Characterization of the mechanisms involved in the gender differences in hepatic taurocholate uptake

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Characterization of the mechanisms involved in the gender differences in hepatic taurocholate uptake. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G556–G565, 1999.—Gender differences in the hepatic transport of organic anions is well established. Although uptake of many organic anions is greater in females, sodium-dependent taurocholate uptake is greater in hepatocytes from male rats. We examined the hypothesis that endogenous estrogens alter the number of sinusoidal bile acid transporters and/or decrease membrane lipid fluidity. The initial sodium-dependent uptake of [3H]taurocholate was 75% greater in hepatocytes from males than from either intact or oophorectomized females rats. Taurocholate maximal uptake was increased twofold (P < 0.03) without a significant change in the Michaelis-Menton constant. Sinusoidal membrane fractions were isolated from male and female rat livers with equal specific activities and enrichments of Na+K+−ATPase. Males had a significant (P < 0.05) increase in cholesterol esters and phosphatidylethanolamine-to-phosphatidylcholine ratio. Fluorescence polarization indicated decreased lipid fluidity in females. In females, expression of the sodium-dependent taurocholate peptide (Ntcp) and mRNA were selectively decreased to 46 ± 9 and 54 ± 4% (P < 0.01), respectively, and the organic anion transporter peptide (Oatp) and Na+K+−ATPase α-subunit were not significantly different. Nuclear run-on analysis indicated a 47% (P < 0.05) decrease in Ntcp transcription, without a significant change in Oatp. In conclusion, these studies demonstrated that decreased sodium-dependent bile salt uptake in female hepatocytes was due to decreased membrane lipid fluidity and a selective decrease in Ntcp.

THE HEPATOCYTE IS PRIMARILY responsible for transport, metabolism, and excretion of many organic anions, including bile acids, bilirubin, fatty acids, and organic dyes. Sexual differences have been described for hepatic transport as well as for enzymatic activities (22), drug detoxification (13), and lipid metabolism (47, 53). Both exogenous and endogenous compounds including indocyanine green (31), bromosulfophthalein (BSP) (39, 40, 48), bilirubin (38), and fatty acids (27, 30, 49) have been reported to be transported to a greater extent by female hepatocytes. In contrast, the initial uptake of taurocholate was more rapid in males than in females (10, 11).

Bile acids undergo an enterohepatic circulation involving secondary active transport processes at the ileal brush border and the sinusoidal domain of hepatocytes (reviewed in Ref. 50). Bile acids are synthesized exclusively in the liver from cholesterol, secreted into bile where they are the major osmotic driving force for bile flow, and subsequently participate in the digestion and absorption of various lipophilic dietary constituents. In the ileum, bile acids are efficiently reabsorbed, transported in the portal circulation to the liver, and very efficiently cleared by hepatocytes (15).

The hepatic uptake of bile acids has been extensively examined (34). Taurocholate, the major bile acid, has been shown to obey Michaelis-Menton kinetics, to require energy, and to be primarily sodium dependent. Recently, two sinusoidal membrane bile acid transporters have been cloned and their functions characterized in heterologous systems (33). The results were consistent with the view that the majority of taurocholate uptake is mediated by the sodium-dependent taurocholate transporter peptide (Ntcp) and to a lesser extent by the organic anion transporter peptide (Oatp) (23).

The hepatic uptake of taurocholate in vivo is regulated by a number of factors, including development (24), diet (16), bile acids (26), hormones (18, 44), and gender (10). Although some of these factors have been shown to regulate Ntcp at the pretranslational level, the mechanism(s) involved in the gender differences is unclear. It has been suggested that differences in liver plasma membrane lipid fluidity and electrogenic driving forces may be involved (4, 49, 54). However, sexual dimorphic differences in cytochrome P-450, enzymatic activities, and receptors have generally been demonstrated to be related to pretranslational regulation of protein content (55). In addition, because estrogens have been shown to decrease the expression of Ntcp and Oatp (44), we hypothesized that the decreased taurocholate uptake was due to transcriptional differences in the expression of the sinusoidal bile acid transporters, Ntcp and/or Oatp. Therefore, the present studies were undertaken to examine whether the sexually dimorphic hepatic uptake of taurocholate was due to differences in membrane lipids, sodium driving forces mediated by Na+K+−ATPase, or, rather, the level of bile acid transporters. The results demonstrated that Ntcp but not Oatp protein content was significantly greater in males and expression of Ntcp was transcriptionally controlled.
MATERIALS AND METHODS

Experimental animals. Male and female Sprague-Dawley rats weighing 180–200 g were purchased from Harlan (Indianapolis, IN) and were allowed to acclimatize in our animal facility for at least 5 days before initiation of experiments. The temperature of the animal room was maintained at 22°C. Fluorescent lighting in the room was controlled by an automatic timer so that animals were exposed to 12 h of light and darkness. Rats were fed Purina rat chow and allowed free access to food and water. Female rats were either gonadectomized or sham operated at 200 g by the breeder. Studies were performed 5–10 days after surgery.

Hepatocyte isolation and taurocholate transport. Hepatocytes were harvested from nonfasted rats under pentobarbital sodium anesthesia by the collagenase perfusion method (7). Viability was assessed by trypsin blue exclusion. Hepatocytes were suspended in 3 ml of DMEM–Ham’s F-12 medium (1:1) containing 8% FCS on 60-mm petri dishes and placed in a 37°C, 5% CO2 incubator. Medium was aspirated after 3 h of incubation, and attached cells were washed twice in either sodium-containing or choline substituted for sodium Hanks’ balanced salt solution (HBSS). After cells were prewarmed for 5 min, buffer was aspirated and 1.5 ml of prewarmed buffer with 100 µM taurocholate (specific activity 3.47 Ci/mmol at 6.65 × 105 dpm/plate) were added for specified times in a 37°C water bath equipped with a shaking platform. Uptake was performed in duplicate at 15, 30, 45, and 70 s. Buffer was aspirated, and cells were washed rapidly four times with HBSS-HEPES at 4°C, scraped, and dissolved in 0.5 N NaOH. Taurocholate uptake was calculated from the slope of the uptake time course. Sodium-dependent taurocholate uptake was determined from the difference measured between sodium-containing and sodium-free buffers. Results are expressed as picomoles of taurocholate per microgram of DNA per minute. DNA was measured by the method of Burton (12). Kinetic parameters of [3H]taurocholate uptake were determined as previously described (6) using concentrations of 1–100 µM taurocholate. Duplicate measurements of uptake were measured at 60 s for each concentration. Radioactivity was counted in a liquid scintillation counter with 10 ml of Econo-Scint scintillation fluid from Research Products (Mt. Prospect, IL).

Isolation of sinusoidal fractions and enzyme measurements. After being fasted overnight, rats were killed under ether anesthesia, and their livers were removed rapidly to isolate sinusoidal membrane fractions as previously described (41). Briefly, 25 mM MgCl2 was added to homogenized liver tissue in 15 ml of buffer containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.5) to give a final concentration of 15 mM MgCl2. After 10 min on ice, the sample was centrifuged at 2,400 g for 15 min. The pellet was resuspended in the same buffer, and sinusoidal membrane fractions were separated through a discontinuous sucrose gradient, floating at 37.5% sucrose after centrifugation at 88,000 g for 2.5 h. Fractions used for immunoblotting were stored in the original homogenization buffer with the following additions (all in mM): 0.5 ZnCl2, 1 sodium orthovanadate, 100 sodium fluoride, and 10 potassium phosphate monobasic. These substances were also added to the 100 mM sodium carbonate (pH 11.0) extraction buffer used to remove nonintegral proteins before Western blotting analysis as described by Bergwerk et al. (5).

Na+-K+-ATPase activity was measured after overnight freezing at −20°C by an enzyme-coupled kinetic assay with pyruvate kinase and lactate dehydrogenase, assuming that ouabain (2.5 mM) inhibition measured the Na+-K+-ATPase activity (41). Cytochrome c reductase, succinate dehydrogenase, and leucine aminopeptidase activities were determined as previously described (41). Activities were reported as micro moles per milligram of protein per hour. Protein was measured with BSA (Sigma) as standard by the method of Lowry et al. (29).

Lipid composition and fluidity measurement. Total lipids were extracted from sinusoidal membrane fractions by the method of Bligh and Dyer (8). Free and esterified cholesterol was quantitated by chromatographic methods after derivatization, as previously described (14). An automatic integrator analyzed gas-liquid chromatogram peak areas after identification of individual peaks by co-tailing with standard compounds. Total phospholipids were quantitated by the method of Ames and Dubin (1), and individual species were measured after separation by two-dimensional TLC as previously described (17).

Fluorescence polarization and lifetime measurements were done on a 4800 phase-correlation nanosecond polarization spectrofluorimeter (SLM Industries, Urbana, IL) with fixed emission and excitation polarization filters. The fluorescence intensity was measured perpendicularly and parallel to the polarization of the exciting light to eliminate incidental scattered light by using 1.5- and 4-mm slits for excitation and emission filters, respectively. All samples were run at 35°C with slits at 4, 4, and 8. The probes, 1,6-diphenyl-1,3,5-hexatriene (DPH), trimethylammonium-DPH (TMA-DPH), and DL-12-(9-anthroyloxy)stearic acid (12-AS) (Molecular Probes, Juntion City, OR), were dissolved in tetrahydrofuran to a final concentration of 0.6 µg/ml. Probes were added to intact membranes in a total volume of 1.2 ml (containing ~72 µg of protein) and frequently vortexed. Fluorescence lifetimes and dynamic depolarization measurements were determined at a frequency of 30 MHz with slits from lamp to sample of 16, 0.5, and 0.5 nm by using DPH in hexadecane (9.62 ns) as a lifetime reference solution. Both phase and modulation lifetimes were measured. The maximal limiting anisotropy taken for DPH and TMA-DPH was 0.365 and 0.285 for 12-AS. Fluorescence polarization measurements were also carried out on multilamellar vesicles prepared from total lipid and polar lipid fractions. The lipid extracts were dried under a stream of nitrogen, fluorescent probes were added, and vesicles were formed in PBS by vortexing and sonication, as described previously (41). The amount of total lipid extract or polar lipid fraction used was calculated to be equivalent to the amount contained in 72 µg of initial membrane protein. Measurements were performed in triplicate, and results were analyzed as described previously (41).

PAGE and immunoblotting. SDS-PAGE and immunoblotting were carried out using minigels for enhanced chemiluminescence (ECL) or standard size gels for Ntcp using alkaline phosphatase. Total liver proteins were prepared by Na2CO3 extractions of liver homogenates and resuspended in PBS, pH 7.5, containing inhibitors for protease (2 µg/ml each of antipain, pepstatin, and chymotrypsin, 5 µg/ml each of leupeptin and aprotinin, 10 µg/ml trypsin inhibitor, and 2 mM PMSF) and phosphatase (10 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5 mM zinc chloride, and 10 mM KH2PO4) as described by Bergwerk et al. (5). Immunoblotting was also performed on proteins from sinusoidal membrane fractions isolated in protease and phosphatase inhibitors and suspended in 1 mM NaHCO3 before storage at −80°C. After electrophoresis, proteins were transferred [using the procedure of Towbin et al. (51)] at 167 V for 1 h using a high-transfer apparatus by Ideal. Gels were blocked for 1 h using 5% Tween 20-Tris buffered saline (TBS). Blots were processed for ECL (Amersham) detection of specific antibodies using
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1% milk in TBS for antibody diluent. For Ntcp, blots were visualized by 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt-nitro blue tetrazolium substrate system (Kirkgaard & Perry Laboratories). All washes were done with 0.5% Tween 20-TBS for 5 min (3 times). ECL blots were placed in plastic film and exposed to Amersham Hyperfilm for ECL for 30–60 s. Autoradiograms were quantitated by densitometry using a Bio-Rad laser densitometer. Immunoblots were probed with monospecific antibodies to Ntcp, Oatp, and Na\(^{+}-K^{+}\)-ATPase \(\alpha\)-subunit (Upstate Biotechnology (UBI)). The charac-
teristics of Oatp and \(\alpha\)-subunit (UBI) antibodies have been previ-
ously described (5, 46).

Polyclonal antibodies to the rat Ntcp fusion peptide (span-
ing 421–1186 bp of the rat cDNA) were prepared in rabbits in our laboratory. Specificity of the antibody to Ntcp was determined using the following criteria: 1) gel electrophoresis revealed a doublet at 51 and 56 kDa as previously shown (44), 2) immunolocalization occurred to the sinusoidal membrane fraction, 3) ethyleneglycol treatment resulted in decreased density (44), 4) antigen adsorption resulted in loss of activity, and 5) molecular mass was reduced to 32 kDa after treatment with glycosidases (44).

RNA isolation, analysis, and transcriptional elongation assay. Total RNA was extracted from whole liver using a RNeasy mini kit (Qiagen). The RNA was fractionated in 1.2% agarose-formaldehyde gels in borate buffer at 140 V for 4 h. RNA was transferred to Hybond-N+ (Amersham) with high-
efficiency transfer solution (Tel-Test) by capillary action and fixed by ultraviolet cross-linking. cDNA probes were labeled with \([\text{32P}]\)dCTP (Amersham) using the Decaprime II (Ambion) random-primed labeling system. Unincorporated label was removed with Probequant G-50 microcolumns (Phar-
macia). Membranes were hybridized using a high-efficiency hybridization system (Tel-Test) for 16 h at 62°C. Membranes were washed twice in 2\(\times\) SSC-0.1% SDS and then twice in 0.1\(\times\) SSC-0.1% SDS (all at 55°C) for 20 min with each wash. Membranes were exposed to Hyperfilm MP with intensifying screen at –70°C for 30 min to 3 days. Autoradiograms were quantitated with an imaging densitometer (Bio-Rad). The following probes were used: Ntcp (provided by B. Hagenbuch), Oatp, Na\(^{+}-K^{+}\)-ATPase \(\alpha\)-subunit (provided by J. Lingrel), and 18S rRNA (Ambion). Relative density of mRNA was normalized to 18S RNA and is expressed as a percentage of the male control.

Transcriptional activity was determined using the run-on assay. RNA transcripts that have already been initiated are fully elongated when using nuclei isolated as described by Gorski et al. (20) from fresh livers of male and female rats. Livers from male and female rats were homogenized in an ice-cold homogenization buffer (10 mM HEPES, pH 7.6, 15 mM KCl, 0.15 mM DTT, and 0.5 mM PMSF) using a Dounce homogenizer. Nuclei (1 \(\times\) 10\(^8\)) were separated by sucrose gradient centrifugation, resuspended in 100 µl of glycerol storage buffer (50 mM Tris, pH 8.0, 40% glycerol, 5 mM MgCl\(_2\), and 0.1 mM EDTA), and used immediately for tran-
scription assays (21, 32). After precipitation by ethanol, purified nuclear RNA was resuspended, hybridized with cDNAs to Ntcp and \(\beta\)-actin, and washed at 37°C successively in 2\(\times\) SSC-0.1% SDS, 2\(\times\) SSC-10 µg RNase A/ml, and 0.1\(\times\) SSC-0.1% SDS and exposed to film with screens at –70°C. Density of the slot blots was determined by densitometry and expressed relative to \(\beta\)-actin.

Data analysis. Taurocholate uptake was calculated by the least-squares regression program, and the kinetic param-
eters were determined by the GraphPad Instat program.

One-way ANOVA and a two-tailed t-test were used to deter-
mine statistical significance. P values < 0.05 were considered significant. Results are expressed as means ± SE.

RESULTS

Taurocholate transport. The uptake of \([\text{3H}]\)taurocho-
late into hepatocytes isolated from male and female rats was determined. Initial uptake of taurocholate was linear for at least 70 s in both sexes (data not shown). The results were compared with oophorectomized (ovx) rats to determine whether endogenous estrogens inhibited taurocholate uptake. Table 1 shows the viability of hepatocyte preparations and the initial uptake rate of sodium-dependent and -independent taurocholate. Vi-
ability of isolated rat hepatocytes was similar in all three groups. The initial sodium-dependent uptake of \([\text{3H}]\)taurocholate in males compared with females was greater by 71%, as previously reported by Brock and Vore (10). Furthermore, in hepatocytes from ovx female rats, \([\text{3H}]\)taurocholate uptake was not significantly different from intact females, suggesting that the decreased transport was not related to a direct effect of endogenous estrogens on the taurocholate transport process. On the other hand, sodium-independent \([\text{3H}]\)taurocholate, which represented 13% of the total in male hepatocytes, was not significantly different in the three groups.

Additional experiments were carried out to deter-
mine the kinetic parameters, the Michaelis-Menten constant (\(K_m\)) and maximal uptake (\(V_{max}\)), for tauro-
cholate uptake into hepatocytes isolated from male and ovx female rats. The initial uptake of sodium-depen-
dent taurocholate uptake is shown over a range of concentrations (Fig. 1). At every concentration, tauro-
cholate uptake was greater in male hepatocytes. Both \(K_m\) and \(V_{max}\) values in male hepatocytes were similar to values previously reported from our laboratory (6). Hepatocytes from ovx females demonstrated significantly (P < 0.01) reduced \(V_{max}\) for taurocholate to 54% without a significant change in \(K_m\). The results are consistent with reduced expression of sodium-dependen-
t sinusoidal bile acid transporters. However, re-
duced \(V_{max}\) may also reflect gender differences in either membrane lipid composition and physical structure or Na\(^{+}-K^{+}\)-ATPase activity, which is partially respon-
sible for generation of the sodium driving forces. There-

Table 1. Initial uptake of \([\text{3H}]\)taurocholate

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>(V_{max}) (\pm SE) pmol·µg DNA (^{-1})·min(^{-1})</th>
<th>(K_m) (\pm SE) µM</th>
<th>Viability, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>3</td>
<td>2.4 ± 0.1</td>
<td>0.32 ± 0.01</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>Female</td>
<td>3</td>
<td>1.4 ± 0.1*</td>
<td>0.26 ± 0.01</td>
<td>93 ± 4</td>
</tr>
<tr>
<td>Oophorectomy</td>
<td>3</td>
<td>1.3 ± 0.1†</td>
<td>0.31 ± 0.02</td>
<td>95 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\) = no. of separate experiments. Hepato-
cytes were isolated, and initial transport rates were determined as described in MATERIALS AND METHODS. Significant changes were determined by ANOVA. *P < 0.05 compared with males. †No significant difference compared with females.
METHODS

Relationship between initial uptake velocity and taurocholate concentration, and fluidity were determined.

Sinusoidal membrane fractions were isolated from male (♂) and oophorectomized (ovx) female (♀) rats and plated for 3 h, and initial uptake of $[^3]$Htaurocholate was measured at 60 s in the presence and absence of sodium, as described in MATERIALS AND METHODS. Relationship between initial uptake velocity and taurocholate concentration fit the Michaelis-Menten equation in both male and female experiments. These data were used to estimate the maximal uptake ($V_{max}$) and the Michaelis-Menten constant ($K_m$) values using GraphPad. Results are means ± SE of 3 independent experiments in each group.

Therefore, these possibilities were examined in the next series of experiments.

Sinusoidal membrane lipid composition and fluidity. Previous studies have suggested that the physical state of sinusoidal membrane lipids contributes a small but significant role in the regulation of taurocholate transport (44). Although fluidity measurements of male hepatocyte plasma membranes were reported to be less fluid than those in females (4), these changes were demonstrated only in 12-wk-old rat hepatocytes using the TMA-DPH probe. Because hepatocyte plasma membrane domains showed marked polarity of their lipid composition and fluidity (43), we reexamined whether gender differences are present in lipid composition and fluidity of sinusoidal liver plasma membrane fractions. Sinusoidal membrane fractions were isolated from male and female livers by differential sucrose density gradient centrifugation, and enzyme activities, lipid composition, and fluidity were determined.

Comparison of membrane enzyme-specific activities and enrichments between male and female is shown in Table 2. Na$^+$-K$^+$-ATPase, a marker of the sinusoidal domain, was markedly enriched to 59- and 53-fold in male and female sinusoidal membrane fractions, respectively. Also, immunoblots of the Na$^+$-K$^+$-ATPase α-subunit showed similar densities between the sexes (Fig. 2). Enrichment of leucine aminopeptidase, a marker of the bile canalicular domain, was lower in female sinusoidal membranes, whereas succinic dehydrogenase and cytochrome c reductase activities, markers of the mitochondria and endoplasmic reticulum, respectively, were similarly enriched or deenriched. Thus neither Na$^+$-K$^+$-ATPase activity nor the isolation of membrane fractions was significantly affected by gender.

Lipid analysis of sinusoidal fractions is shown in Table 3; similar values to our previous reports using sinusoidal fractions from male rat liver (41) were demonstrated. In particular, the free cholesterol and total phospholipid contents of sinusoidal fractions from females were not significantly different. In contrast, the cholesterol ester content in female sinusoidal membrane fractions was significantly lower. The free cholesterol-to-total phospholipid molar ratio, a major determinant of membrane fluidity, was not significantly different. These results suggested that differences in lipid composition by themselves did not account for decreased taurocholate transport in females.

Sinusoidal membrane lipid fluidity using both steady-state and dynamic depolarization measurements was determined. Table 4 reports the physical parameters of sinusoidal membrane lipids from male and female rats measured with three different fluorescence probes. Steady-state fluorescence polarization measurements with the DPH probe, which senses the interior of the lipid bilayer, demonstrated significantly greater polarization values in females compared with those in males. On the other hand, TMA-DPH, which reports on the fluid state and dynamic depolarization measurements was determined. Table 4 reports the physical parameters of sinusoidal membrane lipids from male and female rats measured with three different fluorescence probes. Steady-state fluorescence polarization measurements with the DPH probe, which senses the interior of the lipid bilayer, demonstrated significantly greater polarization values in females compared with those in males.

We next determined the structural (order) and dynamic (rotational rate) components directly using time-resolved fluorescence polarization techniques. Table 4 shows the value for the mean fluorescence lifetime, which was similar in membrane fractions from male and female sinusoidal membrane fractions.
and female rats. This indicated that alterations in lipid physical properties did not represent quenching of the DPH probe. The dynamic depolarization results demonstrated that structural and kinetic parameters were significantly different in female sinusoidal membrane fractions compared with those in males. The differences in both order parameter and rotational rate demonstrated that the fluidity of female sinusoidal membrane fractions was less than that of males. Furthermore, differences in bile canalicular membrane fluidity measurements were not significantly different (data not shown), suggesting that gender differences in lipid structure were specific to the sinusoidal fraction.

To identify the lipid fraction responsible for the differences in DPH and 12-AS fluorescence polarization measurements, sinusoidal membrane fractions were extracted first to remove proteins and subsequently to separate cholesterol from phospholipids. The results are shown in Table 5. Removal of proteins and disruption of the lipid structure still resulted in increased fluidity measurements with DPH and 12-AS in female sinusoidal fractions. Similar gender differences in fluorescence polarization values were also measured in phospholipid vesicles, which are devoid of cholesterol. The results indicated that the gender differences in sinusoidal membrane fluidity were due to changes in phospholipid and/or fatty acid composition rather than in protein content or lipid structural organization.

Sinusoidal membrane phospholipids were extracted and separated by two-dimensional TLC to quantitate individual species. The gender differences in phospholipid species are shown in Table 6. The results demonstrated that, although no significant species differences were measured between male and females phospholipids, the phosphatidylethanolamine-to-phosphatidylcholine ratio was significantly (P < 0.05) greater in the

Table 3. Lipid composition of sinusoidal membrane fractions from male and female rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Free Cholesterol, nmol/mg protein</th>
<th>Cholesterol Esters, μg/mg protein</th>
<th>Total Phospholipids, nmol/mg protein</th>
<th>Cholesterol-to-Phospholipid Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>413 ± 37</td>
<td>7.1 ± 0.5</td>
<td>665 ± 41</td>
<td>0.62 ± 0.06</td>
</tr>
<tr>
<td>Female</td>
<td>379 ± 53</td>
<td>1.5 ± 0.4*</td>
<td>633 ± 22</td>
<td>0.60 ± 0.08</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of separate experiments. Sinusoidal membrane fractions and lipid content were measured as described in MATERIALS AND METHODS. *P < 0.01.

Table 4. Fluorescence polarization measurements in sinusoidal membrane fractions from male and female rats

<table>
<thead>
<tr>
<th>Probe</th>
<th>Male (n = 4)</th>
<th>Female (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH</td>
<td>0.235 ± 0.002</td>
<td>0.254 ± 0.003*</td>
</tr>
<tr>
<td>TMA-DPH</td>
<td>0.332 ± 0.001</td>
<td>0.330 ± 0.003</td>
</tr>
<tr>
<td>12-AS</td>
<td>0.126 ± 0.001</td>
<td>0.162 ± 0.003*</td>
</tr>
<tr>
<td>Lifetime, ns</td>
<td>8.8 ± 0.1</td>
<td>8.6 ± 0.1</td>
</tr>
<tr>
<td>Order</td>
<td>0.149 ± 0.007</td>
<td>0.164 ± 0.003*</td>
</tr>
<tr>
<td>Rotation</td>
<td>0.143 ± 0.004</td>
<td>0.183 ± 0.011*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of separate animals in each group. Fluidity was measured in sinusoidal membrane fractions as described in MATERIALS AND METHODS. DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA, trimethylammonium; 12-AS, DL-12-(9-anthroyl-oxy)stearic acid. *P < 0.01.

Table 5. Differences in fluorescence polarization of male and female sinusoidal membrane fractions after selective lipid extractions

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Lipids</th>
<th>Phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPH</td>
<td>12-AS</td>
</tr>
<tr>
<td>Male</td>
<td>0.196 ± 0.008</td>
<td>0.101 ± 0.002</td>
</tr>
<tr>
<td>Female</td>
<td>0.236 ± 0.002</td>
<td>0.149 ± 0.007</td>
</tr>
<tr>
<td>%Change</td>
<td>20 48</td>
<td>40 60</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of separate experiments. Sinusoidal membrane fractions were isolated by sucrose density centrifugation. Lipid and phospholipid vesicles were prepared as described in MATERIALS AND METHODS.
female sinusoidal membrane fraction. Because the phosphatidylethanolamine-to-phosphatidylcholine ratio is an important determinant of lipid fluidity, these results suggested that gender differences in membrane fluidity were isolated to changes in the phospholipid fraction, and these lipid changes may possibly be related to the decreased capacity of female hepatocytes to transport taurocholate.

Measurement of sinusoidal membrane proteins. Taurocholate is transported at the sinusoidal membrane predominantly by Ntcp and to a lesser extent by Oatp (23). In previous studies, differences in the content of the hepatic sinusoidal taurocholate transport proteins Ntcp and Oatp were more important than lipid fluidity in regulating bile acid transport (44). Therefore, the content of Ntcp and Oatp was measured in liver extractions and in sinusoidal membrane fractions. As shown in Fig. 2, Ntcp protein content was selectively reduced in female liver compared with male liver. Ntcp was present as a doublet in male liver extractions as previously reported for sinusoidal membrane fractions (Fig. 2A) (44). In contrast, only a faint 56-kDa band was demonstrated in liver extractions from female liver. This band represented only 5% of male values. Because the female protein content seemed unphysiologically low, we postulated that this result might have been due to low sensitivity of the antibody (a threshold effect) or selective loss of Ntcp during extraction. Therefore, we prepared sinusoidal membrane fractions and determined the content of Ntcp. In males, density of the higher 56-kDa band was modestly greater than the lower 51-kDa band, but both bands were approximately equally distributed in female sinusoidal fractions (Fig. 2B). However, the distribution was variable from experiment to experiment, so one could not conclude the physiological relevance of this observation. To quantify the gender differences in Ntcp, density of the two bands was combined (Fig. 2C). The protein density of Ntcp in sinusoidal membrane fractions from males was twofold ($P < 0.05$) greater than in those from females.

Table 6. Comparison of phospholipid species in male and female sinusoidal membrane fractions: percent composition

<table>
<thead>
<tr>
<th>Species</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPC</td>
<td>1.6 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>SPH</td>
<td>8.0 ± 1.7</td>
<td>7.4 ± 1.7</td>
</tr>
<tr>
<td>PI</td>
<td>5.3 ± 0.7</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>PC</td>
<td>50.6 ± 0.6</td>
<td>48.3 ± 0.9</td>
</tr>
<tr>
<td>PS</td>
<td>8.1 ± 0.3</td>
<td>8.6 ± 0.4</td>
</tr>
<tr>
<td>PE</td>
<td>26.1 ± 0.2</td>
<td>27.8 ± 1.2</td>
</tr>
<tr>
<td>PG</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>PA</td>
<td>0.16 ± 0.03</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>SPH-to-PC</td>
<td>0.52 ± 0.01</td>
<td>0.58 ± 0.01*</td>
</tr>
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</table>

Values are means ± SE of 4 separate animals in each group. Sinusoidal membrane fractions were isolated, and phospholipids were measured after separation by TLC. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; SPH, sphingomyelin; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PA, phosphatidic acid. *$P < 0.05$.

Oatp, the other major bile acid transporter in liver, was also determined in liver extractions and sinusoidal membrane fractions from male and female livers (Fig. 2). As previously reported, Oatp is present in liver as a single 80-kDa band. In contrast to Ntcp, the protein content of Oatp was not significantly reduced in female liver extractions (80 ± 8%) or sinusoidal membrane fractions (75 ± 7%) compared with those from males (Fig. 2, A and B). Furthermore, no quantitative differences in the density of the Na$^+$-K$^+$-ATPase α-subunit were measured. These results indicated that the dimorphic expression of Ntcp was not due to a generalized change in sinusoidal membrane transporters. Moreover, the decrease in protein density for the principal bile acid transporters was consistent with differences in sodium-dependent taurocholate $V_{max}$.

Analysis of mRNA levels for bile acid transporters. The gender differences in Ntcp might reflect a change in the stability of the protein and/or in the steady-state level of Ntcp mRNA. To distinguish between the two regulatory mechanisms, Northern blot analysis was performed (Fig. 3). Hepatic Ntcp mRNA levels from female rats were 54 ± 4% ($P < 0.01$) of the value obtained in males. On the other hand, Oatp (84 ± 10%) and Na$^+$-K$^+$-ATPase α-subunit mRNA (101 ± 3%) levels were similar in male and female livers. These results suggested a selective pretranslational control of Ntcp gene expression.

The increase in male Ntcp expression might be a consequence of a modification of gene transcription and/or of mRNA stability. To address this question, transcription run-on assays were performed on nuclei isolated from male and female livers. Because β-actin mRNA was unchanged compared with ribosomal 18S, it was used as a control. The results of nuclear run-on studies comparing transcription rates for Ntcp, Oatp, and CYP2C12 are shown in Fig. 4. Ntcp mRNA transcription rate was 47% higher ($P < 0.05$) in males compared with females. In contrast, Oatp transcription rate did not show a gender difference, whereas the female-dominant gene, CYP2C12, was significantly increased. These results supported the hypothesis that the gender difference in Ntcp expression was transcriptionally regulated. Also, decreased Ntcp transcription in female liver was specific and did not reflect a generalized decrease in transcription, since CYP2C12 was increased.

**DISCUSSION**

Gender differences in the hepatic transport of organic anions including taurocholate (10), bilirubin (38), BSP (39), indocyanine green (31), fatty acids (49), and some steroid hormones (10) have been described. Only taurocholate has been shown to have greater transport capacity in males. The mechanism(s) responsible for increased taurocholate transport is unclear but may involve gender differences in cell size, driving forces, or membrane lipid fluidity, as well as in the density of bile acid carriers. The physiological regulation of bile acid transport is well understood. However, few studies have addressed the molecular mechanisms involved in
regulation of either Ntcp or Oatp. The recent cloning of sinusoidal bile acid transporters and the development of specific antibodies have provided the tools to dissect these mechanisms (37). We have combined molecular, biochemical, and physical approaches to examine the mechanisms involved in the gender differences in hepatic taurocholate uptake. The results demonstrated that the lipid physical properties of sinusoidal membranes from female rats were less fluid than those from males. However, more importantly, males selectively expressed greater amounts of Ntcp, the major hepatic bile acid transporter. Sex differences in Ntcp mRNA were due to transcriptional regulation of the steady-state mRNA levels.

A previous study reported that initial uptake of [3H]taurocholate in isolated hepatocytes from male, female, and female ovx rats. The initial uptake of sodium-dependent taurocholate was significantly (P < 0.01) lower in intact (42%) and ovx (46%) female hepatocytes, confirming and extending the studies of Brock and Vore (10). Moreover, because taurocholate transport in hepatocytes from ovx females was similar to that in intact females, endogenous estrogens did not contribute to the gender differences. The kinetic parameters in [3H]taurocholate transport into isolated hepatocytes indicated a significant (P < 0.03) reduction in Vmax (54%) in ovx female hepatocytes without a significant difference in Km. This suggested that differences in the number of taurocholate transporters may account for sexual dimorphic taurocholate transport.

It has been postulated that changes in organic anion uptake, for example BSP and fatty acids, might be due to differences in membrane lipid fluidity, since Vmax was unchanged but Km was greater in males (40, 49). Membrane lipid fluidity is established to be important in the regulation of liver transport processes (43).
number of studies have demonstrated gender and hormonal differences in hepatic lipid metabolism (47, 53), but only two preliminary studies have examined possible sex differences in hepatic membrane fluidity (3, 4). Because taurocholate transport has been shown, in part, to be regulated by membrane lipid fluidity (35), we determined the lipid composition and fluidity parameters in female sinusoidal membrane fractions.

Fluidity measures the bulk physical properties of membrane lipids, which are important determinants of transport properties (43). Benedetti et al. (4) reported (using TMA-DPH in isolated hepatocytes) that there is an age-related increase in female fluidity values. On the other hand, Bellentani et al. (3) (using DPH as a probe) failed to demonstrate gender differences in fluidity using liver plasma membrane vesicles. To study this question, we used both steady-state and dynamic depolarization techniques to measure membrane lipid fluidity components. Steady-state determinations were measured with three different probes to analyze the different components and domains of membrane lipid properties. DPH and TMA-DPH polarization values largely determined static components of membrane lipids in the interior and outer leaflet, respectively, whereas the 12-AS probe reflects the dynamic parameters of lipid fluidity (43). Although TMA-DPH was not significantly different, both DPH and 12-AS measurements indicated that female sinusoidal membranes were less fluid than those isolated from male rat livers. These differences were confirmed using dynamic fluorescence depolarization, in which the structural as well as the dynamic components of membrane fluidity were decreased in female sinusoidal membrane fractions. These results differed from those previously reported, which may be due, in part, to the use of multiple probes, the increased sensitivity of dynamic depolarization measurements, and the use of well-characterized highly purified sinusoidal fractions. Thus the present results suggested that differences in membrane fluidity might account, in part, for the reduced taurocholate transport in female hepatocytes.

In addition to membrane lipid fluidity, it has been suggested that differences in organic anion transport may result from gender differences in cell size or driving forces. However, Sorrentino et al. (49) have shown that male and female hepatocytes are similar in size. On the other hand, Weisiger and Fitz (54) demonstrated a modestly greater potential difference in female hepatocytes. However, this reported difference could only account for ~20% of the difference in taurocholate uptake. In addition, these authors did not explore the biochemical mechanisms, which might account for this difference. Possibilities included activity of the sodium pump as well as membrane potassium currents. Our studies did not uncover gender differences in sinusoidal Na\(^+-\)K\(^+-\)ATPase activity or mRNA content of the α-subunit. Because activities of the sodium pump were similar in both genders, it is suggested that the potential difference reported is related to changes in parameters other than the sodium pump.

Previous studies have demonstrated sexually differentiated functions for sinusoidal organic anion transport; however, no apparent common mechanistic theme has emerged. Taurocholate uptake kinetics demonstrated increased \(V_{\text{max}}\) and unaltered \(K_m\) in males, whereas both fatty acids and BSP-glutathione transport kinetics demonstrated increased \(K_m\) with unaltered \(V_{\text{max}}\) in females. Because decreased taurocholate \(V_{\text{max}}\) suggested, but did not prove, a difference in the expression of Ntcp, we measured the density of the transporter in both total liver and sinusoidal membrane fractions. Ntcp expression was sexually dimorphic, being present in female sinusoidal membranes at only 46% of the level found in males. In contrast, neither Oatp nor the Na\(^+-\)K\(^+-\)ATPase α-subunit was significantly different.

To determine if the difference in Ntcp protein content was due to pre- or posttranslational differences, we first measured the steady-state mRNA levels of Ntcp. The mRNA content of Ntcp in female livers was reduced to 54% of that found in males, consistent with the difference in protein content, and indicated that the protein differences were due to pretranslational processes rather than altered protein turnover. Furthermore, the difference was selective, since neither Oatp nor Na\(^+-\)K\(^+-\)ATPase α-subunit was significantly different. Furthermore, nuclear run-on assays indicated that the gender differences in steady-state mRNA levels resulted from a selective decrease in transcription of Ntcp in livers of females compared with that in males.

It is well established that many hepatic functions are expressed in a sexually dimorphic fashion (36). In particular, the sexual dimorphic expression of cytochrome P-450 enzymes have been thoroughly characterized (22). However, a number of other hepatic enzymes (45), transcription factors (28), and liver plasma membrane receptors (36) have also been identified to be sexually dimorphic. For the most part, these proteins are regulated by the dimorphic secretory pattern of growth hormone rather than the direct effect of sex steroid hormones (42). These gender differences are regulated at the transcriptional level and may be determined by hormonal control of transcription factors such as the signal transducer and activator of transcription (STAT)-5b (52) and hepatic nuclear factor-6 (28). Recently, studies have demonstrated that prolactin-mediated postpartum upregulation of Ntcp is through the Janus kinase (JAK)-STAT pathway of intracellular signaling (19). Growth hormone, similar to prolactin, also utilizes the JAK-STAT signaling pathway to induce hepatic genes containing the STAT or interferon-γ activation site-like DNA binding domains (2). In preliminary studies, we have reported that the intermittent administration (‘‘male-like’’ pattern) of growth hormone to hypophysectomized rats is an important physiological determinant of the dimorphic expression of Ntcp (9).
In conclusion, the present studies have demonstrated that Ntcp was dimorphically expressed in rat liver due to gender differences in transcription. The lower expression of Ntcp in association with decreased sinusoidal fluidity accounts for the decreased taurocholate uptake in female hepatocytes compared with that in males. These studies suggest that sex hormones possibly working through growth hormone significantly contribute to the regulation of sinusoidal transport of taurocholate.

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