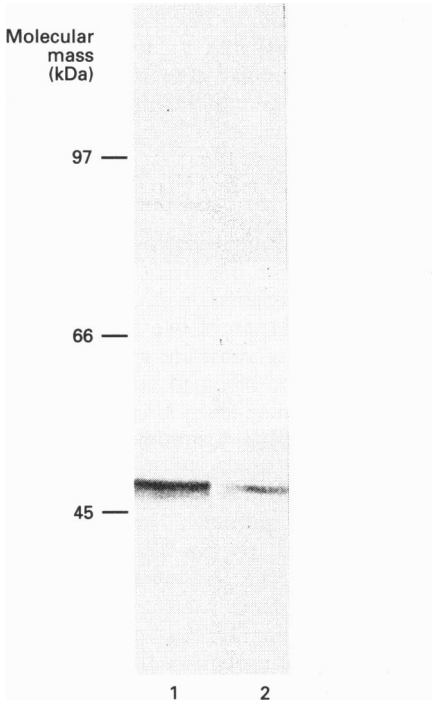

Figure 5 Western-blot analysis of bile-acid transport-negative hepatoma Fao and AS30D cells

For details see legend to Figure 1. Lane 1, 20 μg cellular protein of Fao cells; lane 2, 20 μg cellular protein of AS-30D cells.

mass. This signal is comparable to the signal found for hepatocytes. Because it had been proposed that mEH mediates the uptake of bile acids and is localized on the surface of the plasma membrane [7,9], we attempted to determine the subcellular localization of heterologously expressed mEH, followed by a functional analysis of the protein for bile-acid transport. To evaluate whether heterologously expressed mEH is localized on the plasma membrane, we performed immunofluorescence studies on intact and on permeabilized cells. Monoclonal anti-mEH antibodies reacted as expected from our previous results [14] with vesicular structures in permeabilized BHK21-mEH/Mz1 cells. Unpermeabilized BHK21-mEH/Mz1 cells revealed a fuzzy signal that appeared to be brighter than that in control cells (results not shown). However, given the haziness of the signal, which is most probably due to the intact three-dimensional structure of the cells, we cannot decide whether or not mEH is localized on the surface of the BHK21-mEH/Mz1 plasma membrane.

To determine the function of mEH as a bile-acid transporter, we investigated the transport of taurocholate into BHK21-mEH/Mz1 and the parental BHK21 cells. As depicted in Figure 2, the uptake of taurocholate was no greater in the transfected cell line than in the non-transfected parental cell line. These data clearly demonstrate that mEH is not a carrier system for this conjugated bile acid. Because it was shown by expression cloning that different bile-acid carriers exist and that these carriers have different substrate specificities [2,19,20], we tested the uptake of substrates that are specific for already-characterized transport systems. It is known that the oatp transporter mediates the sodium-independent uptake of various organic anions [20] and of the important bile acid cholate. However, until now the sodium-dependent uptake of this bile acid has remained unknown. We therefore tested the hypothesis that mEH mediates the sodium-dependent uptake of cholate (Figure 3). Our data clearly demonstrate that the cell line BHK21-mEH/Mz1 does not transport cholate. Bumetanide has been shown to be a substrate of a third organic anion transport system, which is

different from oatp and Ntcp. Figure 4 shows that this substrate is also not taken up by heterologously expressed mEH. Summarizing the data, it is obvious from Figures 2–4 that the uptakes of taurocholate, cholate and bumetanide are very similar in BHK21 cells, which do not express mEH, and in BHK21-mEH/Mz1 cells, which express high levels of this enzyme. In all cases the transport activity of the transfected cell line is in the range of the non-transfected cell line and the amount of the radiolabelled substrates does not increase over the incubation time. To exclude artefacts during the uptake studies, the uptake of the test substrates was determined in parallel in hepatocytes. These cells displayed a high transport activity for all substrates tested (results not shown). Because the different organic anions represent the typical substrates for the known hepatocellular carrier systems (Ntcp, oatp and the bumetanide transport system) it is evident that mEH is not a carrier for these organic anions.

Furthermore, Western-blot analysis of two cell lines, namely Fao− and AS30D cells, that show no transport activity for bile acids and bumetanide [15,21,22] revealed that mEH is expressed in these cell lines (Figure 5). If mEH were able to mediate sodium-dependent uptake of taurocholate these cell lines should display a transport activity for this substrate.

DISCUSSION

The question of how many organic anion transport carriers exist with a specificity for bile acids is at present not completely solved. Data from expression cloning in *Xenopus laevis* oocytes have led to the isolation of two different bile-acid transporters with an overlapping substrate specificity [4,5]. Recent papers have shown that the bile acid taurocholate is transported in a sodium-dependent manner by Ntcp and in a sodium-independent manner by oatp [19]. A second potential carrier system for the sodium-dependent uptake of taurocholate was identified by biochemical methods with a protein that was immunoprecipitated by a monoclonal antibody. The amino acid analysis of the immunoprecipitated protein [7] showed an amino-terminal identity with mEH [13]. However, this paper [7] unfortunately gave no direct evidence for the transport function of mEH. Another paper from the same group demonstrated that the immunoprecipitated protein mediates the uptake of taurocholate in reconstituted proteoliposomes [12]. Furthermore, it was shown that the immunoprecipitated protein, which showed identity to the amino terminus of mEH, not only mediates uptake of taurocholate through the plasma membrane of hepatocytes but also through the smooth ER [8]. The authors suggested that mEH mediates sodium-dependent uptake at the plasma membrane and that the same enzyme in an inverse orientation is also able to transport taurocholate in a sodium-independent manner into the smooth endoplasmic reticulum, in addition to metabolizing different xenobiotics. Therefore three different functions, two subcellular localizations and two different membrane orientations have been proposed for this enzyme. With respect to the subcellular localization of mEH, data from our previous study [14] and from the present study employing methanol/acetone and detergent-permeabilized BHK21-mEH/Mz1 cells clearly demonstrated that heterologously expressed mEH is localized in vesicular structures. To detect any additional localization of mEH on the surface of BHK21-mEH/Mz1 cells, we reacted anti-mEH antibodies with non-permeabilized BHK21-mEH/Mz1 cells. On the basis of the results of this experiment we cannot decide whether or not heterologously expressed mEH is localized on the surface of the plasma membrane. Several other groups have attempted to define the subcellular localization of mEH [7,9,23,24]. In one very thorough study, immunofluorescence and immunoelectron microscopy were used to determine the subcellular localization of mEH in rat liver [24]. That study concluded that mEH is not localized at the plasma membrane but in fact is a marker protein for smooth ER. These data conflict with the group claiming that mEH is a bile-acid transporter localized on the surface of the plasma membrane [7,9]. Also, with respect to the membrane topology of mEH, the reports of the group claiming that the mEH bile-acid transporter has two topological orientations, with the same epitopes being exposed to the lumen as well as to the ER [8], are not supported by other reports [25,26]. In the latter studies, employing either chemical probes or immunoelectron microscopy, mEH was not found to be localized on the luminal site of the ER.

Given the conflicting results about the subcellular localization of mEH, we tried to prove the transport function of mEH by using a stably transfected cell line expressing high levels of mEH [14] for uptake studies with different organic anions. The organic anions used (taurocholate, cholate and bumetanide) represent the main substrates for the known hepatocellular bile-acid transporters. However, we were unable to detect any transport of these substrates by heterogenously expressed mEH. It might be argued that mEH expressed in fibroblasts is not functionally equivalent to mEH expressed in hepatocytes. However, this is highly unlikely as we have found that the enzymic activity and the enzyme specificity in the transfected BHK21 cells were not altered [14]. In addition, we have found in the present work that mEH is also expressed in the transport-negative AS-30D− and in the hepatoma cell line Fao [15,21,22]. If mEH were able to transport bile acids, these cell lines should display transport activity. From our data we conclude that the mEH as described by Porter et al. [13] is not an additional carrier for the uptake of taurocholate on the plasma membrane of hepatocytes. Another argument against mEH's transport activity is that mEH is inducible by different substrates such as phenobarbital and aroclor, but in contrast the uptake of bile acids such as cholate is not increased after administration of these xenobiotics (E. Petzinger, personal communication). Because it is known that the mRNA for this enzyme is differentially spliced [27], it could be speculated that the immunoprecipitated protein of von Dippe et al. [7] is encoded by one of these alternatively spliced mRNAs. However, nuclease S1 protection and primer extension analysis [27] clearly demonstrated that these three or more alternatively spliced mEH mRNAs differ only in their 5′ untranslated regions but otherwise encode the same mEH protein as described recently [13]. It is therefore highly unlikely that these alternatively spliced mEH mRNAs would encode the taurocholate transport protein described by von Dippe et al. [7], because the amino-terminal amino acid sequence of this portein is identical to mEH.

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