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Influence of Obstructive Cholestasis and Sex Hormones on the Ratio of mRNA of Two Alternative Prolactin Receptor Isoforms in Rat Hepatocytes

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Abstract—The effects of obstructive cholestasis and sex hormones on the total content of prolactin receptor mRNA and ratio of mRNAs of its short and long isoforms have been studied in rat hepatocytes. Obstructive cholestasis caused insignificant changes in total content of prolactin receptor mRNA, but the proportion of mRNA of the long isoform increased. Comparison of prolactin receptor mRNA levels in gonadectomized and intact animals revealed opposite effects of male and female sex hormones on total mRNA, but both groups of these hormones increased the proportion of prolactin receptor short form mRNA. Changes in ratio of mRNA of receptor isoforms found in rat hepatocytes under obstructive cholestasis did not depend on levels of sex hormones. Obstructive cholestasis and sex hormones are suggested to regulate the content of long and short prolactin receptor isoforms in hepatocytes independently.

Key words: hepatocytes, obstructive cholestasis, sex hormones, prolactin receptor, isoforms, alternative splicing, reverse transcription, PCR

Prolactin is a pituitary hormone playing an important role in regulation of various biochemical processes and proliferative activity in many human and mammalian cell types under normal and pathological conditions. Prolactin signaling involves formation of a membrane complex, which consists of a prolactin molecule and two molecules of prolactin receptor. Most mammalian cells contain several isoforms of prolactin receptor differing in length and biological activity [1]. The long form of the prolactin receptor activates various signal cascades and JAK/STAT is the main pathway responsible for prolactin signal transduction. The short form of the prolactin receptor does not activate STAT proteins. Certain evidence exists that during hormone-dependent formation of prolactin receptor heterodimers the short isoform acts as a negative modulator of prolactin receptor-dependent signaling [2]. This form of prolactin receptor is also involved in transduction of some tissue-specific signals (including activation of serine/threonine protein kinase cascades and interaction with 17-hydroxysteroid dehydrogenase [3, 4]). Thus, tissue sensitivity to prolactin and mode of tissues response to this hormone depend on both the level of prolactin receptor and the ratio of its short and long isoforms. Prolactin receptor isoforms are formed by alternative splicing of pre-mRNA: the formation of long and short isoforms employs proximal exon 10 and distal exon 11, respectively [5]. This implies regulation of prolactin receptor isoform ratio at the stage of pre-mRNA splicing.

Hepatocytes are a convenient model for investigation of tissue-specific regulation of prolactin receptor isoform expression: from 80 to 90% of total content of prolactin receptor is represented by the short isoform [6].

Total level of prolactin receptor in hepatocytes depends on sex hormones: estrogens increase and androgens decrease content of prolactin receptor mRNA and its protein product (prolactin receptor) [7, 8]. Obstructive cholestasis accompanying various liver pathologies is

Abbreviations: JAK) Janus kinase; PCR) polymerase chain reaction; SR) serine- and arginine-rich splicing factors; STAT) signal transducer and activator of transcription.

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characterized by impaired bile outflow. This causes intoxication with bile acids, inflammation, necrosis/apoptosis, hepatocyte proliferation, and jaundice. Hepatic pathologies accompanied by obstructive cholestasis cause changes in total content of prolactin receptor and ratio of its isoforms. Altered prolactin receptor content was found in experimental hepatic fibrosis, and cirrhosis in rat [9], cholelithiasis, secondary hepatic cancer [10], and hepatocyte malignization in man [11]. Previously we found that cholestasis did not influence total content of prolactin receptor in rat female hepatocytes but significantly increased the proportion of long isoform [12].

Impairments in hepatocyte functioning induced by cholestasis are accompanied by altered content of sex hormones. In this connection, it is important to elucidate possible dependence of prolactin receptor gene expression under conditions of obstructive cholestasis on sex hormones.

In order to determine specific (i.e., sex hormone independent influence on prolactin receptor expression) effect of cholestasis on prolactin receptor expression we have investigated the effect of obstructive cholestasis and sex hormones on the level of prolactin receptor and ratio of its isoforms in rat hepatocytes.

MATERIALS AND METHODS

Female and male albino rats (200-250 g) were used in the experiments. Animals were subdivided into the following groups: intact rats, rats with obstructive cholestasis (14 days after ligation of the common bile duct), gonadectomized animals (one month after gonadectomy), and gonadectomized animals with obstructive cholestasis. There were 4-5 rats in each group.

Hepatocytes were isolated from livers of anesthetized animals using perfusion (via portal vein) with collagenase (0.5 mg/ml, Sigma, USA) followed by cell sedimentation (5 min at 50*g*) from the suspension formed. Purity of the isolated hepatocytes was controlled during counting cell number in a Fuchs–Rosenthal chamber; and cell viability was evaluated using Trypan blue [13].

Testosterone propionate (3 mg/injection, Sigma) was injected for three days to castrated male rats. Estradiol (10 μg/injection, Sigma) was injected for three days to gonadectomized female rats. Control and sham-operated animals received injections of equivalent volume of vehicle (propylene glycol, Sigma) [14]. Two equal pieces of liver (50 mg each) were taken from two different lobes and frozen in liquid nitrogen. Qualitative effect of sex hormone administration was evaluated in groups of animals (three rats in each groups), and the semi-quantitative effect was evaluated for one rat from each group.

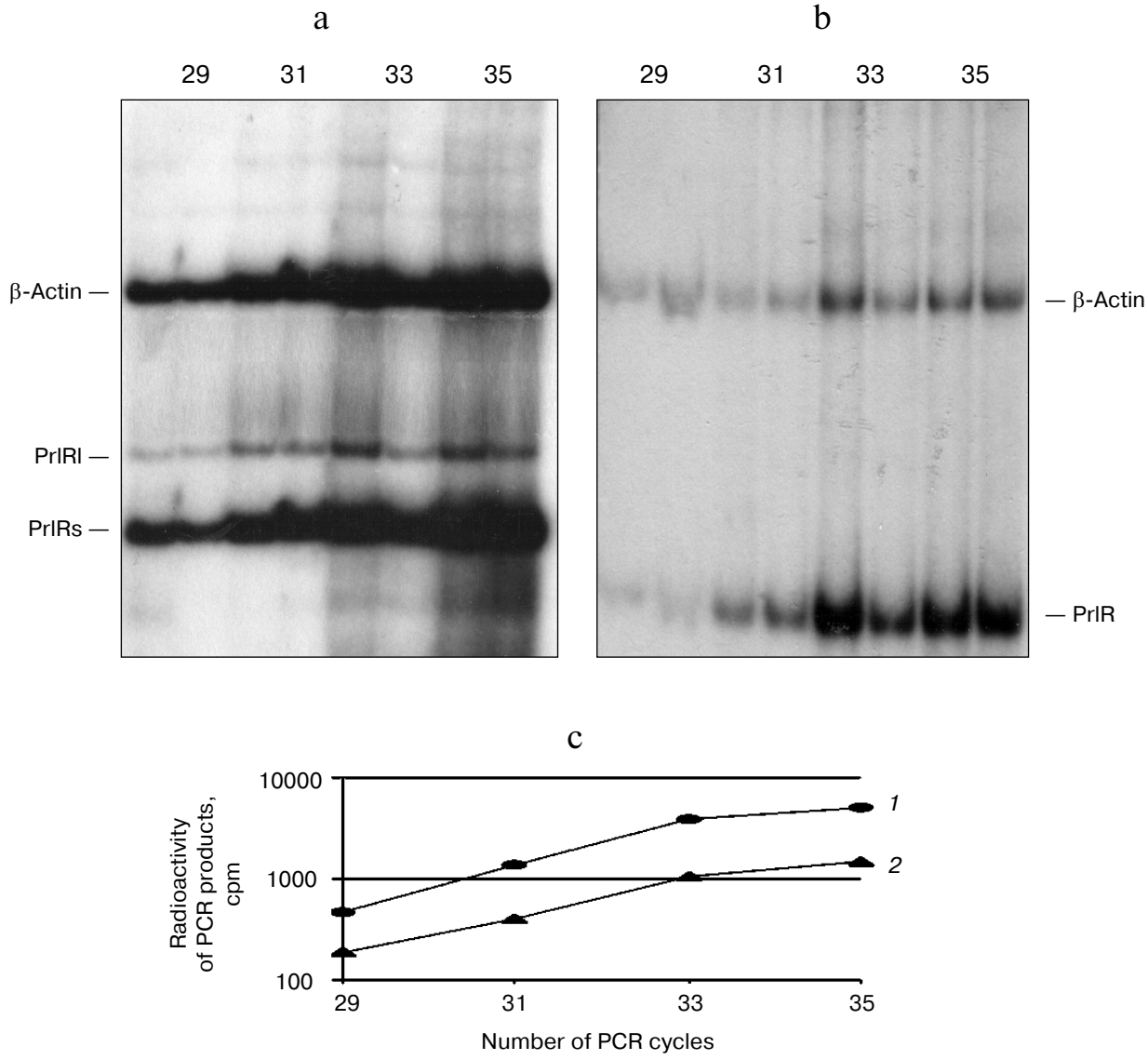
Total RNA was isolated from hepatocytes (10^6) or frozen liver (0.1 g) and resuspended in 4 M guanidine thiocyanate (ICN, USA) using phenol–chloroform extraction [15]. cDNA was synthesized using SuperscriptIIRT RnaseH$^-$ kits (Invitrogen, USA) and 465 ng of total RNA after annealing with 1.5 μg dT_{15} following the supplier's recommendations. Resultant cDNA was used for PCR with the following oligodeoxynucleotides (Syntol, Russia): 1) 5′-gtagatggagccaggagagttc-3′ as forward primer for cDNA amplification of both prolactin receptor isoforms (For); 2) 5′-ggtggaaagatgcaggtcatcat-3′ as reversed primer for cDNA amplification of both prolactin receptor isoforms (Rc); 3) 5′-tgagtctgcagcttcagtagtca-3′ as reversed primer for prolactin receptor short isoform cDNA amplification (Rs); 4) 5′-accagagtcactgtcgggatct-3′ as reversed primer for long isoform cDNA amplification (Rl); Ac. M19304 [16]. Amplification of referent β-actin cDNA (the housekeeper gene) was carried out using 5′-atctggcaccacaccttctacaatgagc-3′ and 5′-actcctgcttgctgatccacatctgc-3′, as forward (Fba) and reversed (Rba) primers, respectively; Ac. NM031144 [16].

PCR was carried out in incubation medium (25 μl) containing 1X buffer for Taq-DNA polymerase, 100 μM dNTP (Fermentas, Lithuania), 1 U Taq polymerase (Dialat, Russia), and 2 μl (1/30) of the reaction mixture for reverse transcription. Samples contained 20 pmol of For, Fba, and Rba oligonucleotides and 1 pmol of For and Fba oligonucleotides labeled with [γ-^{32}P]ATP in the polynucleotide kinase reaction. For competitive kinetic analysis of mRNA content of alternative prolactin receptor isoforms Rl and Rs, oligonucleotides (20 pmol each) were added to the mixture. Prolactin receptor total mRNA content was evaluated in the presence of 20 pmol Rc. There were 29, 31, 33, and 35 PCR cycles (for two parallel samples) using the following scheme: 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec.

PCR products were separated by electrophoresis in 5% polyacrylamide gel. Radioactivity was counted using an Intertechnique SL4000 counter (France).

Prolactin receptor mRNA content was expressed in arbitrary units (a.u.), defined as ratio of radioactivity (cpm) of PCR products of prolactin receptor and β-actin cDNAs. Mean value for several cycles of two independent PCRs are given. The ratio of mRNA for two prolactin receptor isoforms was determined as ratio of radioactivity of PCR products of short and long isoform cDNA.

Prolactin receptor mRNA content was evaluated by a semi-quantitative method using for amplification kinetic analysis β-actin cDNA amplification as internal standard [17]. In parallel experiments fragment of prolactin receptor cDNA common for both receptor isoforms and β-actin cDNA (or prolactin receptor cDNA fragments specific for long and short isoforms and β-actin cDNA fragment) were amplified. The dependence of products of RT-PCR on number of cycles was analyzed in two independent reactions (29th, 31st, and 33rd cycles) using a region of linear and parallel change of logarithm of accumulated product, log(cpm) [12]. Under these conditions, amounts of all four amplification products are compara-



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Number of PCR cycles	Mean value of radioactivity of bands corresponding to common form of prolactin receptor determined in two parallel reactions, cpm	Mean value of radioactivity of bands corresponding to β-actin determined in two parallel reactions, cpm	Ratio of radioactivity of prolactin receptor and β-actin amplification products, a.u.
29	469	189.5	2.48
31	1381	402	3.43
33	3942	1056	3.74
35	5115.5	1479.5	3.46
Mean			3.27

Fig. 1. Kinetics of cDNA amplification (cycles from 29 to 35): a) autoradiogram of PCR products of short (PrlRs) and long (PrlRl) prolactin receptor (PrlR) isoforms and β-actin of hepatocytes from intact female rat; b) autoradiogram of PCR products of prolactin receptors and β-actin of hepatocytes from intact female rat; c) the dependence of accumulation of amplification products (prolactin receptor (*1*), β-actin (*2*)) (see Fig. 1b) of intact female rat hepatocytes on number of PCR cycles; d) calculation of relative content of prolactin receptor mRNA using results of one experiment with intact female hepatocytes.

ble, and this allows comparing mRNA content in initial preparations (Fig. 1).

Hepatic prolactin receptor expression in intact female and female rats with induced cholestasis was detected by indirect immune peroxidase method using 3-μm liver sections and monoclonal antibodies against extracellular domain of the prolactin receptor (clone U5, Affinity Bioreagents, USA) [18]. The level of prolactin receptor was then evaluated semi-quantitatively using cytophotometry. Two experimental (with anti-prolactin receptor antibodies) and two control (without anti-prolactin receptor antibodies) sections from each animal were analyzed by cytophotometry. These measurements were carried out using a LUMAM I-3 photometry microscope (objective magnitude ×40) at 520 nm with light-transmitting probe 5 μm in diameter built into the objective. Intensity of prolactin receptor-specific staining of cells was evaluated by optical density (D) value calculated as D = log(Tk/T), where T and Tk are optical absorbance of cells in experimental and control sections, respectively. Fifty hepatocytes were analyzed in each section.

Statistical analysis was carried out using STATISTICA v. 6, StatSoft, Inc. (2001). Statistical significance of differences was evaluated by Kruskal–Wallis and Mann–Whitney criteria.

RESULTS

Prolactin receptor mRNA content in hepatocytes of various experimental groups of rats. Pilot analysis revealed that sham operated (sham gonadectomy or ligation of common bile duct) female and male rats did not significantly differ from intact animals in liver prolactin receptor mRNA content and ratio of its isoforms (Kruskal–Wallis ANOVA test, $p > 0.9$).

Obstructive cholestasis did not influence prolactin receptor mRNA content in hepatocytes of both intact and ovariectomized female rats. The content of prolactin receptor protein product in hepatocytes of normal female rats and female rats with obstructive cholestasis was also nearly the same, D = 0.203 ± 0.03 and 0.199 ± 0.02, respectively ($p > 0.4$). The development of obstructive cholestasis in male rats was accompanied by some reduction in prolactin receptor mRNA; this decrease was more pronounced in intact rats (44%) than in castrated males (17%) (Fig. 2).

PCR-analysis of prolactin receptor mRNA content revealed that the level of prolactin receptor total mRNA is 4.2 times lower in male than in female rats (Fig. 3). Gonadectomy caused 1.7-fold increase in prolactin receptor total mRNA content in males and 2.5-fold reduction of this parameter in female rats. Thus, the difference in total content of prolactin receptor mRNA between female and male rats become insignificant ($p > 0.65$) (Fig. 3). Administration of corresponding sex hormones to the gonadectomized animals restores prolactin receptor mRNA levels typical for intact rats of both sexes (Fig. 4, a and b). This coincides with observations of other authors [7].

Ratio of short and long isoforms (S/L) of prolactin receptor mRNA in hepatocytes of various experimental groups of rats. Figure 5 shows ratio of short isoform mRNA and long isoform mRNA of prolactin receptor

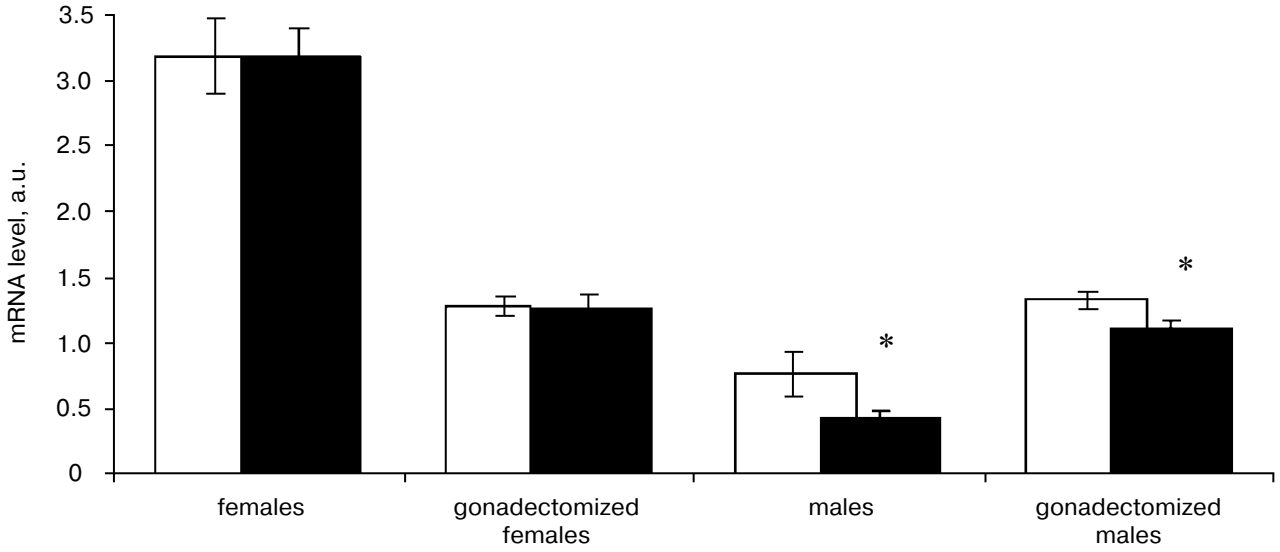

Fig. 2. Effect of obstructive cholestasis on mRNA content in rat hepatocytes. Light columns show groups of animals without obstructive cholestasis; dark columns show groups of animals with obstructive cholestasis; a.u., arbitrary units (see "Materials and Methods" section). * Statistically significant difference ($p < 0.05$) between male rat with and without obstructive cholestasis.

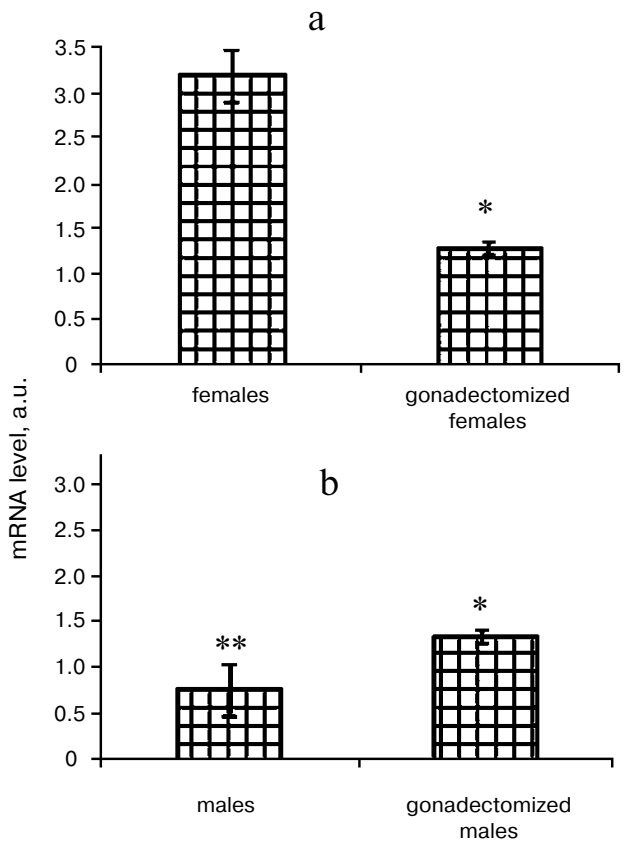

Fig. 3. Effect of gonadectomy on prolactin receptor mRNA content in rat hepatocytes: a) females; b) males. * Statistically significant difference ($p < 0.05$) between intact and gonadectomized rats. ** Statistically significant difference ($p < 0.05$) between intact female and male animals; a.u., arbitrary units (see "Materials and Methods").

reflecting relative efficacy of distal exon employment during splicing of primary prolactin receptor transcript in animals of various experimental groups. In all groups of animals with obstructive cholestasis the ratio of short to long prolactin receptor isoforms (S/L ratio) decreases and becomes almost identical: 1.5-1.6 to 1.

In females the S/L ratio was higher than in males; however, gonadectomy caused marked reduction of this value and in the gonadectomized females it did not significantly differ from castrated males (2.6-2.7 to 1, $p > 0.68$, Fig. 5). Administration of sex hormones to corresponding animals reversed this effect of gonadectomy (Fig. 4, a and c).

Content of prolactin receptor short and long isoforms mRNA in hepatocytes of rats from different experimental groups. Calculation of the prolactin receptor short and long isoform mRNA levels has shown that in all groups of animals the short form of prolactin receptor predominates. However, under conditions of obstructive cholestasis significant reduction of the short form of prolactin receptor occurs (table).

DISCUSSION

For analysis of the effects of sex hormones and obstructive cholestasis on prolactin receptor isoform mRNA content in hepatocytes from rats of various groups, it is convenient to consider the effects of the employed treatments on sequential steps of gene expression separately. It should be taken into consideration that the content of prolactin receptor total mRNA in cells is determined by transcriptional activity of the prolactin receptor gene while the ratio of prolactin receptor iso-

Content of prolactin receptor isoforms mRNA in rat hepatocytes under various conditions (a.u ± SEM)[a]

Group	Rats without obstructive cholestasis		Rats with obstructive cholestasis	
	short isoform	long isoform	short isoform	long isoform
Females	1.30 ± 0.22	0.13 ± 0.01	0.87 ± 0.02[b]	0.56 ± 0.12[b]
Males	0.27 ± 0.06[c]	0.07 ± 0.02[c]	0.12 ± 0.02[b]	0.07 ± 0.01
Ovariectomized females	0.42 ± 0.04[d]	0.15 ± 0.02	0.33 ± 0.03[b]	0.24 ± 0.01[b]
Castrated males	0.43 ± 0.02[d]	0.16 ± 0.01[d]	0.30 ± 0.04[b]	0.19 ± 0.01[b]

[a] a.u., arbitrary units (see "Materials and Methods").
[b] Statistically significant differences ($p < 0.05$) between prolactin receptor isoforms mRNA in hepatocytes of rats with and without obstructive cholestasis.
[c] Statistically significant differences ($p < 0.05$) between prolactin receptor isoforms mRNA in hepatocytes of intact female and male rats.
[d] Statistically significant differences ($p < 0.05$) between prolactin receptor isoforms mRNA in hepatocytes of gonadectomized and intact rats.

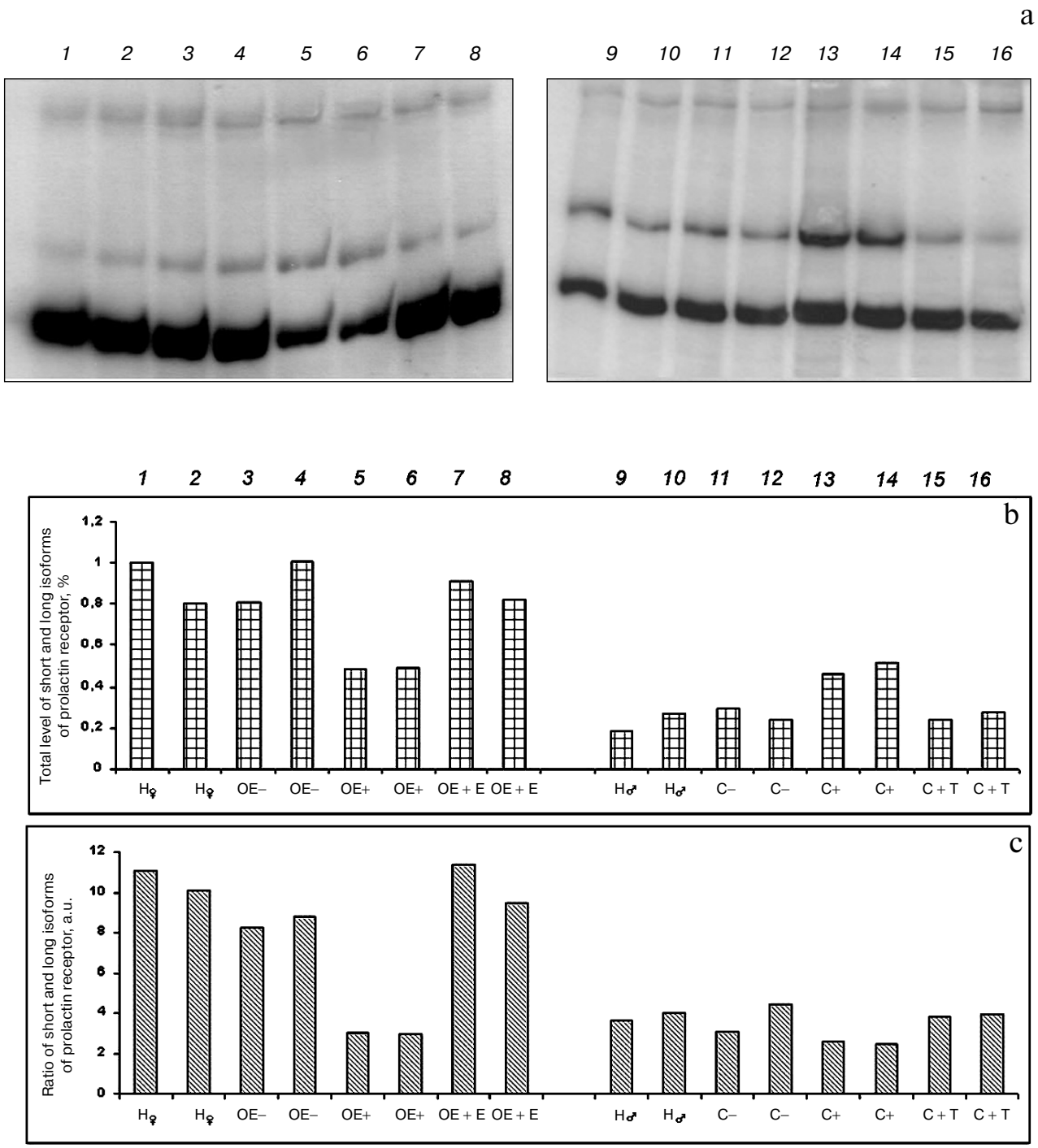

Fig. 4. Effect of sex steroids on total level and ratio of prolactin receptor isoforms in rat liver. a) Autoradiogram of amplification products (31 cycles of PCR) of β-actin; short and long prolactin receptor isoform cDNAs separated in 5% polyacrylamide gel under non-denaturing conditions. b) Relative total content of short and long isoforms of prolactin receptor: sum of ratios of radioactive amplification products of prolactin receptor isoforms to radioactivity of β-actin product radioactivity, normalized to that level in hepatocytes of intact female rat. c) Ratio of short and long receptor isoforms of prolactin receptor. *1*, *2*) (H♀) hepatocytes of intact female rat; *3*, *4*) (OE–) liver of sham-operated female rat treated with propylene glycol; *5*, *6*) (OE+) liver of gonadectomized female rat treated with propylene glycol; *7*, *8*) (OE + E) liver of gonadectomized female rat treated with estradiol; *9*, *10*) (H♂) hepatocytes of intact male rat; *11*, *12*) (C–) liver of sham-operated male rat treated with propylene glycol; *13*, *14*) (C+) liver of gonadectomized male rat treated with propylene glycol; *15*, *16*) (C + T) liver of gonadectomized male rat treated with testosterone propionate.

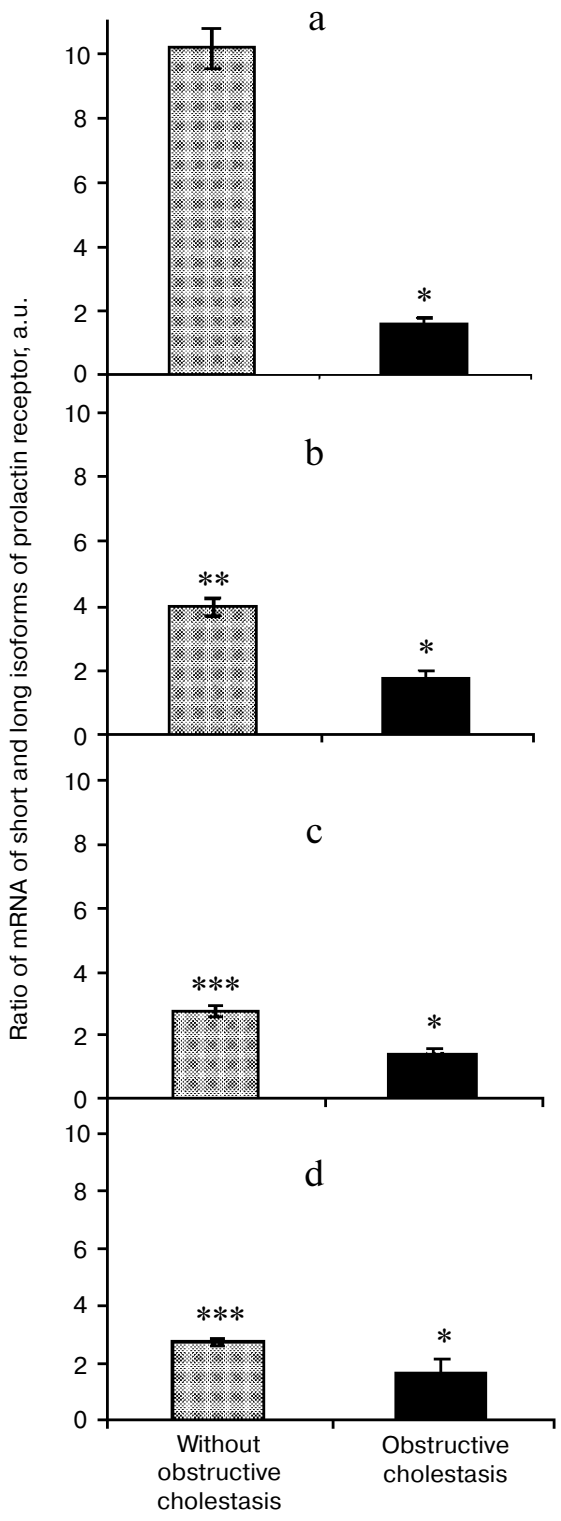

Fig. 5. Ratio of mRNA of short and long forms of prolactin receptor in hepatocytes of rats without (light columns) and with obstructive cholestasis (dark columns): a) females; b) males; c) gonadectomized females; d) gonadectomized males. * Statistically significant difference ($p < 0.05$) between rats with and without obstructive cholestasis. ** Statistically significant difference ($p < 0.05$) between intact females and males. *** Statistically significant difference ($p < 0.05$) between gonadectomized and intact rats.

forms is characteristic feature for relative efficacy of use of one of the alternative exons during splicing of prolactin receptor primary transcript. (In the case of prolactin receptor pre-mRNA, the S/L ratio reflects efficacy of selection between distal and proximal exons, respectively.)

Rat prolactin receptors are encoded by a single gene; its transcription is controlled by one of three alternative promoters I, II, and III [19]. In rat liver promoter II is the most active; it neighbors a regulatory site containing elements responsive to estrogens, transcription factor AP-1 (dimer of c-jun and c-fos), nuclear hepatocyte factor 4, and STAT proteins [19-21]. Results of the present report demonstrate that estrogens increase level of prolactin receptor total mRNA in hepatocytes (Figs. 3a and 4a). Taking into account that hepatocytes express estrogen receptors [22] and this promoter contains estrogen-response element, it is relevant to suggest that estrogens directly influence promoter II activity. In addition, the effect of estrogens on promoter II activity has already been recognized in hypothalamus [17]. We found that androgens decrease prolactin receptor gene in hepatocytes (Figs. 3b and 4a). Although regulatory sites of the prolactin receptor gene lack androgen-response elements, it is possible that these hormones indirectly influence gene transcription via interaction of androgen receptor with various cofactors. For example, androgen receptor suppresses transcription of genes dependent on factor AP-1, androgen receptors interact with this factor involving several protein-mediators; this involves several messengers. Androgen receptor may also decrease activity of STAT proteins by interacting with protein inactivating active STAT protein (PIAS-3) [23]. We demonstrated that obstructive cholestasis did not influence transcription of prolactin receptor gene in hepatocytes of both intact and gonadectomized females. However, in intact and gonadectomized male the level of prolactin receptor total mRNA is reduced with obstructive cholestasis (Fig. 2). Different effects of obstructive cholestasis on prolactin receptor gene transcription in gonadectomized males and female rats suggest involvement of some hepatic systems with androgen-programmed activity as is known for hepatocyte proliferation, rate of synthesis, and composition of bile acids which are targets for androgen imprinting [22]. The "presence" of functionally competent gonads in normal males additionally as compared with castrated animals increases negative effect of obstructive cholestasis on the level of prolactin receptor total mRNA.

Processing of prolactin receptor pre-mRNA involves alternative splicing [19]. The ratio of prolactin receptor isoform mRNA is determined by relative efficiency of insertion of exon 10 (long isoform) or exon 11 (short isoform) into mature mRNA. A number of *in vivo* and *in vitro* experiments revealed that selection of alternative exon is determined by ratio and activity of factors responsible for alternative splicing. These include serine and

arginine-rich proteins, increasing probability of proximal exon incorporation into mRNA, and heterogeneous nuclear ribonucleoproteins increasing preferential selection of distal exon [24]. The effect of splicing *trans*-factors depends on their interaction with *cis*-elements of pre-mRNA. In the case of prolactin receptor pre-mRNA, it is possible that such *cis*-elements may be localized in non-coding sequences of three alternative first exons, transcribed from alternative promoters of the prolactin receptor gene. However, the interrelationship between use of alternative promoters of prolactin receptor gene and preferential formation of certain isoform of prolactin receptor remains to be demonstrated [19].

Results of the present study provided the first evidence that female and male sex hormones exert unidirectional effect on alternative splicing of prolactin receptor pre-mRNA in rat hepatocytes increasing preferential use of the distal exon (Fig. 4); although with different efficacy. However, data of other authors [7] (obtained without using internal standard) may serve as evidence of opposite effects of androgens and estrogens on alternative splicing of prolactin receptor primary transcripts; however, they did not use an internal standard for PCR. Estrogen effect on alternative splicing resulting in preferential usage of the distal exon was demonstrated for mouse hepatocytes growth hormone receptor pre-mRNA [25] and rat hepatocyte prolactin receptor pre-mRNA [7]. It is possible that these effects are realized via interaction of estrogen receptor with co-activator of activating protein-1 (CoAA) [26]. Moreover, it should be noted that prolactin as a hormone positively regulated by estrogens influences splicing of pre-mRNA of its own receptor in rat corpus luteus by increasing preferential use of the distal exon [27]. Since gonadectomy reduced prolactin content in males and females [28] it is relevant to suggest that prolactin is the principal factor determining ratio of prolactin receptor isoform mRNA, whereas estrogens potentiate its effect in females.

Obstructive cholestasis significantly increased effectiveness of use of the proximal exon of prolactin receptor pre-mRNA in rat hepatocytes irrespectively to the level of sex steroids in blood. In the present study we have demonstrated that obstructive cholestasis and sex hormones cause opposite effects on ratio of long and short forms of prolactin receptor. Taking into consideration that obstructive cholestasis is accompanied by the increase of sex steroids avoiding their liver metabolism [29] we can conclude that obstructive cholestasis influences alternative splicing of prolactin receptor pre-mRNA irrespectively to changes in level of sex hormones. The development of obstructive cholestasis is accompanied by necrosis/apoptosis of hepatocytes due to intoxication, inflammation, and compensatory hepatocyte proliferation. Each of these processes may influence pre-mRNA splicing. For example, it was demonstrated that hepatocyte proliferation is accompanied by augmentation of level and activity of SR-proteins. This results in increased insertion of proximal exon into fibronectin mRNA [30]. It is possible that a similar mechanism is responsible for changes in the ratio of prolactin receptor isoform mRNA.

Content and ratio of prolactin receptor isoforms directly influence efficacy of prolactin action (and action of other members of growth hormone family [31]) on hepatocytes. Since estrogens and androgens cause opposite effects on magnitude of cell response to prolactin, but increase content of the short form of prolactin receptor, it is possible that sex hormones specify cell response to prolactin (i.e., they make the prolactin effect more "hepatospecific") by increasing proportion of signal cascades unrelated to JAK/STAT pathway. On the contrary, obstructive cholestasis increases relative proportion of the long form of prolactin receptor and shifts the key point of cellular response to prolactin to the JAK/STAT pathway, which is more typical for most tissues.

Thus, we conclude that sex hormones and obstructive cholestasis regulate the level of long and the short prolactin receptor isoforms in rat hepatocytes in different ways, modifying both the efficacy and the mode of cell response to prolactin.

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