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Regulation of basolateral organic anion transporters in ethinylestradiol-induced cholestasis in the rat

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Abstract

Background/Aims: Estrogen-mediated cholestasis is an important clinical entity, but its molecular pathophysiology is still not fully understood. Impaired sodium-dependent uptake of bile acids has been associated with diminished expression of a basolateral Na^+/bile acid cotransporter (Ntcp), whereas sodium-independent uptake is maintained despite a down-regulation of the organic anion transporter Oatp1. Thus, expression of the two other rat Oatps (Oatps2 and -4) was determined in estrogen-induced cholestasis. In addition, known transactivators of Oatp2 and Ntcp were studied to further characterize transcriptional regulation of these transporter genes. *Methods*: Hepatic protein and mRNA expression of various Oatps (1, 2, 4) in comparison to Ntcp were analyzed after 0.5, 1, 3 and 5 days of ethinylestradiol (EE) treatment (5 mg/kg) in rats. Binding activities of *Oatp2* and *Ntcp* transactivators were assessed by electrophoretic mobility shift assays. *Results*: All basolateral Oatps (1, 2 and 4) were specifically down-regulated at the protein level by 30–40% of controls, but less pronounced than Ntcp (minus 70–80%). In contrast to unaltered Oatp4 mRNA levels, Oatp1 and Oatp2 mRNAs were reduced to various extents (minus 40–90% of controls). Binding activity of known transactivators of Ntcp and Oatp2 such as hepatocyte nuclear factor 1 (HNF1), CAAT enhancer binding protein α (C/EBPα) and pregnane X receptor (PXR) were also diminished during the time of cholestasis. *Conclusions*: Estrogen-induced cholestasis results in a down-regulation of all basolateral organic anion transporters. The moderate decline in expression of Oatp1, -2 and -4 may explain the unchanged sodium-independent transport of bile acids due to overlapping substrate specificity. Reduction in transporter gene expression seems to be mediated by a diminished nuclear binding activity of transactivators such as HNF1, C/EBP and PXR by estrogens.

Keywords: Organic anion transporter; Estrogen; Cholestasis

1. Introduction

Estrogens are well known to cause reversible intrahepatic cholestasis in humans and rodents. Intrahepatic cholestasis occurs in susceptible women during pregnancy, administration of oral contraceptives and postmenopausal hormone replacement therapy [1]. Since its first description in 1954 [2] cholestasis of pregnancy has been of major clinical and experimental interest for almost five decades. Despite differences in estrogen levels between the rat model and pregnant women, 17α-ethinylestradiol (EE) treatment of rats has been widely accepted as one animal model of the human disease entity [3]. However, its molecular pathomechanisms are multifactorial and still largely unknown.

Physiologically, organic anions are taken up from portal blood by a sodium-dependent taurocholate cotransporter (rat: Ntcp, Slc10a1; human: NTCP, SLC10A1) and several members of a growing family of sodium-independent organic anion transporters (Oatps). After rapid transhepatic transport to the canalicular membrane, organic anions are

Abbreviations: AP-1, activating protein-1; Bsep, bile salt export pump; C/EBP, CAAT enhancer binding protein; EE, ethinylestradiol; ER, estrogen receptor; HNF, hepatocyte nuclear factor; Mrp, multidrug-resistance protein; Ntcp, sodium/taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor

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secreted into the bile by two ATP-dependent export pumps, the bile salt exporting pump (rat: Bsep, Abcb11; human: BSEP, ABCB11) and the multidrug-resistance protein 2 (rat: Mrp 2, Abcc2; human: MRP2, ABCC2) [4].

Estrogens induce cholestasis by reducing both bile salt-dependent (BSDF) and bile salt independent bile flow (BSIF) [1]. Several studies indicate that early generalized abnormalities of the sinusoidal membrane fluidity, Na(+)–K(+)–ATPase activity and reduced Ntcp, Oatp1 and Mrp2 expression contribute to EE-induced cholestasis [3,5,6]. Reduction in BSIF parallels the impairment in glutathione and bicarbonate excretion and might be a correlate of decreased Mrp2 protein expression [7,8]. However, decreased canalicular ATP-dependent taurocholate transport in hepatocyte canalicular membrane preparations [5] is thought to result rather from *trans*-inhibition of Bsep following Mrp2-dependent transport of EE into the canaliculus than from changes in gene expression [9–12].

At the basolateral membrane, uptake of bile salts and certain organic anions such as sulfobromophthalein is markedly impaired under these conditions [5,13,14] and might result, at least in part, from the down-regulation of *Ntcp* and *Oatp1* expression. However, differential down-regulation of the three members of the Oatp family expressed in the liver has been shown during extrahepatic cholestasis [15]. Oatp1 (Slc21a1) decreases by 90% 7 days after ligation of the common bile duct whereas Oatp2 (Slc21a5) and Oatp4 (Slc21a10) expression is unaffected [15]. This differential down-regulation may affect the excretion of important endogenous and xenobiotic substrates. Oatp1 (Slc21a1), Oatp2 (Slc21a5) and Oatp4 (Slc21a10) [16–19] are involved in the absorption and elimination of a wide variety of structurally unrelated amphipathic organic compounds from sinusoidal blood and represent multispecific transport systems with distinct but partially overlapping transport specificities [20–23].

Despite the long interest in estrogen-induced cholestasis, the mechanisms of altered sinusoidal uptake in estrogen-associated cholestasis have not fully been elucidated. Neither the excretion of potential substrates including a broad range of clinically relevant drugs nor the expression of the exclusively digoxin transporting Oatp2 and the ‘‘liver specific’’ Oatp4 at both protein and mRNA level has been studied in estrogen-related cholestasis yet.

To get further insights into the mechanisms by which these basolateral organic anion transporters might be regulated under the influence of estrogen, we have characterized the expression of Oatp2 and Oatp4 in comparison to Oatp1 and Ntcp. In addition, nuclear binding activities of known transactivators of these transporter genes such as hepatocyte nuclear factor 1 (HNF1), CAAT enhancer binding protein (C/EBP), retinoid X receptor:retinoic acid receptor (RXR:RAR) and retinoid X receptor:pregnane X receptor (RXR:PXR) were determined in EE induced cholestasis.

2. Materials and methods

2.1. Animal models

Male Sprague–Dawley rats (250–300 g) were obtained from the animal facility of Aachen University of Technology. Cholestasis was induced in each animal by continuous daily subcutaneous injections of sterile 17α-ethinylestradiol dissolved in 1,2-propanediol (5 mg/ml, Sigma) for up to 5 days as previously described [3,11]. Control animals were daily injected with 1,2-propanediol alone. Animals were maintained on a 12-h light and 12-h night cycle with free access to standard chow and water. Livers were harvested either 0.5, 1, 3 or 5 days after injection, immediately frozen in liquid nitrogen for protein and RNA-analysis or freshly used for isolation of nuclear extracts. All study protocols were approved by the Federal Government’s Animal Care Committee.

2.2. Northern blot analysis

RNA was isolated from whole liver by Ultraspec™ phenol chloroform extraction procedure (Biotecx Laboratories Inc., Houston, TX) according to the instruction manual, quantified spectrophotometrically at 260 nm and stored at −70 °C. Total RNA (10–20 μg) was denatured, electrophoresed on a 1% agarose/formaldehyde gel, transferred to a nylon membrane (Nytran 0.2; Schleicher&Schüll, Dassel, Germany) by overnight capillary blotting and UV-cross-linked (UV Stratalinker 1800, Stratagene, La Jolla, CA). Ethidium bromide staining of 18S and 28S bands was used to ensure equal loading for each sample. The membranes were prehybridized for 30 min at 60 °C in ExpressHyb™ solution (Clontech, Palo Alto, CA). After replacement with fresh ExpressHyb solution, hybridization was performed at 60 °C for 1 h after addition of specific rat complementary DNA (cDNA) probes labeled with [^{32}P]dCTP (specific activity 10^8 cpm/μg) by a random primed method (High Prime™, Boehringer Mannheim, Mannheim, Germany). Blots were washed twice with 2× SSC/0.05% SDS for 10 min at room temperature, followed by 2× SSC/1% SDS for 20 min at 50 °C. Specific mRNA levels were detected after exposure of membranes to a Phosphoimager screen (Biorad, Munich, Germany) and quantified using a Personal Molecular Imager FX (Biorad). Specific and constitutively expressed probes used were as previously described [19,24,25]: Ntcp cDNA (0.9 kb *Eco*RI fragment), Oatp1 cDNA (0.7 kb *Pfl*MI fragment), Oatp2 cDNA (3.7 kb *Eco*RI/*BSS*HII fragment), Oatp4 cDNA (0.4 kb *Nco*I/*Hin*dIII fragment), ecto-ATPase cDNA (1.3 kb *Xba*I/*Pst*I fragment) and GAPDH cDNA (1.3 kb *Pst*I fragment).

2.3. Western blot analysis

Preparation of liver microsomes and analysis of protein mass of various polypeptides were performed as previously

Table 1
DNA sequences of oligonucleotides used for electrophoretic mobility shift analyses

Oligonucleotide	Sequence (sense strand)
HNF1	5′ -GATCTGCTGGTTAATCTTTTATTT-3′
RXR:RAR	5′ -GATCTCCGGGGCATAAGGTTATGG-3′
C/EBP	5′ -GATCCAGGAAACTTGAGCAAGGTA-3′
RXR:PXR	5′ -AGACAGTTCATGAAGTTCATCT-3′
AP-1	5′ -CGCTTGATGACTCAGCCGGAA-3′
ER	5′ -AGACCCACCCATGACCTGC-3′

described [25,26]. Protein concentrations were determined according to Bradford [27]. After determination of the linear range (25–200 μg), similar amounts of protein (75 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [28], transferred to polyvinylidene fluoride blotting membranes (Biotrace, Gelman Sciences, Roßdorf, Germany), and probed to either anti-Ntcp fusion protein antiserum [29], anti-Oatp1 fusion protein antiserum [30], anti-Oatp2 peptide antiserum [17], anti-Oatp4 peptide antiserum [23] and monoclonal anti-Na^+/K^+-ATPase α-1 antibody (Upstate Biotechnology, Lake Placid, NY). Immune complexes were detected using horseradish peroxidase-conjugated donkey anti-rabbit IgG F(ab′)$_2$ fragments according to the ECL™ Western blotting kit (Amersham, Little Chalfont, England). Immunoreactive bands obtained by autoradiography were quantified by laser densitometry (Fluor S MultiImager, Quantity One™ software, Biorad).

2.4. Electrophoretic mobility shift analysis

Preparation of nuclear extracts and electrophoretic mobility shift assays were performed as previously described [31–33]. Protein concentrations were determined according to Bradford [27]. Nuclear extracts (5–10 μg protein) were incubated on ice for 30 min with a specific [^{32}P]end-labeled oligonucleotide probe (2×10^4 cpm, see Table 1) in a 20-μl reaction containing 8-μl water, 4-μl 5× binding buffer (25 mM HEPES pH 7.6, 50 mM KCl, 0.5 mM DTT, 5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol) and 2-μg poly(dI–dC)–poly(dI–dC) (Pharmacia Biotech, Freiburg, Germany). For competition assays, 100-fold molar excess of specific unlabeled oligonucleotides were added with the labeled oligonucleotide to the binding reaction. Separation of protein–DNA complexes from the unbound labeled probe was performed by electrophoresis through a nondenaturing 6% polyacrylamide gel. Dried gels were exposed both to autoradiography and Phosphoimager screens, the latter used for quantification as described above for Northern blot analysis.

2.5. Statistical analysis

Statistical significance ($P<0.05$) between controls and EE-treated animals was determined by multivariate ANOVA and posttesting. Data represent the mean ± S.D. of at least four animals per group.

3. Results

3.1. Effects of EE on steady-state mRNA levels

To determine whether mRNA expression of hepatocellular organic anion transporters was altered during the cholestatic response to EE injection, we quantified steady-state mRNA levels of transport proteins exclusively localized to the basolateral plasma membrane of hepatocytes (Ntcp, Oatp1, -2 and -4) by Northern blot analysis (Fig. 1). The most extensive down-regulation was observed at the basolateral plasma membrane for Oatp2 mRNA, which declined on average to 8 ± 2% of control levels 5 days after EE treatment ($P<0.05$, $n=4$). Similar to Oatp2, Ntcp and Oatp1 decreased, but to a lesser extent as shown by an average reduction of specific mRNA to 33 ± 4% and 43 ± 6% ($P<0.05$, $n=4$), respectively. In contrast to all other basolateral organic anion transporters, Oatp4 mRNA remained largely unchanged during the whole time course with a slight but significant reduction to 77 ± 6% ($P<0.05$, $n=4$) after 3 days (Fig. 2). Densitometric data shown in Fig. 2 demonstrate that mRNA reduction of Ntcp and Oatp2 followed a rapid time course with a 70% reduction within the first 12 h, while Oatp1 declined slowly to a 5-day minimum after EE treatment. In contrast to reductions in mRNA levels of organic anion transporters, mRNA levels of the housekeeping gene GAPDH remained unchanged at any time point after EE treatment (Fig. 1).

3.2. Protein expression after EE injection

To study whether alterations in steady-state mRNA levels resulted in reduced protein expression of hepatocellular organic anion transporters, protein levels of various transporters including the basolateral Oatp1, -2 and -4 were

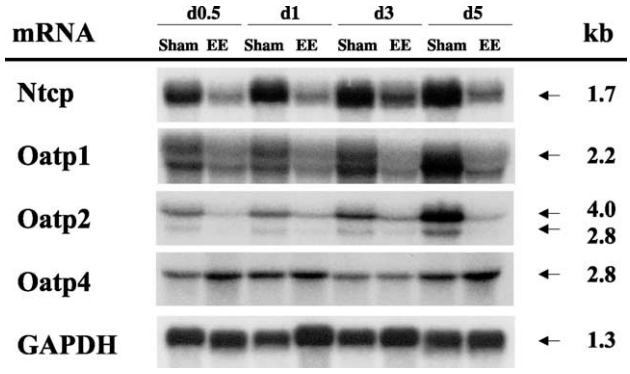

Fig. 1. Effect of EE on steady-state mRNA levels of organic anion transporters. Rats ($n=4$) were treated either with EE or solvent alone (sham-controls) for up to 5 days. Total RNA was isolated from livers as described, blotted onto nylon membrane and hybridized to specific cDNA probes as described in Materials and methods. Blots were then stripped and rehybridized with a GAPDH cDNA probe. Representative autoradiographs are shown for each treatment period.

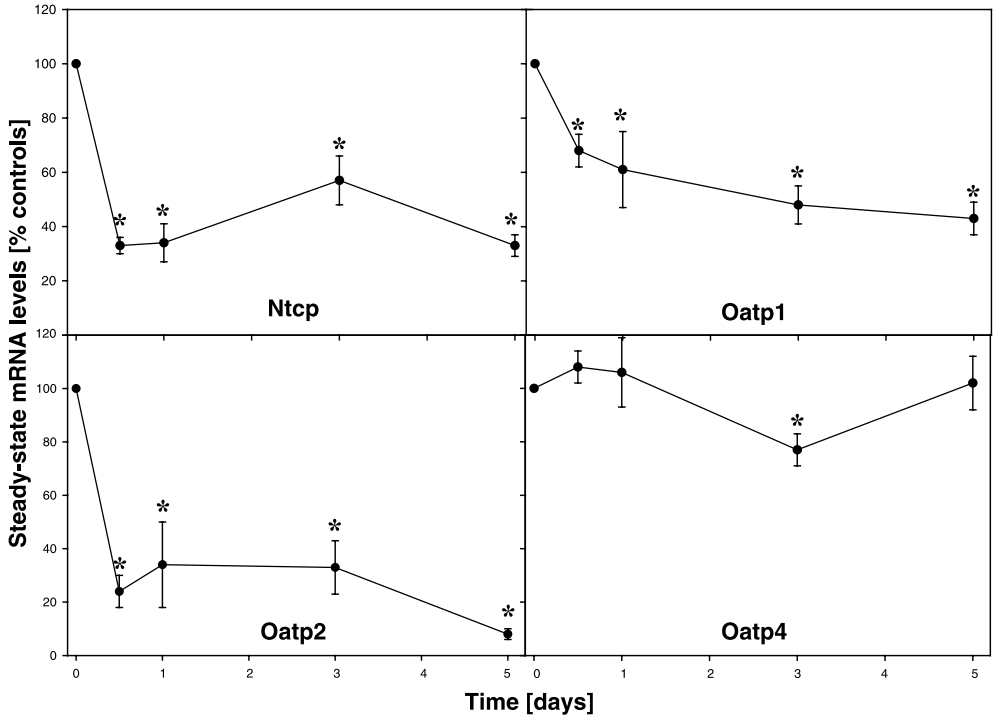

Fig. 2. Densitometric analysis of basolateral organic anion transporter mRNA in EE-treated rats. Autoradiographs were quantified by phosphoimaging and data were expressed as percentage of sham-controls ($n=4$). Data are given as mean ± S.D. *$P<0.05$ as compared to sham-controls.

quantified by Western blotting (Fig. 3). Protein levels of the basolateral Ntcp was significantly reduced between 0.5 and 5 days (17 ± 4% at day 3 and 33 ± 2% at day 5, respectively, $P<0.05$, $n=4$) in parallel to mRNA levels (Fig. 4). In contrast, Oatp1 and -2 protein levels remained statistically unaffected for the first 24 h of treatment and thereafter declined to a lesser extent compared to Ntcp (59 ± 10% and 63 ± 7%, respectively, at day 5, $P<0.05$, $n=4$; Figs. 3 and 4). In contrast to largely unchanged mRNA levels, Oatp4 protein slightly but significantly declined between 3 and 5 days of EE treatment to 72 ± 11% ($P<0.05$, $n=4$) at day 5 as shown in Fig. 3. The decrease in organic anion transporter protein mass appeared to be specific since the basolateral inorganic ion exchanger Na^+, K^+-ATPase remained unchanged (Fig. 3).

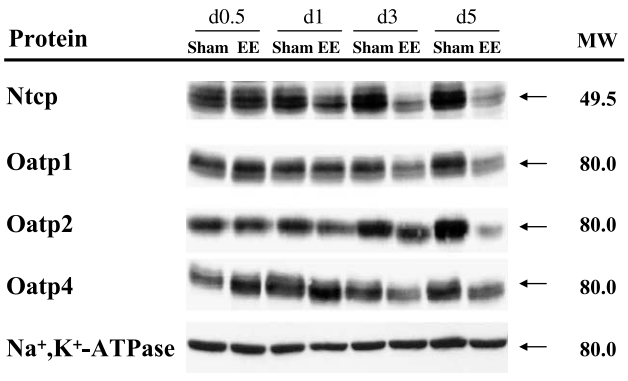

Fig. 3. Effect of EE on organic anion transporter protein levels. Membrane fractions were isolated from the same animals described in Fig. 1, subjected to SDS-PAGE (75 μg protein/lane), and subsequently blotted onto polyvinylidene fluoride blotting membranes as described in Materials and methods. Representative immunoblots are shown for each treatment period. Molecular weight (MW) markers are given in kilodaltons.

3.3. Effects of EE on binding activity of transcription factors

To further elucidate the mechanisms of EE-induced differential down-regulation of basolateral organic anion transporters at the mRNA level, we performed electrophoretic mobility shift assays with nuclear extracts isolated from animals at two different time points (24 and 72 h) after EE application. Oligonucleotides representing consensus binding sites for previously identified nuclear transactivators of the *Ntcp* gene [34,35] including HNF1, C/EBP and RXR:RAR as well as RXR:PXR, a transactivator of rat *Oatp2*, were used for EMSA (Fig. 5). Since estrogen receptors (ER) not only bind to estrogen response elements (ERE) but also affect transcription at activating protein-1 (AP-1) sites [36,37], oligonucleotides with the binding site for ER in the rat Ntcp promoter (nt −184/−165) and a consensus binding sequence for AP-1 were included in this study. Analysis of nuclear extracts isolated 24 h after EE showed a decrease in binding activity of the known Ntcp transactivator C/EBP to 46 ± 16% ($P<0.05$, $n=4$), with a further decline to 35 ± 13% after 3 days ($P<0.05$, Figs. 5

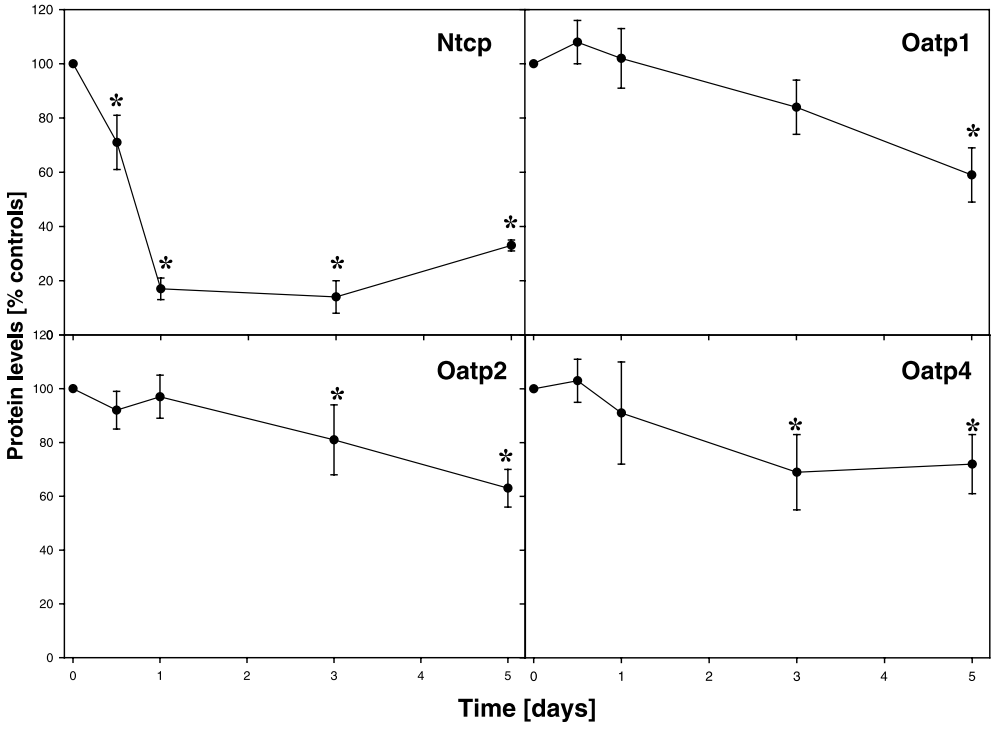

Fig. 4. Densitometric analysis of transporter protein levels in EE-treated rats. Autoradiographs were quantified by laser densitometry, and data were expressed as percentage of sham-controls ($n=4$). Data are given as mean ± S.D. *$P<0.05$ as compared to sham-controls.

and 6). In contrast, HNF1 binding activity remained unchanged at day 1 and declined to 61 ± 21% ($P<0.05$, $n=4$) at day 3. Binding to an RXR:RAR responsive element was largely unaffected by EE treatment at day 1 and even increased at day 3 as shown in Fig. 6. In contrast to RXR:RAR, nuclear binding activity of RXR:PXR, a transcription factor that induces Oatp2 expression, was initially unchanged (103 ± 10%) but thereafter decreased to 40 ± 4% compared to controls ($P<0.05$, $n=4$). AP-1 binding activity appeared to be increased at both 1 and 3 days after EE treatment to 217 ± 40% and 258 ± 64%, respectively ($P<0.05$, $n=4$). Interestingly, nuclear protein binding to the ERE of the Ntcp gene promoter was largely unchanged by EE treatment with a decrease to 55 ± 21%, which was statistically not significant ($P>0.05$, $n=4$) (Figs. 5 and 6).

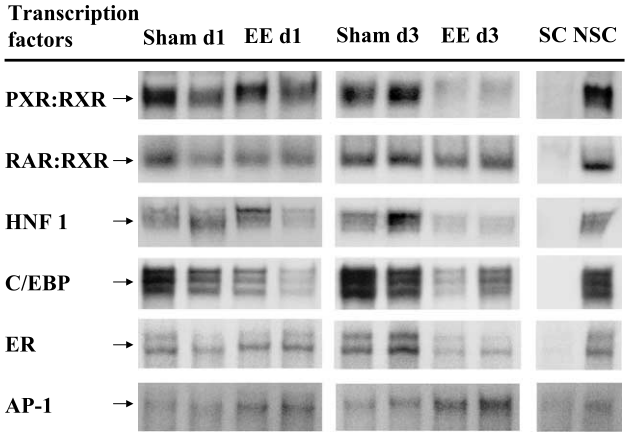

Fig. 5. Effect of EE on DNA binding activity of various transcription factors. Hepatic nuclear extracts were prepared from sham-controls and EE treated rats ($n=4$) 24 and 72 h after either treatment. Nuclear extracts (5–10 μg protein) were incubated with radiolabeled oligonucleotides, electrophoresed through a 6% nondenaturing polyacrylamide gel (EMSA) and autoradiographed. Representative autoradiographs of hepatic DNA binding proteins from two independent samples are shown. Differences in band intensity between control samples isolated at day 1 or day 3 are explained by separate gel analysis and exposure of the X-ray film. SC: specific competition; NSC: nonspecific competition.

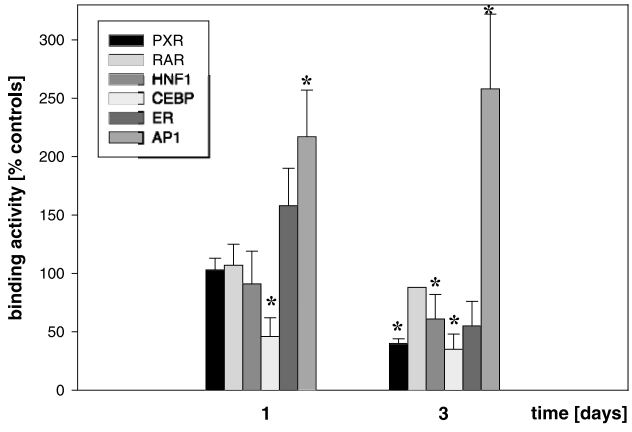

Fig. 6. Densitometric analysis of DNA binding activity. Autoradiographs obtained after EMSA were quantified by phosphoimaging and data were expressed as percentage of sham-controls ($n=4$). Data are given as mean ± S.D. *$P<0.05$ as compared to sham-controls.

4. Discussion

Intrahepatic cholestasis can be induced by ethinylestradiol (EE) with a reduction in overall bile flow to about 60% and an increase of bile acids in serum but not within hepatocytes [5,3,38]. Functional studies show both diminished sinusoidal taurocholate (Tc)-uptake as well as canalicular Tc-secretion within 5 days of EE application [5]. Impaired Tc-uptake is primarily caused by diminished sodium-dependent Tc-transport, whereas sodium-independent Tc-uptake is almost unaffected [3]. In parallel to these effects caused by exogenous estrogens, female gender and pregnancy are also associated with reduced Tc-uptake in hepatocytes [39–41]. Previous studies revealed that changes in transport activity are associated with a decrease in expression of Ntcp and Oatp1 at the basolateral plasma membrane and Mrp2 at the canalicular membrane, whereas Bsep is relatively well preserved [3,6,11].

In the present study, we have extended our knowledge of transporter expression to Oatp2 and Oatp4 as two recently identified members of the Oatp family to determine why sodium-independent transport remains unchanged in EE cholestasis despite down-regulation of Oatp1 expression [3]. In addition, we have analyzed various transcription factors in this model of cholestasis to delineate the molecular mechanisms by which down-regulation of organic anion transporters might occur.

In regard to mRNA expression, Oatp2 showed a similar pattern as Ntcp with a rapid down-regulation within 12 h after the first EE injection with similar levels even at later time points (Figs. 1 and 2). In agreement with previous data, Oatp1 mRNA also declined, but at a less pronounced rate, probably due to strain-specific differences [3]. At the protein level Oatp2 declined at a much slower rate than Ntcp ($33 \pm 2\%$) with a final reduction in protein levels to $63 \pm 7\%$ of controls (Figs. 3 and 4). Similar to Oatp1 and Oatp2, Oatp4 protein declined to the same extent although its mRNA was largely unaffected in contrast to the other basolateral organic anion transporters (Figs. 1–4). These data suggest that Oatp4 is mainly regulated by posttranscriptional mechanisms as previously shown in a model of toxic liver injury [33]. The modest changes of all Oatps by approximately 30% to 40% reduction in protein expression may explain the unaffected overall sodium-independent Tc-uptake due to their partially overlapping substrate specificity.

Down-regulation of Ntcp protein and mRNA levels after EE treatment is in accordance with previous studies in EE-associated cholestasis [3] and in female rats compared to male controls [39]. In the latter study, reduced mRNA expression occurs by diminished transcriptional activity of the *Ntcp* gene under the influence of estrogen as shown by nuclear run off assays [39]. In regard to basolateral organic anion transporters, gene regulation has only been further elucidated for *Ntcp* after cloning its promoter and identification of transactivators driving its basal expression [31]. In endotoxin-induced cholestasis and toxic liver injury, reduced gene transcription was due to diminished binding activity of Ntcp transactivators such as HNF1, C/EBP and a heterodimer of RXR and RAR [33,34]. In particular, decreased binding activity of HNF1α and C/EBPα has been crucial in both toxic hepatitis and endotoxin-induced cholestasis, whereas reduced RXRα:RARα DNA interaction was confined to endotoxin-induced cholestasis [34,33].

In estrogen-induced cholestasis, nuclear binding activity of the *Ntcp* transactivator RXR:RAR remained unchanged, whereas both HNF1 and C/EBP were significantly reduced after 1 and 3 days of treatment (Figs. 5 and 6). Transcriptional regulation of target genes by estrogens occurs by binding of the ligand to the ER, which is located in the cytoplasm and translocated to the nucleus [42]. In the nucleus the activated ER mediates gene regulation either directly by binding to estrogen response elements (ERE) in target genes (“classical pathway”) or indirectly by activation or suppression of gene transcription at alternative DNA elements (“nonclassical” pathway) [37]. DNA sequences for binding of the transcription factor AP-1 represent such alternative binding sites, by which ER may affect transcriptional activity after protein–protein interaction with AP-1 [43,36]. To our knowledge, the mechanisms by which estrogens finally affect *Ntcp* gene transcription are presently unknown. Sequence analysis revealed a potential ERE in the *Ntcp* gene at nt $-184/-165$, which, however, did not show any changes in binding activity when assayed by EMSA (Fig. 5). In addition, multiple potential AP-1 sites are present within the *Ntcp* gene, even within the minimal promoter region [34]. Since a direct binding of ERE on the Ntcp promoter sequence could not be demonstrated by EMSA, we determined AP-1 binding activity after EE injection, which was increased at both 1 and 3 days after treatment (Figs. 5 and 6). However, direct interaction of ERE and AP-1 has been difficult to demonstrate since these interactions are not strong enough to be detected by a supershift of AP-1 bound to ER by EMSA or coimmunoprecipitation studies [43]. Alternatively to an indirect regulation of *Ntcp* by EE via AP-1/ERE, EE may affect expression of Ntcp by modulation of its transactivators such as HNF1 and C/EBPα. *HNF1* contains putative ERE binding sites in its promoter region, whereas *C/EBP*α does not exhibit these consensus sequences in its 5′ flanking region by TRANSFAC analysis. Which mechanisms finally regulate *Ntcp* gene *expression* in EE cholestasis needs to be determined in more details in future studies.

Transcriptional regulation of Oatps has not been studied in great details due to the lack of cloning its various genes except for the very recent characterization of the *Oatp2* 5′ flanking region [44]. Studies in PXR $-/-$ mice suggested that the PXR, which acts as a physiological sensor for toxic bile acids including lithocholic acid, controls the inverse transcriptional regulation of cholesterol 7α-hydroxylase (*Cyp7a1*) as the key enzyme of bile acid synthesis and *Oatp2* [45]. These in vivo data are confirmed by the

identification of several consensus sequences of PXR in the *Oatp2* gene promoter, which are transactivated by PXR ligands in cotransfection studies [44]. EE has also been shown to act as a PXR ligand in addition to several other natural steroids and moderately activates PXR-mediated reporter gene expression [46]. Thus, PXR binding activity was assessed in nuclear extracts from EE-treated animals (Fig. 5). In addition, the *Oatp2* 5′ flanking region contains several putative binding sites for HNF1 [44]. However, the discrepancy between rapid down-regulation of Oatp2 mRNA (Figs. 1 and 2) and sustained binding activity of both PXR and HNF1 (Figs. 5 and 6) 24 h after EE injection suggests that other factors are even more important in regard to Oatp2 gene expression at this early time point of EE-induced cholestasis than HNF1 and PXR. Later, decreased binding activity of both HNF1 and PXR may contribute to sustained down-regulation of *Oatp2* gene expression (Figs. 5 and 6).

In summary, estrogen-induced cholestasis results in moderate down-regulation of all known basolateral organic anion transporting polypeptides including Oatp1, Oatp2 and Oatp4 in addition to the more pronounced decline in Ntcp expression. The largely maintained expression of the Oatps may provide an explanation at the molecular level for the unaltered sodium-independent transport of bile acids in this model of intrahepatic cholestasis. In case of *Ntcp*, decreased gene expression seems to be mediated by decreased nuclear binding activity of transactivators such as HNF1 and C/EBP. In regard to Oatp2, PXR and HNF1 may play a role in sustained down-regulation of gene expression during the later period of EE-induced cholestasis.

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