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Isocaloric maternal low-protein diet alters IGF-I, IGFBPs, and hepatocyte proliferation in the fetal rat

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Fetal growth is a complex process controlled by specific interactions among nutrients, hormones, and growth factors. IGFs and their regulatory binding proteins (IGFBPs) play an important role in fetal growth and development (29). IGFs (I and II) are structurally similar to proinsulin and are key mitogens that affect cell growth and cellular metabolism, acting as endocrine, paracrine, and autocrine factors (23). Deletion of IGF-I or IGF-II genes in mice results in severe growth retardation or postnatal lethality in most animals (36–38). Bioactivity and availability of IGFs are modulated by specific binding proteins, and at present there are six well-characterized IGFBPs with high affinity for IGFs (8). During fetal life, the predominant IGFBPs are IGFBP-1 and IGFBP-2, which are expressed in most tissues (6). In mice overexpressing IGFBP-1, perinatal mortality or postnatal growth retardation is observed (15, 43).

In models of intrauterine growth retardation due to maternal malnutrition throughout gestation, the regulation of IGFs and IGFBPs has been poorly studied. Woodall et al. (57) demonstrated that a 30% nutritional restriction reduces fetal plasma IGF-I levels while it enhances levels of IGFBPs. In the study of Muaku et al. (42), a severe protein restriction (5% instead of 20% in controls) applied during gestation decreased plasma IGF-I level in the progeny at birth, but no changes were observed in the profile of IGFBPs. Rivero et al. (45) also showed that a 35% reduction in the diet of rats from day 16 of gestation decreased serum IGF-II levels at 18 and 19 days of gestation in fetuses from undernourished mothers, but not at the last day of gestation. Conversely, serum IGFBPs were found unchanged after this caloric restriction (45).

It has been suggested that hormones such as insulin (45) and glucocorticoids (18, 35), rather than growth hormone (56), may modulate the IGF system in such a period of life. Indeed, insulin and glucocorticoids play an essential role in modulating somatic growth in utero. An altered availability and/or activity of these hormones may lead to intrauterine growth retardation. In the fetus, severe insulin deficiency is associated with lower growth (39). In contrast, several studies in vivo in humans, as well as in animals, have demonstrated...
that fetal exposure to glucocorticoid excess induces intrauterine growth retardation (34, 47). Maternal malnutrition during pregnancy may increase exposure of the fetus to maternal glucocorticoids (3, 30).

Maternal low-protein (LP) diet, 8% instead of 20% in controls, given during gestation induces deleterious effects in the endocrine pancreas of the offspring, because β-cell mass, islet cell proliferation, islet vascularization, and pancreatic insulin content and secretion are reduced at birth (7, 10, 49). The effect of such a protein restriction on the regulation of IGFs and IGFBPs has not yet been evaluated. The aim of this research was to study the effect of a moderate and isocaloric protein deficiency in maternal diet on fetal hepatocyte growth by using a culture model of highly purified primary hepatocytes, and also to examine the role of changes in IGFs, IGFBPs, insulin, and glucocorticoids in liver growth retardation.

MATERIALS AND METHODS

Animals and diets. All animal studies were performed according to the guidelines of the animal ethics committee of the Catholic University of Louvain, Belgium. Wistar rats bred in our laboratory were maintained under controlled conditions (25°C, 12:12-h dark-light cycle) with free access to food and water. Virgin females were caged with males overnight, and mating was confirmed the next morning by the presence of vaginal plug or spermatozoa after a vaginal smear. Midnight was considered the time of mating. Dam were then housed individually and fed throughout gestation a 20% protein diet, representing the control (C) group, or an 8% isocaloric protein diet, representing the low-protein (LP) group. Both diets were isocaloric, because the protein deficiency in the LP diet was compensated by the addition of carbohydrates. Diets were purchased from Hope Farms (Woerden, Netherlands), and their composition has been previously described by Snoeck et al. (49).

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). Insulin was from Novo Nordisk (Copenhagen, Denmark). William’s E (WE) medium, 10× HBSS, antibiotics, and FBS were from Gibco-BRL (Merelbeke, Belgium). Deoxyribonuclease I was from Boehringer (Mannheim, Germany), and Vitrogen 100 was from Collagen (Palo Alto, CA). Human recombinant IGF-I and IGFBP-1 were purchased from UBI (Lake Placid, NY), tritiated thymidine (deoxyribose-6-[3H]thymidine, sp act 24–30 Ci/mmol) from Amersham (Ghent, Belgium), and 125I-labeled IGF-II (sp act 344 μCi/μg) from ICN (Irvine, CA). Percoll was purchased from Pharmacia (Uppsala, Sweden). Goat anti-rat IgGFP-B and anti-rat IGFBP-2 polyclonal antibodies were obtained from San- vertec (Santa Cruz, CA). Rabbit anti-goat immunoglobulin was from Dako (Merelbeke, Denmark). 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was from Serva (Heidelberg, Germany), and DNA from calf thymus used as standard was from Boehringer.

Blood and liver collection. On day 21.5 of gestation, dams were anesthetized with pentobarbital sodium (55 mg/kg body wt), and fetuses were exposed after abdominal incision along the midline. The feto-maternal circulation was maintained when blood was collected from fetal axillary arteries and kept in heparinized tubes chilled on ice. Plasma was then separated by centrifugation at 4°C for 2 min at 3,000 g and stored at −20°C until analyzed for IGF-I and IGFBPs. Fetal livers were removed, rapidly weighed, and homogenized in 0.1 M Tris-HCl buffer at pH 7.4 with added Triton X-100 (0.5%). After centrifugation, the supernatant was collected and its protein content determined, and then the aliquots were stored at −20°C for IGFBP measurements.

Isolation of fetal hepatocytes. Fetal hepatocytes were isolated from time-pregnant Wistar rats at 21.5 days of gestation by a modification of the method of Freemark and Handwerger (19). Fetal livers from 2 or 3 litters were removed aseptically and pooled, rinsed in a balanced salt solution (BSS; in mM: 142 NaCl, 6.7 KCl, and 10 HEPES, pH 7.4), and coarsely minced. Liver fragments were incubated in BSS containing 1 mM EDTA (8 ml/g wet wt) at 37°C under constant agitation for 7–10 min. The suspension was centrifuged at 100 g 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 (~8 ml/g wet wt) for 7–10 min at 37°C under constant agitation. After disassociation, the cell suspension was collected, diluted in WE medium supplemented with 0.2% BSA, and centrifuged for 5 min at 35 g. This procedure for digesting liver was repeated twice more until most of the tissue was digested. At the last digestion, the supernatants were pooled, filtered through nylon meshes (100 and 30 μm, respectively), washed with WE-BSA medium, and centrifuged for 5 min at 35 g. Fetal hepatocytes were further purified by a modified procedure of Dunn et al. (17), as follows. The cell pellet was resuspended in WE-BSA medium, and 15 ml of cell suspension were added to 14.4 ml of diluted Percoll solution (9:1 Percoll-10× HBSS, vol/vol). The mixture was centrifuged at 400 g for 8 min. The pellet (hepatocyte fraction) was resuspended, washed once in WE-BSA medium, and centrifuged at low speed (15 g, 5 min) to eliminate the residual hematopoietic cells. Purified fetal hepatocytes were resuspended in WE medium supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and cell number was estimated in a hemocytometer. Routinely, 10 × 106 hepatocytes/g liver were obtained with viability >90% as assessed by Trypan blue exclusion. In some experiments, the upper fraction of the gradient (heterogeneous fraction) was also recovered to obtain cultures enriched with nonparenchymal cells. This heterogeneous fraction was washed twice with WE-BSA (300 g, 5 min), diluted, and counted as described above for purified fetal hepatocytes.

Hepatocyte culture. Fetal hepatocytes were seeded and cultured on dishes coated with a type I collagen gel. Collagen gels were prepared by diluting 1 part of Vitrogen 100 stock solution, 1 part of HCl (1 M), and 0.5 part of 5× WE medium. Collagen solution was chilled on ice and mixed just before use and distributed evenly over culture dishes (1 ml/60 mm, 0.34 ml/35 mm, 0.1 ml/well, and 0.4 ml/200 mm in 6- or 24-well plates) and was allowed to polymerize at 37°C one night before use. Cells were seeded at a mean density of 104 cells/cm2 in WE medium supplemented with 10% FBS and antibiotics. Four hours after plating, the cells were washed 3 times and transferred into hormone-defined medium (insulin 10 nM, dexamethasone 10−7 M, EGF 50 ng/ml, ascorbic acid 0.1 mM, linoleic acid 10 μg/ml) with the exception of the experiments concerning mitogen-independent growth and IGFBP regulation by insulin and dexamethasone, where cells were transferred into control serum-free and hormone-free WE medium supplemented with antibiotics. After 24 h, the medium was replaced, and hormones were added as specified in RESULTS. Cells were cultured for a further 48–72 h, the media being replaced every 24 h. Nonparenchymal cells, obtained as we mentioned from the heterogeneous fraction, were plated and cultured as described for fetal hepatocytes.
Quantification of cell proliferation. Cell proliferation was evaluated either by incorporation of triitated thymidine or by measurement of DNA concentration at day 3 of culture. To measure DNA content in cultures at day 3, 500 µl of cellular extracts were transferred into tubes, and 2 ml of a buffer containing (in mM) 12 NaCl, 5 HEPEs, and 5 EDTA, pH 7, were added to each tube. Thereafter, 500 µl of DAPI solution (120 ng/ml) were added. The fluorescence was measured in a Kontron fluorimeter (Zurich, Switzerland) with calf thymus DNA as standard. The excitation and emission wavelengths were 372 and 454 nm, respectively.

DNA synthesis was estimated by the incorporation of triitated thymidine (0.25 µCi, or 0.25 ml/well; 24-well plates) performed over 24-h periods of time from day 0 to day 4. After incorporation, the cells were rinsed 3 times with serum-free medium containing an excess of nonradioactive thymidine and incubated for 10 min at 37°C in the last washing medium. After incubation in the presence of collagenase (1 mg/ml) for 5 min at 37°C to digest the collagen gel, the cells were collected and sonicated for 30 s. Eight milliliters of scintillation fluid were added for β-emission counting. The values per mg of protein were expressed as a percentage of the mean control value. Cell proliferation was also evaluated by cell counting performed on 35-mm dishes fixed at day 4 of culture with Bouin’s fluid. Dishes were stained with oil red O and Mayer’s hemalun. Cells were counted microscopically in duplicate or triplicate for each experiment with a randomized sampling procedure derived from the method of Cure and Boué (9). A similar procedure was used at day 1 to estimate the proportion of parenchymal vs. nonparenchymal cells in purified and heterogeneous cultures.

Preparation of conditioned media and cellular extracts. Media were conditioned over 24-h periods of time from day 0 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 trichloracetic acid (10% wt/vol final concentration) and concentrated 15 times upon reconstitution in Laemml sample buffer without β-mercaptoethanol. The corresponding cellular extracts were harvested in parallel, as follows. Cells were washed 3 times with PBS and incubated in the presence of collagenase (1 mg/ml, 1 ml/dish) for 10 min at 37°C to digest the collagen gel. The cell suspension was washed twice with PBS at 4°C (300 g for 5 min). Pellets were resuspended in the extraction solution (Tris·HCl 0.1 M, pH 7.4, containing 0.5% Triton X-100). After 30 min of incubation on ice, the extracts were centrifuged at 20,000 g for 15 min, and the supernatants were collected. Proteins in the cell extracts were determined. The extracts were then diluted in Laemmli sample buffer without β-mercaptoethanol to obtain 50 µg of protein in 15 µl of sample buffer.

Western ligand blot and immunoblot analysis. To examine the number and molecular size of IGFBPs, aliquots of serum, liver supernatants, or conditioned medium and cellular extracts from cultures were subjected to ligand blot analysis as previously described (16). Briefly, samples were boiled in Laemmli buffer and loaded on 12% SDS polyacrylamide gels under nonreducing conditions. After separation, proteins were transferred electrophoretically to nitrocellulose membranes, washed, and incubated with 750,000 counts·min⁻¹ membrane⁻¹ of 125I-IGF-II overnight at 4°C on a rocking platform. After incubation, the membranes were thoroughly washed, air dried, and autoradiographed with enhancing screens.

For immunoblotting, membranes were probed with a 1:500–1:1,000 dilution of antiserum against rat IGBP-1 and IGBP-2. An exception was the detection of IGBP-1 in plasma, with a dilution between 1:50 and 1:100 of primary antibody. Complexes of antigen antibody were identified with rabbit anti-goat immunoglobulin G labeled with horseradish peroxidase. Specific binding was visualized by 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.

Measurement of IGF-I. IGF-I in plasma and culture medium was measured by enzyme immunoassay (EIA) with a rat IGF-I EIA kit from Diagnostic System Laboratories (Webster, TX). The IGF-I EIA is a homologous assay that uses a pretreatment step to eliminate the interference of IGFBPs. After the pretreatment step, neither IGBP-1 nor IGBP-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 RT-PCR analyses. Total RNA was isolated from the livers of 21.5-day-old rat fetuses with TRIzol reagent according to the manufacturer’s instructions (Invitrogen Life Technologies, Carlsbad, CA). Reverse transcription (RT) 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). PCR amplification was carried out in a final volume of 40 µl by using 1 µl of RT product in the following conditions: 94°C for 4 min, 22 cycles at 94°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s, with a final elongation step of 5 min at 72°C. Absence of nonspecific or genomic amplification was assessed by including a nontemplate control and minus RT controls. Rat β-actin was used to normalize the IGF-II signal in each sample. The optimum number of PCR cycles was determined by serial dilutions of the same reverse-transcribed product and by subjecting them to a range of amplification cycles. Optimal detection of both IGF-II and β-actin mRNAs was obtained after 22 amplification cycles (not shown). PCR products were separated in 1× Tris-borate-EDTA, or TBE, electrophoresis buffer on 1.5% agarose gels containing ethidium bromide. Mean intensity of the bands in the agarose gel was measured with a Kodak Digital Science Image Station 440CF (NEN Life Science Products, Boston, MA) and analyzed with the Kodak digital science 1D image analysis software (NEN Life Science Products). Primer sequences were: 1) IGF-II sense, 5’-CAC GCT TCA GGT TCT GTG TCC-3’; antisense 5’-CGG GGT CTT TGG GTG GTA AC-3’, product size 385 bp; 2) β-actin sense, 5’-GAT GGT GGT TAT GGG TCA GAA GGA-3’; antisense 5’-GCT CAT TGC CAG TAG TGA TGA CCT-3’, product size 634 bp.

Statistical methods. The significance of mean differences was calculated by Student-Newman-Keuls tests after analysis of variance (ANOVA 1). Significance was assumed when P < 0.05. Results were expressed as means ± SE.

RESULTS

Effect of maternal isocaloric LP diet on liver weight, IGFs, and IGFBP levels in plasma and liver of fetuses. The liver weight measured in 21.5-day-old fetuses from 4–5 different mothers was significantly more reduced in LP animals than in controls (C: 319 ± 6 mg; LP: 280 ± 6 mg; n = 35–38, P < 0.01). The level of IGF-I was evaluated by EIA in plasma of C and LP fetuses at 21.5 days of gestation (Fig. 1A). Plasma IGF-I in LP fetuses was significantly lower than in controls (P < 0.01). To detect all of the IGBP species in fetal plasma
at the last day of gestation, analysis was performed by Western ligand blot using 125I-IGF-II (Fig. 1B). In both C and LP groups, three bands were detected, including a major band in the 29- to 32-kDa range, a group of bands of lower intensity between 40 and 49 kDa, and a very faint band at 24 kDa. Densitometric quantitation showed that maternal protein undernutrition provoked a significant increase in the abundance of 29- to 32-kDa (\(P < 0.01\)) and 40- to 49-kDa IGFBPs (\(P < 0.05\)). Because it is known that in the fetus the predominant IGFBPs are IGFBP-1 and IGFBP-2 (50), we focused our study on the analysis of this band. To specify the increase observed in the 29- to 32-kDa band in LP animals, immunoblot analysis was performed using polyclonal antibodies to detect IGFBP-1 and -2. Both immunoblots of IGFBP-1 and IGFBP-2 showed a line at 29–32 kDa that corresponds to the band revealed by ligand blot. For IGFBP-2, it showed the same tendency to increase as the ligand blot in the LP group, albeit it was not statistically significant (Fig. 1C, \(P = 0.08\)). The immunoblot of IGFBP-1 showed clearly that maternal LP diet augmented significantly (\(P < 0.05\)) the amount of IGFBP-1 in plasma of LP fetuses compared with C animals.

In liver homogenates of 21.5-day-old rat fetuses, one major band corresponding to the 29- to 32-kDa IGFBPs was revealed by Western ligand blot using 125I-IGF-II and was analyzed by densitometry (Fig. 2A). Consistent with results obtained for plasma, its abundance was higher in the LP group compared with controls (\(P < 0.01\)). The immunoblots of IGFBP-1 and IGFBP-2 (Fig. 2B) showed increased, although not significant, levels in binding proteins in LP liver homogenates. Of note, IGFBP-1 in liver appeared as a doublet on the Western blot, which may correspond to phosphorylated isoforms of IGFBP-1 (24, 25).

We have analyzed the abundance of IGF-II mRNA from both C and LP liver rat fetuses at the last day of gestation. RT-PCR analyses revealed that the mean ratio of IGF-II to \(H_{9252}\)-actin mRNAs was not statistically different in C and LP groups (C: 100 ± 15%, LP: 98 ± 15%, \(n = 15–16\)).

Characterization of primary culture of hepatocytes isolated from fetal liver. To study the effect of LP diet in vitro, we first validated the primary fetal hepatocyte culture system. Purified hepatocyte cultures consisted of 96.4 ± 0.6% of hepatocytes (Fig. 3A, \(n = 13\)), whereas cultures derived from the upper part of the gradient (heterogeneous fraction) contained mainly nonparenchymal cells (Fig. 3B, 82.1 ± 3.2%, \(n = 8\)). The IGFBPs secreted in these cultures were analyzed at day 3 and revealed that the pattern of IGFBP secretion was very different between hepatocytes and nonparenchymal cell cultures. Only one band situated at 29–32 kDa was detected in purified hepatocyte cul-
tured, whereas a major band located at 40–49 kDa and a minor band at 29–32 kDa were detected in the nonparenchymal cells (Fig. 3C).

Effect of maternal isocaloric LP diet on cultured fetal hepatocytes. Fetal hepatocytes cultured in serum-free WE medium in absence of hormones or growth factors were characterized by mitogen-independent growth, since a progressive increase in DNA synthesis was observed up to day 3 (Fig. 4A). In view of the reduced liver weight in LP fetuses and the altered IGF-I and IGFBP profile observed in the LP group and their ability to modulate cell growth, we studied the effect of maternal LP diet on hepatocyte proliferation. DNA content as measured by the DAPI method at the last day of culture (Fig. 4B) was significantly reduced by ~30% in the LP group compared with control values (P < 0.01). As in the plasma in vivo, the level of IGF-I measured by EIA at day 3 of culture (Fig. 5A) was significantly reduced in medium of LP fetal hepatocytes compared with controls (P < 0.01). Hepatocyte-conditioned culture medium was collected after 24 h of incubation to evaluate IGFBP levels during each day of culture. Densitometric quantitation of the Western ligand blots (Fig. 5B) showed that dietary protein restriction caused a significant increase in the 29- to 32-kDa band. This increase was significant (P < 0.01), from the 2nd day of culture until the end (Fig. 5B). The immunoblots of IGFBP-1 (Fig. 5C, P < 0.05) and IGFBP-2 (Fig. 5C, P < 0.01) confirmed the same pattern of increase in the LP group as that obtained by the ligand blot.

Hormonal regulation of proliferation and of IGFBPs in control fetal hepatocytes. Treatment of control hepatocytes with 10 nM of dexamethasone or 10 nM of insulin led to significant alterations in cell proliferation as measured by tritiated thymidine incorporation.

Fig. 2. A: Western ligand blotting analysis of IGFBPs in fetal liver homogenates at 21.5 days of gestation. Proteins were detected by 125I-IGF-II. Histogram represents quantification of the 29- to 32-kDa band after image analysis of 6 autoradiograms. Values are means ± SE (**P < 0.01) corresponding to AU of optical density expressed in %, 100% representing the mean value of IGFBP content in fetal control liver homogenates. B: IGFBP-1 and IGFBP-2 immunoblots from liver homogenates of rat fetuses at 21.5 days of gestation. Histograms represent quantification of the 29- to 32-kDa bands after image analysis of 4 and 3 autoradiograms for IGFBP-1 and IGFBP-2, respectively. Values are means ± SE, corresponding to AU of optical density (OD) expressed as OD/ug of protein in cellular extracts, 100% representing the mean value of IGFBP content in fetal control liver homogenates.

Fig. 3. A-B: light micrographs showing heterogeneous vs. purified primary cultures of fetal hepatocytes. Cultures were fixed 24 h after plating and stained with oil red O and hemalun. Bar, 35 μm. A: purified fetal hepatocyte culture. When a Percoll gradient step was used, homogeneous cultures containing mostly fetal hepatocytes (h) are obtained. Only a few fibroblast-like cells (arrowhead) were present in these conditions. B: heterogeneous fraction, recovered after the Percoll gradient step and plated in the same conditions used for purified fetal hepatocytes. Numerous fibroblast-like cells (arrows) and megakaryocytes (m) were present, whereas only a few hepatocyte clusters (h) were observed. C: Western ligand blot of IGFBPs detected by 125I-IGF-II. Comparison between IGFBPs produced by purified fetal hepatocyte cultures (H), nonparenchymal cell cultures (NP), and a 5x diluted sample of fetal bovine serum as control (S).
Dexamethasone potently inhibited (54 ± 2.3%, \( P < 0.01 \)), whereas insulin significantly stimulated DNA synthesis (127 ± 5.5% and \( P < 0.01 \)). The values are reported as a percentage of the mean value obtained at day 1 and taken as 100%. They are expressed as means ± SE (n = 28). A: DNA content in C and LP fetal hepatocytes as performed by DAPI at day 4 of culture in presence of hormono-defined medium (see MATERIALS AND METHODS). Values are means ± SE (n = 7, **\( P < 0.01 \)).

Analysis of IGFBP secretion in cultures treated with insulin (200 nM) or dexamethasone (100 nM) (data not shown). Cell counting also validated these results (data not shown).

Fig. 4. A: mitogen-independent growth of fetal hepatocytes cultured in William’s E (WE) medium in absence of serum or any additional factor from day 1 (3 h postplating) to day 4. For measurement of DNA synthesis, cells were incubated with \(^{3}H\)thymidine for 24-h periods, which ended at times shown. Values are reported as a % of the mean value obtained at day 1 and taken as 100%. They are expressed as means ± SE (n = 28). B: DNA content in C and LP fetal hepatocytes as performed by DAPI at day 4 of culture in presence of hormono-defined medium (see MATERIALS AND METHODS). Values are means ± SE (n = 7, **\( P < 0.01 \)).

Dexamethasone markedly increased its abundance (Fig. 6A, \( P < 0.01 \)). In corresponding cellular extracts, the hormonal treatments affected similarly the intensity of the 29- to 32-kDa band (Fig. 6B, \( P < 0.01 \)). The role of IGFs and IGFBPs in fetal hepatocyte proliferation was also investigated by testing the effects of exogenous recombinant human (rh)IGF-I and rhIGFBP-1. As shown in Table 1, IGF-I alone (1 and 10 nM) was unable to stimulate DNA synthesis above control level. By contrast, IGFBP-1 alone (4 and 16 nM) or in the presence of IGF-I (1 nM) led to a significant decrease in \(^{3}H\)thymidine incorporation at the highest dose tested (\( P < 0.05 \)).

Fig. 5. A: IGF-I levels as measured by EIA in culture medium of C and LP hepatocytes isolated from fetuses at 21.5 days of gestation. Values are means ± SE (n = 6, **\( P < 0.01 \)), expressed in ng/\( g \) of protein content in cellular extracts. B: Western ligand blotting of IGFBPs detected by \(^{125}I\)-IGF-II in culture medium of C and LP fetal hepatocytes after 24-h incubation in hormono-defined medium during each day of culture. Histograms represent quantification of the 29- to 32-kDa band after densitometric analysis of 4–12 autoradiograms. C: immunoblots of IGFBP-1 (\( * P < 0.05 \)) and IGFBP-2 (**\( P < 0.01 \)) in culture medium of fetal hepatocytes at day 3 of culture. Histograms represent quantification of the 29- to 32-kDa bands after image analysis of 3 autoradiograms. Values are means ± SE relative to AU of OD expressed as OD/\( g \) of protein in cellular extracts, 100% representing the mean value of IGFBP content in fetal C culture medium.

Fig. 6. A: IGF-I levels as measured by EIA in culture medium of C and LP fetal hepatocytes isolated from fetuses at 21.5 days of gestation. Values are means ± SE (n = 6, **\( P < 0.01 \)), expressed in ng/\( g \) of protein content in cellular extracts. B: Western ligand blotting of IGFBPs detected by \(^{125}I\)-IGF-II in culture medium of C and LP fetal hepatocytes after 24-h incubation in hormono-defined medium during each day of culture. Histograms represent quantification of the 29- to 32-kDa band after densitometric analysis of 4–12 autoradiograms. C: immunoblots of IGFBP-1 (\( * P < 0.05 \)) and IGFBP-2 (**\( P < 0.01 \)) in culture medium of fetal hepatocytes at day 3 of culture. Histograms represent quantification of the 29- to 32-kDa bands after image analysis of 3 autoradiograms. Values are means ± SE relative to AU of OD expressed as OD/\( g \) of protein in cellular extracts, 100% representing the mean value of IGFBP content in fetal C culture medium.
DISCUSSION

The primary objective of the present study was to investigate whether isocaloric maternal protein restriction modulates the proliferative capacity of fetal hepatocytes. We also investigated the contribution of IGFs and IGFBPs, as well as insulin and glucocorticoids, in this modulation. For this purpose we validated a culture system of purified fetal hepatocytes to compare the proliferative behavior of hepatocytes originated from control and LP animals. Our results show that DNA synthesis was markedly lower in the LP cultures. Because it is known that the IGF system plays a determinant role in overall fetal growth, abnormalities in IGFs and their binding proteins could therefore play a crucial role in the alteration of hepatocyte proliferation observed in LP fetuses. Because of these observations, it was interesting to study whether isocaloric maternal low protein affects the IGF-IGFBP axis, both in vivo and in vitro. Thus, in vivo, we found that the moderate protein restriction in the diet of the dam reduces the level of IGF-I in the fetal plasma, despite the fact that the diet was isocaloric and the food intake was unchanged or only slightly increased according to the experimental series (49). Very limited data are available about the impact of maternal protein undernutrition on the IGF axis in the fetus. When a hypocaloric protein-restricted diet was given to pregnant rats, Muaku et al. (42) reported lower plasma IGF-I in offspring at birth. In the rat models in which general (calorie) food restriction was applied during gestation, the level of fetal IGF-I in serum was also reduced and associated with impaired body weight at birth. By contrast, we found that the abundance of IGF-II mRNA in the liver is not affected by the isocaloric maternal LP diet. This is in agreement with previous studies in which the concentration of circulating IGF-II in the progeny, as well as its mRNA abundance in the liver, appeared less affected, or not affected, by maternal malnutrition or starvation (42, 45, 48), which indicates that nutrition may not be the principal modulator of this peptide and that IGF-I should be the peptide most affected by maternal malnutrition.

Different hypotheses could be proposed to explain the reduced IGF-I in fetal LP plasma. Nutrients and hormones are known to regulate IGF expression. Physiological concentration of insulin increases the IGF (I and II) peptide secretion, as well as their mRNA abundance in cultured hepatocytes isolated from fetuses at the last day of gestation (21). On the other hand, glucocorticoids appear to decrease IGFs in serum and their principal modulator of this peptide and that IGF-I should be the peptide most affected by maternal malnutrition.

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Table 1. Tritiated thymidine incorporation into DNA by fetal hepatocytes after incubation with insulin, IGF-I, and/or rhIGFBP-1

<table>
<thead>
<tr>
<th>Treatments</th>
<th>% vs. Control</th>
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<tbody>
<tr>
<td>Control</td>
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</tr>
<tr>
<td>IGF-I (1 nM)</td>
<td>103.04 ± 2.77</td>
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<tr>
<td>IGF-I (30 nM)</td>
<td>113.15 ± 6.95</td>
</tr>
<tr>
<td>IGFBP-1 (4 nM)</td>
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</tr>
<tr>
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<td>IGF-I (1 nM) + IGFBP-1 (16 nM)</td>
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IGF-I and/or IGF-binding protein-1 (IGFBP-1) were added 1 day after plating. rh, recombinant human. DNA synthesis was measured by a 24-h tritiated thymidine incorporation performed from day 2 to day 3 of culture. Values are reported as a percentage of the mean value obtained for cells maintained in control medium, William's medium (WE), and taken as 100%. They are expressed as means ± SE (n = 4–12). *P < 0.05.
study using caloric restriction (57). However, the amount of 28- to 34-kDa IGFBPs appeared unchanged in the experiments of Muaku et al. (42) after a hypocaloric protein-restricted diet. The reason for this discrepancy is unknown, but it might be due to differences in diet composition and/or the hormonal status of the animals. Insulin tended to be lower in the plasma of our LP fetuses (4) compared with the levels reported by Muaku et al.

To clarify which 29- to 32-kDa IGFBP species was affected by the maternal diet, we used specific rat IGFBP-1 and IGFBP-2 antibodies. In plasma derived from LP fetuses, IGFBP-1 levels were significantly increased, whereas IGFBP-2 levels were higher but not significantly. By Western blot, IGFBP-2 was easily detected in the plasma, but, interestingly, IGFBP-1 was observed only when the primary antibody was concentrated 10- to 20-fold more than the dilutions used for the in vitro studies. This suggests that this protein may be present in low amounts in rat serum at the late stages of fetal life. Some authors have reported that, in fetal rat serum, the most abundant IGFBP is IGFBP-2 (14, 54). However, it was IGFBP-1 that was markedly enhanced by the maternal LP diet. This is not so surprising, since serum IGFBP-1 is known to be rapidly regulated by nutrient intake, showing acute elevations during fasting and declining during postprandial periods (26). Moreover, IGFBP-1 was undetectable in the plasma of normally fed young rats but appeared in plasma of rats fed a protein-restricted diet during 8 days after weaning (33). In fetal liver, increased expression of IGFBP-1 and -2 in ligand blot was also confirmed to a certain extent by immunoblot. This is in agreement with a previous study reporting increased expression of liver IGFBP-1 and IGFBP-2 in neonatal rats fed a protein-restricted diet (33).

In vitro, when LP and C cells were kept under similar conditions of culture for 4 days, LP cells still produced less IGF-I and more IGFBPs than C cells. This indicates that impaired IGF-I and elevated IGFBPs are not the result of extemporaneous regulation by the maternal milieu but have been submitted to some programming in utero due to fetal malnutrition.

Because of previously reported actions of IGFs and IGFBPs regarding cell growth (23), we hypothesized that these proteins might modulate the fetal hepatocyte proliferation in our cultures. Thus we checked the effect of IGF-I and IGFBP-1 on DNA synthesis in control fetal hepatocytes. IGF-I had no effect, whereas addition of rhIGFBP-1 inhibited DNA synthesis. This appears contradictory to previous studies, in which IGF-I has been reported to stimulate fetal hepatocyte proliferation (53). The inability of IGF-I to stimulate DNA synthesis may be due to the presence of IGFBPs in the culture medium, since they can decrease bioactivity of IGFs and therefore autocrine and paracrine action. Menuelle et al. (40) indeed demonstrated that, in the absence of IGFBPs (fresh medium), DNA synthesis was stimulated by IGF-II and insulin, whereas in the presence of IGFBPs (cell-conditioned medium), IGF-II stimulation of DNA synthesis was inhibited whereas that induced by insulin was not. Taken together, it seems that elevated IGFBPs reduce the availability of IGF-I to its receptor, which may lead to impaired proliferation of fetal hepatocytes.

Insulin and glucocorticoids may also regulate the replicative capacity of fetal hepatocytes. We found that DNA synthesis was stimulated by insulin and inhibited by dexamethasone in hepatocytes isolated from control animals. Insulin has been reported to enhance DNA synthesis in both fetal and adult rat hepatocytes (27, 40), but the role of glucocorticoids in hepatocyte proliferation is unclear: they have been found to potentiate DNA synthesis in the fetus (40) or inhibit it in the adult (52). The effect of insulin on DNA synthesis seems to be dominant by comparison with other hepatic mitogens, such as TGF-α and hepatocyte growth factor (22). In adult rat hepatocytes, insulin is also capable of reversing the inhibitory effect of glucagon and glucocorticoids on DNA synthesis (52).

In our model of maternal malnutrition, fetal plasma insulin tends to be lower than normal, and anyway, less insulin is secreted in response to secretagogues (7). We have not assessed the glucocorticoid status of our animals, but in case of growth retardation induced by general calorie restriction (3), fetal corticosterone levels were increased. In the case of a low-protein diet, the higher activity of glucocorticoid-inducible enzymes in fetal/neonatal brain and liver also suggested increased fetal glucocorticoid exposure (31), corresponding to attenuated activity of the placental glucocorticoid-inactivating enzyme 11β-hydroxysteroid dehydrogenase in these animals (30). It is then possible that low insulin and high glucocorticoid levels in low-protein animals in vivo have contributed to the lower capacity for proliferation, which still remains apparent in culture. In fact, insulin and dexamethasone may have an indirect effect on fetal hepatocyte proliferation by modulating IGFs and IGFBPs. A pathway that is affected in vitro is the IGF system, because IGF-I secretion is diminished in the culture medium of low-protein hepatocytes, and both IGFBP-1 and -2 are secreted in higher amounts.

In the literature, experimental studies have already demonstrated the modulatory role of insulin (21) and glucocorticoids (2, 18, 28, 35, 46) regarding IGFs and IGFBPs. In this study, we have confirmed that insulin inhibited, whereas dexamethasone stimulated, 29- to 32-kDa IGFBPs in both culture medium and cellular extracts of fetal hepatocytes, indicating a regulation of synthesis rather than secretion. As already mentioned, the 29- to 32-kDa band has been demonstrated to correspond to IGFBP-1 and IGFBP-2 as revealed by immunoblotting. Using cultured hepatocytes from fetuses at 15 and 18 days of gestation, Menuelle et al. (40) showed that cortisol stimulated both IGFBP-1 and IGFBP-2 peptides. Also, IGFBP mRNA was regulated positively by cortisol and negatively by insulin (40). In adult rat hepatocytes, similar data have been reported, at least for IGFBP-1 (1, 55). In vivo, an enhanced expression of liver IGFBP-1 and/or IGFBP-2 has been described in the case of fetal growth retardation in-
duced by dexamethasone (44). It is not clear whether this was due to excessive glucocorticoids or simply reflects alteration in insulin action, since insulin secretion was decreased in this model. A dominant effect of insulin vs. dexamethasone in regulation of IGFBPs has been noted (41, 55), and a predominant action of insulin has also been mentioned in the presence of other hormones such as glucagon and tri-iodothyronine (11, 12).

In summary, the results demonstrate that a moderately restricted isocaloric protein diet alters the IGF axis and the growth capacity of fetal hepatocytes. These alterations persist ex vivo in cultured low-protein hepatocytes, supporting the concept of programing in utero. Furthermore, insulin and glucocorticoids, which are modified by maternal malnutrition, may contribute to the altered IGF axis, as well as to fetal hepatocyte proliferation.

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DISCLOSURES

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REFERENCES


LOW-PROTEIN DIET ALTERS IGF-I, IGFBPs, AND PROLIFERATION


