RESEARCH COMMUNICATION

Prolactin increases hepatic Na⁺/taurocholate co-transport activity and messenger RNA *post partum*

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We have shown that Na⁺/taurocholate co-transport activity is decreased in pregnancy, but rebounds *post partum* relative to non-pregnant controls, and that activity can be increased by treatment with ovine prolactin [Ganguly, Hyde and Vore (1993) *J. Pharmacol. Exp. Ther.* **267**, 82–87]. To determine the basis for these effects, Na⁺/taurocholate co-transport was determined in purified basolateral liver plasma-membrane (bLPM) vesicles and compared with steady-state mRNA levels encoding the Na⁺/taurocholate-co-transporting polypeptide (Ntcp) in non-pregnant controls, pregnant rats (19–20 days pregnant), rats *post partum* (48 h *post partum*) and rats *post partum* treated with bromocryptine to inhibit prolactin secretion. Na⁺/taurocholate co-transport activity (nmol/5 s per mg of protein) in bLPM was decreased from 10.4 ± 1.8 in non-pregnant controls to 7.9 ± 0.6 in

bLPM in pregnant rats, but rebounded to 17.5 ± 1.3 *post partum*; treatment of rats *post partum* with bromocryptine to inhibit prolactin secretion decreased activity to 14.1 ± 0.9. Northern and slot-blot analyses revealed similar changes in mRNA for Ntcp, so that a positive correlation was observed between Na⁺/taurocholate co-transport activity and Ntcp mRNA. Furthermore, treatment of ovariectomized rats with ovine prolactin increased Ntcp mRNA 10-fold compared with solvent-treated controls, consistent with the 2-fold increase in $V_{\max}$ for Na⁺/taurocholate co-transport in isolated hepatocytes. These data are the first to demonstrate endogenous physiological regulation by prolactin of Ntcp mRNA in parallel with Na⁺/taurocholate co-transport activity.

INTRODUCTION

Secretion of bile is a major function of the liver, and serves as the major excretory route for cholesterol, bilirubin and other endogenous and exogenous compounds. Bile flow is largely associated with the secretion of osmotically active bile salts into the confined space of the canalicular lumen, followed by the passive movement of water and accompanying counter-ions [1]. The first step in the vectorial transport of bile salts from the blood into the bile has been identified as a Na⁺-dependent bile acid co-transporter localized in the basolateral domain of the hepatocyte plasma membrane [2,3]. This electrogenic secondary active symporter is fuelled by the inwardly directed Na⁺ gradient maintained by the primary active Na⁺,K⁺-ATPase pump and facilitated by the intracellular negative membrane potential [4,5]. A functional Na⁺/taurocholate-co-transporting polypeptide (Ntcp) has recently been cloned and shown to possess similar transport characteristics to the Na⁺/taurocholate co-transporter of hepatocytes [6,7]. However, despite these significant advances, little is known about the mechanisms of regulation of this transport protein under physiological conditions. Pregnancy and oestrogens have long been known to inhibit taurocholate transport across the basolateral domain of isolated hepatocytes [8,9], and this has been shown to be due to inhibition of the Na⁺-dependent component of taurocholate transport [10,11]. We have recently demonstrated that Na⁺/taurocholate co-transport in hepatocytes from rats *post partum* is increased to levels greater than in non-pregnant rats; this increased Na⁺/taurocholate co-transport is blocked by inhibition of secretion of the anterior

pituitary hormone prolactin (PRL) and is reproduced by treatment of rats with ovine PRL (oPRL) [11]. The mechanism by which pregnancy, oestrogens and PRL regulate Na⁺/taurocholate co-transport is not known. However, the cloning of Ntcp has now permitted assessment of the effects of these hormones on the expression of Ntcp at the gene level. The present studies demonstrate that Na⁺/taurocholate co-transport activity in basolateral-membrane vesicles is significantly increased *post partum* and that these changes are paralleled by similar changes in the steady-state Ntcp mRNA levels. Furthermore, inhibition of PRL secretion blocks the increase in Ntcp mRNA *post partum*, and treatment with oPRL increases Ntcp mRNA. These data are the first to demonstrate that PRL acts as an endogenous physiological regulator of Na⁺/taurocholate co-transport.

MATERIALS AND METHODS

Materials

[³H]Taurocholate (2.1 Ci/mmol; 98 % purity) and [α -³²P]dCTP (3000 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). [γ -³²P]ATP (6000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). 2-Bromo- α -ergocryptine methanesulphate (bromocryptine) was given by Sandoz Research Institute (East Hanover, NJ, U.S.A.). Unlabelled taurocholate was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). A 26-mer oligonucleotide complementary to the human 28 S rRNA [12] was synthesized at the Oligonucleotide Core Facility at the University of Kentucky. oPRL (NIDDK-oPRL-19; AGFP-9221a) was kindly provided

Abbreviations used: bLPM, basolateral liver plasma membrane; Ntcp, Na⁺/taurocholate-co-transporting polypeptide; PRL, prolactin; oPRL, ovine PRL.

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by the National Hormone and Pituitary Program (University of Maryland, Baltimore, MD, U.S.A.; Ogden Bioservices, Rickville, MD, U.S.A.) of the NIDDK. All other chemicals were of reagent or molecular-biology grade and were purchased from GIBCO-BRL (Gaithersburg, MD, U.S.A.) or Sigma Chemical Co.

Animals

Female Sprague-Dawley rats weighing 200–250 g (Harlan Industries, Indianapolis, IN, U.S.A.) were housed and fed *ad libitum* for at least 7 days before use. Pregnant rats were timed according to the first day on which sperm were detected (day 0). Non-pregnant rats served as controls, and rats at 19–20 days of pregnancy and between 24 and 48 h *post partum* were used as representative of pregnant rats and rats *post partum* respectively. In a fourth group, pregnant rats were implanted subcutaneously with bromocriptine pellets (7.5 mg, 10 day release; Innovative Research of America, Toledo, OH, U.S.A.) at day 17 of pregnancy to inhibit PRL secretion. Bromocriptine-treated rats were used 24–48 h *post partum*. Ovariectomized rats were purchased as such and infused intravenously for 7 days with either solvent or oPRL (300 µg/day) via osmotic minipumps (Alzet, Palo Alto, CA, U.S.A.) as described [13].

Preparation of liver basolateral plasma membrane (bLPM) vesicles

Rats were decapitated between 08:00 and 09:00 h and bLPM was prepared by using discontinuous-sucrose-density-gradient centrifugation adapted for preparation of vesicles from a single rat liver [14]. Membranes were resuspended in suspension buffer consisting of 250 mM sucrose, 0.2 mM CaCl_2 and 20 mM Hepes/Tris (pH 7.5) at a protein concentration of ≥ 7 mg/ml. Na^+ , K^+ -ATPase activities for non-pregnant rats, pregnant rats, rats *post partum* and bromocriptine-treated rats *post partum* were 20.4 ± 4.89 , 20.6 ± 1.4 , 19.0 ± 2.0 and 25.3 ± 4.2 pmol of phosphate /h per mg of protein respectively.

Vesicle transport studies

Frozen vesicle suspensions were thawed quickly at 37 °C, diluted to 4 mg/ml protein in suspension buffer and re-vesiculated by 20 passages through a 25-gauge needle. Uptake of 1 µM [^3H]-taurocholate was determined in bLPM (20 µl; 80 µg of protein) in the presence of a Na^+ or K^+ gradient at 25 °C. The incubation buffer (80 µl) consisted of 62.5 mM sucrose, 0.2 mM CaCl_2 , 6.25 mM MgCl_2 , 20 mM Hepes/Tris, pH 7.5, and either 125 mM NaCl or KCl. The stop buffer consisted of 100 mM sucrose, 100 mM KCl, 0.2 mM CaCl_2 , 5 mM MgCl_2 and 20 mM Hepes/Tris, pH 7.5. [^3H]Taurocholate associated with the membrane was determined by a routine rapid-filtration assay [15]. The filters (0.45 µm-pore HA filters; Millipore Corp., Bedford, MA, U.S.A.) were assayed for radioactivity in 5 ml of Aquasol (New England Nuclear). All determinations were performed in triplicate in at least three different membrane preparations.

Assays

Blood was collected after decapitation, and serum was isolated by centrifugation for measurement of PRL levels by a standard double-antibody r.i.a. as described [13]. Serum PRL values for non-pregnant rats, pregnant rats, rats *post partum* and bromo-

criptine-treated rats *post partum* were 12.1 ± 3.8 , 25.2 ± 5.7 , 224 ± 54 and 10.3 ± 0.9 ng/ml respectively

Northern-blot analysis

Animals were decapitated between 08:00 and 09:00 h and liver samples were frozen immediately in liquid nitrogen. Total RNA was prepared as described [16] and a 20 µg portion was electrophoresed through a 1% agarose/formaldehyde/Mops gel. The RNA was transferred to a Duralon UV membrane (Stratagen, La Jolla, CA, U.S.A.) in $20 \times \text{SSC}$ ($1 \times \text{SSC} = 150$ mM NaCl/15 mM sodium citrate). The membrane was cross-linked by u.v. (Bio-Rad UV GeneLinker) and baked *in vacuo* at 80 °C for 1 h. The 0.92 kb *EcoRI* fragment (nucleotides 261–1187) of the cloned full-length cDNA of the Ntcp [7] was labelled with [α - ^{32}P]dCTP [$(1-10) \times 10^8$ d.p.m./µg of DNA] by random priming using the Prime-a-gene kit (Promega, Madison, WI, U.S.A.). The single-stranded 26-mer oligonucleotide for the 28 S rRNA was end-labelled with [γ - ^{32}P]ATP by polynucleotide kinase [$(1-10) \times 10^8$ d.p.m./µg of DNA] by using the 5' end-labelling kit (Promega). Hybridization and post-hybridization washes for the Ntcp probe were carried out as described [7]. For the 28 S rRNA oligoprobe, hybridization conditions were the same as for Ntcp, whereas post-hybridization washes were carried out as described in [17]. The membranes were exposed to Kodak XAR-2 film with an intensifying screen at -70 °C for 24 h for the Ntcp probe and for 1–2 h for the 28 S rRNA probe. For slot-blot analysis, 10, 2 and 0.5 µg dilutions of the same RNA samples were used to quantify the Ntcp mRNA by using the same hybridization protocols. The relative amounts of Ntcp mRNA and 28 S rRNA were determined by densitometry using the AMBIS Radioanalysis System software (Northern blots) or a CAMAG densitometer attached to a SP 4290 integrator (slot-blots). Values (in arbitrary units of density) for Ntcp mRNA were then normalized against those of the 28 S rRNA.

Data analysis

All values are given as means $\pm$ S.E.M. Differences among mean values were assessed by using ANOVA, followed by the Student–Newman–Kuels test where appropriate. The minimum criterion for statistical significance was $P < 0.05$.

RESULTS

Na^+ -dependent transport of taurocholate in bLPM

As shown in Figure 1, the uptake of taurocholate was stimulated significantly by an inwardly directed Na^+ versus K^+ gradient, and demonstrated a 3–6-fold accumulation above equilibrium values (overshoot) in the presence of a Na^+ gradient (out > in) in all groups. Initial (5 s) Na^+ -dependent uptake values for taurocholate were decreased slightly in vesicles from pregnant compared with non-pregnant rats (Table 1), but were significantly increased in vesicles from rats *post partum*. Inhibition of PRL secretion with the dopamine agonist bromocriptine, which decreased ($P < 0.01$) PRL levels from 224 ± 54 in rats *post partum* to 10.3 ± 0.9 ng/ml in bromocriptine-treated ones, also decreased Na^+ /taurocholate co-transport somewhat (Table 1). There were no significant differences in the equilibrium values (60 min) among the four groups, nor were any differences observed in taurocholate transport in the presence of an inwardly directed K^+ gradient. These data indicate that the size and integrity of the vesicles and any non-specific binding of taurocholate to the membranes did not differ among the four groups.

Figure 1 Uptake of $1 \mu\text{M}$ [^3H]taurocholate measured in the presence of a Na^+ or K^+ gradient in bLPM vesicles from non-pregnant rats (NP), pregnant rats (P), rats *post partum* (PP) and bromocriptine-treated rats *post partum* (BC)

Data are expressed as means ($\pm$ S.E.M.) of triplicate determinations in each of three membrane preparations: a, $P < 0.05$, significantly different from P; b, $P < 0.05$, significantly different from all other groups.

Figure 2 Representative Northern blots of RNA isolated from non-pregnant rats (NP), pregnant rats (P), rats *post partum* (PP) and bromocriptine-treated rats *post partum* (BC)

Lanes 1–3, NP; lanes 4–6, P; lanes 7–9, PP; lanes 10–12, BC. Each lane represents a single liver RNA sample.

Regulation of mRNA encoding Ntcp

In order to determine whether the increase in Na^+ /taurocholate co-transporter activity seen *post partum* might reflect comparable changes in Na^+ /taurocholate co-transporter synthesis, a cDNA probe was used to quantify Ntcp mRNA. Northern-blot analysis (Figure 2) of total liver RNA revealed a single transcript of approx. 1.7 kb. Normalization of the Ntcp mRNA signal intensity with that for the 28 S rRNA to yield relative-density units showed a 5- and 35-fold increase in Ntcp mRNA in rats *post partum* (39 ± 7) relative to non-pregnant rats (6.7 ± 1.2) and pregnant rats (1.1 ± 0.42) respectively. Most importantly, treat-

Table 1 Comparison of Ntcp mRNA and Na^+ /taurocholate co-transport activity

Ntcp mRNA relative to 28 S rRNA was determined by slot-blot analysis as described in the Materials and methods section. Abbreviations: P, pregnant; NP, non-pregnant; BC, bromocriptine-treated rats *post partum*; PP, *post partum*. Significance: * $P < 0.01$ versus P; $P < 0.05$ versus NP. Ntcp mRNA and Na^+ /taurocholate co-transport were positively correlated ($P < 0.05$; Spearman Rank correlation test).

Rats	Ntcp mRNA	Na^+ /taurocholate co-transport (pmol/5 s per mg of protein)
P	4.9 ± 0.7	8.0 ± 0.6
NP	5.8 ± 0.6	10.4 ± 1.8
BC	8.8 ± 1.5	14.1 ± 0.9
PP	20.4 ± 2.7	$17.5 \pm 1.3^*$

ment of rats *post partum* with bromocriptine to inhibit PRL secretion prevented the increase in Ntcp mRNA (1.2 ± 0.2). Slot-blot analysis showed an excellent correlation ($P < 0.05$; Spearman Rank correlation test) between Ntcp mRNA and initial Na^+ /taurocholate co-transport activity in these four groups (Table 1). Finally, treatment of ovariectomized rats with oPRL increased Ntcp mRNA 10-fold relative to solvent-treated rats (60.7 ± 10.2 and 5.8 ± 2.5 relative-density units respectively). These results are consistent with a 2-fold increase in the V_{max} for Na^+ /taurocholate co-transport activity previously demonstrated [11] in isolated rat hepatocytes from oPRL-treated compared with solvent-treated rats (2 and 1 nmol/min per mg of protein respectively). These data support the hypothesis that the increase in Na^+ /taurocholate co-transport activity seen *post partum* is due to a PRL-induced increase in the steady-state mRNA for Ntcp, which leads to an increased synthesis and incorporation of functional transporter in the basolateral plasma membrane.

DISCUSSION

The present studies report for the first time a positive correlation between the activity of the hepatic Na^+ /taurocholate co-transporter and steady-state levels of its corresponding mRNA, and provide evidence for the physiological modulation of functional Ntcp by the anterior-pituitary hormone PRL. Ntcp mRNA was significantly increased 5–6-fold in rats *post partum*, and, more importantly, inhibition of PRL secretion with bromocriptine decreased Ntcp mRNA levels. Furthermore, ovariectomized rats treated with oPRL exhibited 10-fold higher Ntcp mRNA levels and a 2-fold increase in the V_{max} for Na^+ /taurocholate co-transport activity relative to solvent-treated controls. Taken together, these data indicate that PRL increases Na^+ /taurocholate co-transport at the level of mRNA. Whether the changes in Ntcp mRNA are due to activation of gene transcription *post partum* and after oPRL treatment, or to alteration of mRNA stability, or both, is under investigation.

A second important finding is that PRL-induced increases in Na^+ /taurocholate co-transport activity previously identified in isolated rat hepatocytes [11] are also detected in bLPM vesicles. These data indicate that changes in the Na^+ gradient or membrane potential are not solely responsible for the increased transport seen in isolated hepatocytes from rats *post partum* and oPRL-treated rats. These data support the hypothesis that PRL-induced Ntcp mRNA is associated with increased synthesis of transporter protein. This assumption is supported by studies indicating that the PRL-induced increase in Na^+ /taurocholate co-transport activity in isolated hepatocytes and in bLPM vesicles can be

blocked by treatment of rats with the protein-synthesis inhibitor cycloheximide [18]. It should be noted that the 60% decrease in $V_{\max}$ for Na^+ /taurocholate co-transport in isolated hepatocytes from pregnant rats [11] was more profound than the decrease in Ntcp mRNA in the present studies. Similarly, the 10-fold increase in mRNA after oPRL treatment exceeded the 2-fold increase in $V_{\max}$ for Na^+ /taurocholate co-transport in isolated hepatocytes [11]. Thus other factors in addition to altered mRNA levels, for example the post-translational processing or orientation of the Ntcp protein in the plasma membrane, changes in membrane potential, or concomitant changes in taurocholate efflux mechanisms, may also contribute to the apparent changes in Na^+ /taurocholate co-transporter activity in pregnancy and after oPRL treatment.

Hepatic prolactin receptors are present in large numbers in the female rat liver [19], but their physiological role has been unclear. The present studies strongly suggest that PRL-induced increases in Na^+ /taurocholate co-transport activity are due to the increase in Ntcp mRNA, presumably followed by increased synthesis and incorporation of functional transporters in the membrane. These results indicate that the liver is a target tissue for PRL, and suggest an important role for the hepatic PRL receptor.

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