The regulation of IGFs and IGFBPs by prolactin in primary culture of fetal rat hepatocytes is influenced by maternal malnutrition

Ilham El Khattabi, Claude Remacle, and Brigitte Reusens

Laboratoire de Biologie Cellulaire, Université Catholique de Louvain, Louvain-la-Neuve, Belgium

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El Khattabi, Ilham, Claude Remacle, and Brigitte Reusens. The regulation of IGFs and IGFBPs by prolactin in primary culture of fetal rat hepatocytes is influenced by maternal malnutrition. Am J Physiol Endocrinol Metab 291: E835–E842, 2006.—During perinatal development, the regulation of IGF system appears to be growth hormone (GH) independent. By using highly purified primary fetal hepatocytes, we investigated the role of prolactin (PRL) in the regulation of IGF system and hepatocyte proliferation. We also analyzed the consequence of a maternal low-protein (LP) diet on the regulation of IGF, IGF-binding protein (IGFBP), and hepatocyte proliferation by prolactin. Pregnant Wistar rats were fed a control (C) diet (20% protein) or isocaloric (LP; 8%) diet throughout gestation. On day 21.5, fetal hepatocytes were cultured for 4 days and incubated with rat prolactin. In the C hepatocytes, PRL at 100 ng/ml decreased the abundance of IGFBP-1 and IGFBP-2 by 50 (P < 0.05) and 60% (P < 0.01), respectively. It also reduced by 70% the level of IGF-II mRNA (P < 0.01). By contrast, PRL failed to modulate IGFBP-1 and IGFBP-2 production by LP hepatocytes, and this was associated with reduced abundance of the short form of PRL receptor (P < 0.05). PRL had no effect on either the proliferation or the IGF-I production by C and LP hepatocytes, although it reduced the expression of IGF-II. These results suggest that prolactin influences hepatocyte proliferation in vitro by inhibiting IGFBP-1, IGFBP-2, and IGF-II levels, which may coincide with the decline of IGF-II observed in rodents during late gestation in vivo. On the other hand, maternal LP diet induces a resistance of fetal hepatocytes to PRL.

low-protein diet; fetal hepatocytes; insulin-like growth factors; insulin-like growth factor-binding proteins

THE IGF SYSTEM PLAYS A KEY ROLE in the endocrine regulation of fetal growth, which in turn is largely determined by the availability of nutrients to the fetus (18, 27). The role of IGF system in utero has been well documented by specific gene deletion of IGFs, IGF-binding proteins (IGFBPs), and IGF receptors (9, 37, 41). Previously, we have shown that a maternal isocaloric moderate low-protein (LP) diet (8 vs. 20%) throughout gestation leads to reduced birth weight (54), and this was associated with reduced IGF-I and more IGFBP-1 and -2 in rat fetuses (13). The pancreatic expression of IGF-II mRNA and the fraction of islet area that was immunopositive -2 in rat fetuses (13). The pancreatic expression of IGF-II was lower in LP fetuses and neonates (6, 47). In addition, reduced liver weight in vivo and lower hepatocyte proliferation in vitro were observed in the 8% fetal group (13).

We and others (13, 23, 36, 39) showed that insulin and glucocorticoids modulate fetal IGF and IGFBP secretion as well as fetal hepatocytes proliferation. Prolactin (PRL) and placental lactogens (PLs), also known as “lactogenic hormones,” may also regulate the IGF system during the perinatal period, when such regulation seems to be GH independent (26, 29, 57). PRL and PLs form, along with growth hormone (GH), a group of proteins with structural similarities (20). Depending on the species, PRL or PL circulates at high levels in the fetus (15, 17, 38, 42). In rat, PLs are the major lactogens in the fetal circulation, where at least two isoforms have been identified: PL-I, which appears until mid-pregnancy; and PL-II, detected during the last half of pregnancy (15, 51). Furthermore, the PRL receptor (PRLR) that is closely related to the GH receptor (20) is widely expressed in fetal tissues (16), where at least two forms exist: a long form (LPRLR) and a short form (SPRLR) that differ in the length of the intracellular domain. These proteins are coded by a single gene and generated by alternative splicing (20). Among tissues examined, liver is one tissue where PRLR mRNA is highly expressed (5). Although the LPRLR can transmit a well-defined intracellular signal, that of the SPRLR is not yet clearly identified. It has been demonstrated that the short form of the rat PRL receptor cannot mediate cell proliferation but acts as a “dominant negative” form by inhibiting the function of the PRLR (3, 46, 52). However, others reported antagonistic results with regard to one of the three short forms of the mouse PRL receptors (PR1). Transfection of NIH 3T3 cell line with the full-length cDNA of PR1 stimulates proliferation (11). Also, overexpression of PR1 in heterozygous mice that express only one allele of the PRL receptor rescued the reduced mammary gland development and lactation in young females (4).

From these data, it has been postulated that lactogenic hormones play an important role in growth during the perinatal period. Available evidence indicates that PRL or PL-I increases significantly the growth of rat embryos in vitro (31, 32, 33, 53), and this activity could be mediated through IGFs (32, 33). It has been also suggested that these hormones may substitute for GH to modulate the fetal IGF system. The association between PRL/PLs and IGF system has been described in many in vivo and in vitro experiments though conflicting. In vivo, strong positive correlations have been found between human placental lactogen (hPL) and IGFs in human fetal serum (34), and administration of bovine PRL to hypophysectomized adult rats enhanced the serum IGF-I level as well as the liver mRNA for this peptide (40). In contrast, others have reported that administration of ovine PL (oPL) to fetuses of the same species at late gestation did not influence the IGF production, whereas it altered the IGFBP-3, either by

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reducing the protein level in the circulation (44) or by enhancing its expression in the liver (10). In vitro, hPL stimulated IGFs and IGFBPs in fetal human fibroblasts (25). The interpretation of these data was rendered difficult due to the use of heterospecific system and ligands. Moreover, it has been demonstrated that oPL and bovine PL, as well as hPL, bind to the GH receptor (GHR) in addition to the PRLR (19).

Therefore, we were interested in studying the lactogenic regulation of IGFs, IGFBPs, and proliferation by using a homologous system of primary cultured fetal rat hepatocytes described previously (13). For that purpose, we have used rat PRL instead of rat PL-II, since the latter is not available commercially. In rodents, PRL and PL show closer similarity in their primary sequence than in human and bind with strong and similar affinity to the PRLR but do not show any cross-reactivity to the GHR (19). We also sought to determine in vitro whether PRL could restore some of the alterations previously observed in LP-derived hepatocytes.

MATERIALS AND METHODS

Animals and diets. Wistar rats bred in our laboratory were placed in a controlled environment (25°C, 12:12-h dark-light cycle) and allowed free access to food and water. Virgin females were housed with males overnight, and copulation was confirmed the next morning by the presence of vaginal plug or spermatozoa after a vaginal smear. Midnight was considered as the time of mating and day 0 of gestation. Pregnant females were then caged individually and randomly assigned to one of two diets throughout gestation: 20% protein diet representing the control (C) group, 8% isocaloric protein diet representing the PRL group. As a general characteristic, the body weight gain during gestation in LP dams was lower than in C dams [C: 118 Â± 10 g (n = 7); LP: 97.6 Â± 3.4 g (n = 5), P < 0.05; Student’s t-test].

On the last day of gestation, dams were killed and fetuses exposed after abdominal incision. Both diets were identical in fat content and rendered isocaloric by the addition of carbohydrates in the LP group. Diets were purchased from Hope Farms (Woerden, The Netherlands), for which composition has been previously described by Snoeck et al. (54). Ethical approval for the animal study was obtained from the animal ethics committee of the Catholic University of Louvain, Louvain-la-Neuve, Belgium.

Materials. Type II collagenase (sp act 387 U/mg), dexamethasone, HEPES buffer, EDTA, and bovine serum albumin (fraction V) were purchased from Sigma (St. Louis, MO). William’s E medium, 10 mM Hepes, EDTA, and bovine serum albumin (fraction V) were purchased from Gibco BRL (Merelbeke, Belgium). Deoxyribonuclease I was from Boehringer Mannheim (Mannheim, Germany) and Vitrogen 100 from Collagen (Palo Alto, CA). Percoll was purchased from Pharmacia (Upplands, Sweden). Goat anti-rat IGFBP-1 and anti-rat IGFBP-2 polyclonal antibodies were obtained from Novo Nordisk (Bagsvaerd, Denmark). William’s E medium, 10Â× HBSS, anti-biotics, and fetal bovine serum (FBS) were from Gibco BRL (Merelbeke, Belgium). Serum was purchased from Gibco BRL (Merelbeke, Belgium). Desoxyribonuclease I was from Boehringer Mannheim (Mannheim, Germany) and Vitrogen 100 from Collagen (Palo Alto, CA). Percoll was purchased from Pharmacia (Upsala, Sweden). Goat anti-rat IGFBP-1 and anti-rat IGFBP-2 polyclonal antibodies were obtained from Novo Nordisk (Bagsvaerd, Denmark). Mouse anti-rat PRLR U5 mAb was from Affinity Bioreagents (Golden, CO). Rabbit anti-mouse and rabbit anti-goat immunoglobulin were from Dako (Carpinteria, CA). Anti-mouse and rabbit anti-goat immunoglobulin were from Dako (Carpinteria, CA). 4,6-Diamidino-2-phenylindole (DAPI) was from Serva (Heidelberg, Germany) and DNA from calf thymus, used as standard, from Boehringer Mannheim. Protein G-Sepharose beads were from Zymed (San Francisco, CA). The rat PRLR (NIDDK rPRL) was obtained from Sanvertech (Santa Cruz, CA).

Fetal hepatocytes isolation and primary culture. The detailed protocol of isolated fetal hepatocytes and cell culture has been described previously (13). Briefly, minced livers from rat fetuses 1 day before birth were incubated in a balanced salt solution (BSS: 142 mM NaCl, 6.7 mM KCl, 10 mM HEPES, pH 7.4) containing 1 mM EDTA at 37°C under constant agitation. The suspension was centrifuged at 716 rpm for 5 min, and the tissue pellet was then digested in BSS containing 6.7 mM CaCl₂, 0.5 mg/ml collagenase, and 0.1 mg/ml deoxyribonuclease I at 37°C under constant agitation. After dissociation, the cell suspension was collected and diluted in William’s E medium supplemented with 0.2% BSA and centrifuged for 5 min at 424 rpm. Subsequently, the supernatants were filtered through nylon meshes (100 and 30 μm, respectively) and washed with WE-BSA medium (424 rpm, 5 min). Fetal hepatocytes were further purified by adding 15 ml of cell suspension to 14.4 ml of diluted Percoll solution (9 volumes Percoll and 1 volume 10× HBSS). The mixture was centrifuged at 1,433 rpm for 8 min. To eliminate the residual hematopoietic cells, the pellet (hepatocyte fraction) was resuspended in WE-BSA medium at low speed (277 rpm, 5 min). Purified fetal hepatocytes were resuspended in William’s E medium supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and cell number was counted in a hemocytometer. Routinely, 10 × 10⁶ hepatocytes/g liver were obtained with viability above 90–95% as assessed by Trypan blue exclusion.

Fetal hepatocytes were cultured on dishes previously coated with a type I collagen gel at a density of 10⁵ cells/cm² in William’s E medium supplemented with 10% FBS and antibiotics. Four hours after plating, the cells were washed three times and transferred into hormono-defined medium (10 nM insulin, 10⁻⁷ M dexamethasone, 50 ng/ml EGF, 0.1 mM ascorbic acid, 10 μg/ml linoleic acid) without or with rPRL: 10 or 100 ng/ml at final concentrations.

Preparation of conditioned media and cellular extracts. For IGF-I and IGFBP measurements, media were conditioned over 24-h periods of time from day 1 to day 4. Conditioned media were recovered at 4°C, centrifuged to eliminate cells and debris, and kept at −20°C. They were then precipitated with trichloroacetic acid (10% wt/vol final concentration) and concentrated 15 times upon reconstitution in Laemmlli sample buffer under nonreducing conditions containing 0.5% Triton X-100. Purified fetal hepatocytes were resuspended in William’s E medium supplemented with 10% FBS and antibiotics. Four hours after plating, the cells were washed three times and transferred into hormono-defined medium (10 nM insulin, 10⁻⁷ M dexamethasone, 50 ng/ml EGF, 0.1 mM ascorbic acid, 10 μg/ml linoleic acid) without or with rPRL: 10 or 100 ng/ml at final concentrations.

Western immunoblot analysis. Aliquots of conditioned medium from culture were subjected to Western blot analysis according to the method described by Hossenlop et al. (28). Briefly, samples were boiled in Laemmlli buffer (Tris-HCl, 0.625 mol/l, pH 6.8, 10% glycerol, 2% SDS, and 0.0125% bromophenol blue) and loaded onto 12% SDS polyacrylamide gels under nonreducing conditions. Standards were run under similar conditions in adjacent lanes. After separation, proteins were transferred electrophoretically to nitrocellulose membranes. The latter were blocked with 5% nonfat dry milk for 1 h in Tris-buffered saline (TBS: 0.05 mol/l Tris, 0.2 mol/l NaCl, pH 7.4).
7.4) and washed twice in TBS-Tween (TBS with 1 mL of Tween 20). Membranes were then incubated with antiserum against rat IGFBP-1 and IGFBP-2 (1:500 to 1:1,000 dilutions). Antigen-antibody complexes were identified with rabbit anti-goat immunoglobulin G labeled with horseradish peroxidase. Specific binding was visualized by chemiluminescence [enhanced chemiluminescence (ECL) detection system; Amersham]. Autoradiograms were analyzed using the NIH Image 1.55 software. The relative amount of each major band was assessed on the basis of its surface area and mean density.

Liver collection. On the last day of gestation, dams were anesthetized with pentobarbital sodium (55 mg/kg body wt), and fetuses were exposed after abdominal incision along the midline. Fetal livers were removed rapidly, frozen, and stored at −80°C until utilized. Frozen tissues were homogenized with a Polytron in lysis buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 50 mM Na-Fluoride, 30 mM Na pyrophosphate, 1 mM orthovanadate, 10% glycerol, 0.5% Triton-X 100) containing protease inhibitors (1 mM PMSF, 1 mg/ml pepstatin, 2 mg/ml leupeptin, 5 mg/ml apro tin). The homogenate was then centrifuged at 3,000 rpm for 5 min at 4°C. The clear supernatant was carefully removed and its protein content determined, and then the aliquots were stored at −20°C.

Immunoprecipitation and Western blot analysis of PRLR. Liver homogenates were diluted 1:1 in lysis buffer containing protease inhibitors, and total cellular extracts were lysed in 1 ml of the same buffer for 30 min at 4°C. Cell lysates were then centrifuged at 14,000 rpm at 4°C for 10 min and resuspended in 1 ml of lysis buffer. Five microliters per milliliter anti-receptor mAb U5 were added, and all samples were then rotated at 4°C for 1 h. Sixty microliters of protein G-Sepharose were added, and rotation at 4°C was continued over-night. Sixty microliters of protein G-Sepharose were then rotated at 4°C for 1 h. The supernatant was centrifuged again at 12,000 rpm for 30 min at 4°C. The clear supernatant was carefully removed and its protein content determined, and then the aliquots were stored at −20°C.

Measurement of IGF-I. In plasma and in culture medium IGF-I was measured by enzyme immunoassay (EIA), using rat IGF-I EIA kit from Diagnostic System Laboratories (Webster, TX). The IGF-I EIA is a homologous assay that uses a pretreatment step (Diagnostic System Laboratories) to eliminate the interference of IGFBPs. After the pretreatment step, neither IGFBP-1 nor IGFBP-2 was detected by immunoblotting of conditioned culture media (data not shown). Intra- and interassay coefficients of variation were 7.4% and 9.4%, respectively.

RNA extraction and real-time PCR analyses of IGF-II. Total RNA was isolated from cultured hepatocytes derived from livers of rat fetuses 1 day before birth with TRIzol reagent according to the manufacturer’s instructions (Invitrogen Life Technologies, Carlsbad CA). Reverse transcription was performed in a final volume of 20 μl using 3 μg of total RNA in the presence of 500 nM random hexamer primers (Invitrogen). Primer sequences were 1) IGF-II sense 5’-CCTG-TAC-TTC-CGG-ACG-CT-3’, antisense 5’-CTG-CCC-GGG-GAC-TGT-CT-3’; and 2) ARN 185 sense 5’-GAT-CCA-TTG-GAG-GGC-AAG-TCT-3’, antisense 5’-GCA-GCA-CTT-ATA-TAC-GCT-ATT-G-3’. Real-time PCR was performed in a GeneAmp 7000 Sequence Detection System (Applied Biosystems, Foster city, CA) using SYBR Green as the detection format. Amplification was carried out in a total volume of 25 μl containing 20 ng of reverse-transcribed total RNA for IGF-II and 0.4 ng for IGF-I and in a final concentration of 1× qPCR Mastermix Plus for SYBR Green (Eurogentec, Seraing, Belgium), with 300 nM of each primer having the following profile: 2 min for 1 cycle (initial step) at 50°C; 10 min for 1 cycle (Hot GoldStar Activation) at 95°C; 15 s at 95°C and 1 min at 60°C for 40 cycles.

The comparative cycle threshold (Ct) method was used to quantify the results obtained by the real-time PCR. With this method, arithmetic formulas were used to calculate the relative expression levels compared with a calibrator, i.e., the C group. The amount of target, normalized to an endogenous housekeeping gene (18S) and relative to the untreated group, is given as 2−ΔΔCt, where ΔΔCt = ΔCt (sample) – ΔCt (untreated group) and ΔCt = Ct (target gene) – Ct (housekeeping gene).

Statistical methods. The significance of mean differences was calculated by using Scheffé’s test after analysis of variance (ANOVA 1 or 2 as specified). Significance was assumed when P < 0.05. Results were expressed as means ± SE.

RESULTS

C and LP hepatocytes were cultured for 3 days with PRL (10 or 100 ng/ml), and hepatocyte-conditioned culture medium was collected at the end of days 3 and 4. The level of IGF-I was evaluated by EIA in culture medium of C and LP hepatocytes isolated from fetuses 1 day before birth. The addition of PRL at 10 or 100 ng/ml for 48 h from day 1 to day 3 of culture had no effect on the production of IGF-I by C and LP hepatocytes. Because prolactin was reported to stimulate the expression of its own receptors (1), we then prolonged the time of incubation during an additional 24-h period of time, and again no effect was observed on the IGF-I levels in both C and LP groups (data not shown).

The level of IGF-II mRNA was evaluated by real-time PCR on day 4 of culture in cellular extracts of C and LP hepatocytes isolated from fetuses at 21.5 days of gestation (Fig. 1). The addition of PRL at 10 or 100 ng/ml for 72 h from day 1 to day 4 of culture decreased significantly the IGF-II mRNA levels of C and LP hepatocytes relative to untreated hepatocytes (P <
As mentioned elsewhere (13), only one band situated at 29–32 kDa was detected in purified hepatocyte cultures, as performed by Western ligand blot, using 125I-IGF-II to detect all the IGFBP species. Also, a major band in the similar range of 29–32 kDa was detected in vivo. This band had been demonstrated to correspond to IGFBP-1 and IGFBP-2 as revealed by immunoblot.

We therefore investigated whether PRL had any regulatory role on IGFBPs, in particular IGFBP-1 and IGFBP-2, since they represent the predominant binding proteins in the fetal period (21, 55). In our previous work, we had reported that growth retardation caused by maternal protein restriction was associated with increased IGFBP-1 and IGFBP-2 (13). To observe such differences, the C and LP samples had to be analyzed in the same gel. However, in the present study, having focused on the regulation by PRL on IGFBPs in both C and LP groups, these differences are not apparent since we used independent gels for the two groups. The immunoblot analysis was performed using specific polyclonal antibodies to detect IGFBP-1 and -2, and in all groups studied both IGFBP-1 and IGFBP-2 immunoblots showed a line at 29–32 kDa (Fig. 1). In C hepatocytes, densitometric quantitation showed that IGFBP-1 and IGFBP-2 immunoblots showed a line at 29–32 kDa was detected in vivo. This band had been demonstrated to correspond to IGFBP-1 and IGFBP-2 as revealed by immunoblot.

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The present study, both LPRLR and SPRLR were clearly identified as the major lactogenic receptor (30, 45). In mammary epithelial cells, there is a predominance of LPRLR, whereas in liver the short form of PRLR is a predominance in the mammary gland and the liver of adult rats, reverse ratios of tissues, which vary in a tissue-specific manner. For example, in the mammary gland and the liver, LPRLR tended to decrease in liver homogenates of LP fetuses compared with C samples, although it was not significant (Fig. 4A).

To know whether this alteration persists in vitro, we used cellular extracts of cultured hepatocytes isolated from C and LP rat fetuses on the last day of gestation. The C and LP cells were maintained for 4 days under similar conditions (see MATERIALS AND METHODS), and collection of cellular extracts was performed at the end of the last day of culture. After quantification of the immunoblot by densitometry, the statistical analysis of the doublet corresponding to SPRLR immunoblots showed clearly that maternal LP diet reduced significantly the abundance of SPRLR in cultured LP hepatocytes compared with C hepatocytes (P < 0.05; Fig. 4B).

**DISCUSSION**

Our data revealed that in C hepatocytes PRL had no effect on the hepatic IGF-I production, whereas, unexpectedly, it decreased the mRNA level of IGF-II that may reflect a decrease in the production of IGF-II, since a strong correlation has been made between circulating IGFs and abundance of hepatic IGF mRNAs (22, 50). We also found that PRL reduces the production of both IGFBP-1 and IGFBP-2 in a dose-dependent manner. In the literature, very limited results are available about the IGF and IGFBP response to lactogenic hormones. Hill et al. (25) have already reported that hPRL, but not GH, stimulated in vitro release of IGF-I, IGF-II, and IGFBP by human fetal fibroblasts. In vitro, PRL has been found to stimulate IGF-II in mouse primary mammary epithelial cells (8). Previously, Fielder et al. (14) reported that mouse PL-I, PL-II, or PRL stimulated 29-kDa IGFBP (IGFBP-1 and/or IGFBP-2) secretion by cultured mammary epithelial cells isolated from pregnant mice. Also, Phillips et al. (49) noted that PRL stimulated the secretion of IGFBP-2 into the culture medium of a mouse mammary epithelial cell line. Thus, contrary to our study, most of these findings demonstrated a stimulatory response of IGFs and IGFBPs (IGFBP-1 and -2) to lactogenic hormones. These discrepancies in PRL activities may be due to the type and level of PRLR expression in target tissues, which vary in a tissue-specific manner. For example, in the mammary gland and the liver of adult rats, reverse ratios of long and short PRLR exist. In mammary epithelial cells, there is a predominance of LPRLR, whereas in liver the short form has been described as the major lactogenic receptor (30, 45). In the present study, both LPRLR and SPRLR were clearly doublet, exhibited the expected relative mass (in the range of 34–44 kDa) of the short form of PRLR. Densitometric quantification of this doublet showed that the amount of SPRLR tended to decrease in liver homogenates of LP fetuses compared with C samples, although it was not significant (Fig. 4A).

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**Fig. 3. Effect of P on the abundance of IGFBP-2 evaluated by immunoblotting in culture medium of C and LP hepatocytes isolated from fetuses at 21.5 day of gestation. Hepatocytes were cultured for 3 days in the absence (0 ng/ml) or presence of 2 concentrations of prolactin: 10 or 100 ng/ml. Immunoblots of IGFBP-2 were performed in culture medium of C and LP fetal hepatocytes on day 3 (A) or on day 4 (B) of culture. Bars represent the quantification of the 29- to 32-kDa band after densitometry analysis of 5–11 autoradiograms from 3–4 different cultures. Values reported are means ± SE relative to arbitrary units of optical density expressed as follows in % (optical density/g of proteins in liver homogenates or cellular extracts); mean value of IGFBP-2 content in untreated fetal hepatocytes is 100%. ANOVA 1 followed by Scheffe’s test tested differences between means. aP < 0.05, C of prolactin (10 and 100 ng/ml) vs. untreated C. B: **P < 0.01, C of prolactin (100 ng/ml) vs. untreated C.**
expressed in the liver of adult rat, whereas in both fetal liver homogenates and cultured hepatocytes the short PRLR was mainly detected. Contradictory data have been reported with regard to the SPRLR (see the beginning of this article). In the rat, it has been postulated that SPRLR cannot mediate cell proliferation and inhibits the function of the LPRLR (3, 46, 52). Therefore, the inhibitory response that we observed for IGF-II and the IGFBPs (IGFBP-1 and -2) may be due to the largely dominant expression of SPRLR in fetal liver.

Furthermore, because of the role of IGFs and IGFBPs in cell proliferation (35), and the ability of PRL to induce proliferation in many cell types in vitro (2, 5, 7, 8), we examined the effect of PRL on fetal hepatocytes proliferation. PRL was inefficient to enhance DNA synthesis of fetal hepatocytes. Once again, this may be related to the dominant presence of the short form of PRL receptor. Our results indicate that the decrease in IGFBP production would normally lead to an enhancement of the availability of free IGF, and hence, stimulate proliferation. At the same time, we found that PRL decreases the levels of hepatic IGF-II mRNA, which suggests that PRL could maintain low IGF-II levels to limit hepatocytes proliferation. Indeed, in vivo, the proliferative capacity of fetal hepatocytes declines with advancing gestational age, and it has been suggested that inhibitory factors are involved in the growth arrest observed at term (24). Cortisol has also been reported (36) to suppress IGF-II gene expression in the fetal liver near term, and we have previously reported (13) that glucocorticoids inhibited fetal hepatocytes proliferation. Therefore, it is possible that PRL and related placental lactogens act synergistically with glucocorticoids to maintain a low rate of fetal hepatocytes growth towards term.

In hepatocytes of protein-restricted animals, PRL also reduced significantly the IGF-II mRNA, and this effect appears more pronounced than in hepatocytes of normally fed progeny. As we mentioned above, gestational protein restriction increases IGFBP-1 and IGFBP-2 levels in serum and the production by fetal hepatocytes (13). In the present work, PRL failed to modulate the IGFBP production in the LP hepatocytes as it did in control, which indicates that this hormone is unable to restore the alteration. Because control and LP cells were kept under the same culture conditions for 3 days, this indicates a persistence of programming due to fetal malnutrition already observed in basal conditions (13), and it reveals that maternal malnutrition may provoke a resistance to PRL in the response of IGFBPs due to a receptor or postreceptor defect. At this point, it has to be mentioned that in our model, an increase in carbohydrates was necessary to compensate for hypocaloric status in the protein-restricted animals. This fact may influence our results; however, no significant increase in glycermia occurs in LP animals, in either the mothers or their fetuses.

Our results showed the predominance of short PRLR in the liver at late fetal age, and we found that the abundance of this form is reduced in LP hepatocytes, which may explain in part the absence of PRL activity with regard to IGFBPs. A reduced mRNA for the long form of PRLR in adipose tissue has been shown in growth-retarded sheep fetuses, whereas both types of PRLR mRNA were unchanged in the liver (48, 56). In these studies, lowering placental irrigation has induced growth retardation. Finally, our data indicate that PRL may regulate differentially IGF and IGFBPs in fetal hepatocytes, since in protein-restricted animals IGF-II expression is more sensitive to PRL, whereas IGFBP production is blind to the hormone. These results suggest that the IGF-II PRL action could be mediated either through different receptors or via the same receptor but different intracellular signaling pathways.

In conclusion, our study revealed that maternal LP diet might alter the PRL regulation of IGF-II and IGFBPs and added further weight to the concept of programming in utero. This study also demonstrated in vitro a novel possible role of PRL in the regulation of IGF axis that seems to coincide with the requirement during late gestation in vivo, when a decline of IGF-II levels was observed in rodents and sheep (12, 36) and when there is a need to shift from a phase of dominant cellular proliferation to the differentiation of function required after birth.

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